A LATEX AGGLUTINATION METHOD FOR USE IN EVALUATING INSECT PREY-PREDATOR SYSTEMS

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ABSTRACT

Serological techniques currently employed for the evaluation of prey/predator systems as well as their practical application in ecological research are reviewed. A quantitative determination of the amount (10 mg/ml) of insect protein (antigen) necessary for the production of high titred specific antisera, upon which all successful serological identifications depend is reported.

The latex agglutination technique commonly used in medical science was investigated to assess its potential and practicability in use, for evaluating prey-predator relationships. The migratory locust Locusta rnlgratorja (L) was used as the initial test insect and having confirmed that this technique could be used to detect locust antiserum-antigen reactions, it was then applied under laboratory conditions to detect the presence of Acrythosiphon pisum (pea aphid) in the gut of the 7 spotted Coccinellid (Coccinella septempunctata). A further test was conducted using the larvae of the Lepidopterous insect Venessa io (the Peacock butterfly). In all cases, comparisons were made between this method and the standard precipitin test (Capillary ring test). Results indicated that the method was as sensitive as the ring test, but was found to be superior in terms of speed of reaction, (occurring between 1-2 minutes, as opposed to 2 hrs in the Capillary ring test) and utilization of minimum equipment and reagent, an asset to its potential application as a rapid screening method for arthropod predators under field conditions.

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INTRODUCTION

The impact of natural enemies, when successful can be of immense importance in a pest management programme. A rational biological control method which could be incorporated into an integrated control programme could emerge when the extent of control of a pest population by its natural enemies has been quantitatively assessed.

Some of the methods adopted for such an assessment utilise serological techniques. Information on the various serological techniques used for studying insect prey-predator systems is scattered throughout the literature. An attempt has therefore been made here to bring such information together and to review the practical application of the techniques in current use, especially the precipitin test. Like other methods which have been used in studying prey-predator relationships, the precipitin test has several limitations which include among others, the problem of cross-reactions, which limits its use in analysing species which are closely related (Hall et al., 1953; Fox and MacClellan, 1956; Dempster, 1960; Loughton et al., 1963); the non-specificity of the prepared antiserum to all stages of an insect (Telfer and Williams, 1953; Spencer, 1969; Service, 1976); and more so, the inability of the test to distinguish between multiple and single feeds (Dempster, 1960; Rothschild, 1966, 1970; Davies and Reynoldson, 1971). Most of the studies using the precipitin test have as a result of these limitations been qualitative, although a few quantitative estimates of predatory influence on the population change of preys have been attempted (Dempster, 1960, 1966, 1967; Rothschild, 1970; Ashby, 1974). An attempt was made to determine the amount of insect protein necessary to produce an antiserum of high titre and specificity, an essential ingredient to the success of all precipitin test studies (Boreham, 1975). Quantitative information on this aspect is virtually lacking

and various investigators have consequently used varying amounts of antigen in obtaining antisera of varying titres.

For situations in developing countries where capital and expertise are limited, the development of an easy, cheap but effective technique which could be used in the field for prey-predator studies is desirable. This is because most serological techniques in common use are not only expensive in terms of equipment and reagent, but also can hardly be applied in the field. These considerations prompted the search for another method. The latex agglutination test appeared to offer a new opportunity. In addition to its success in the area of forensic science for the identification of the origin of blood stains (Cayzer and Whitehead, 1973; Whitehead et al., 1974), it has also been used in other areas such as in the diagnosis of many clinical conditions including meningococcal infections (Posolescu and Mihalcu, 1974), and rheumatoid arthritis (Singer and Plotz, 1958), for the detection of plant viruses (Abu Salih et al., 1968). In Section IIB of this work there is a report on the results of experiments carried out with the latex agglutination tests for prey-predator identification. Section IIC contains the results of its application in identifying prey in predators under test conditions. Results from these tests are compared with those obtained from the standard precipitin tests. Indications are that this method compares favourably with the standard precipitin test and holds out promise as an effective time-saving technique for the preliminary screening of insect predators in the field.

Section I

Review of Serological techniques used in prey-predator studies

1. Non Serological Methods

Natural enemies of insects include both vertebrate and invertebrate predators, parasitoids which by some are considered to be specialized predators and various pathogens. In insects, mortality caused by predation can in certain situations be the key factor in the regulation of pest population. A classical example being the control of the cottony cushion scale Icerya purchasi by a Coccinellid beetle, Rodolia (= Vadalia) cardinalis in California.

1.1. Direct Observations: There are several methods open to ecologists to assess the effect of a predator on an insect population. One of these is Direct Observation under field conditions (De Bach et al., 1950; Richards and Waloff, 1961; Ashall and Ellis, 1962; Sunderland, 1975). The most obvious limitation of such observations is that they usually describe the minimal effects of predators and rarely take account of multiple feeds. For predators which swallow their prey whole e.g. birds, fairly good quantitative information can be obtained through visual identification of the gut contents, but for a wide range of predators that have sucking or piercing mouthparts, or species that have specialized mandibles and predigest their prey, the method is clearly unsuitable (Service, 1976).

1.2. Experimental Methods: These methods involve comparison between plots in which the prey/predator ratio is modified in some way in some plots, while other plots remain as untreated 'controls'. The difference in numbers of prey between treated and untreated plots gives a measure of the effect of the natural enemies in controlling prey numbers.

1.3. Mechanical Exclusion Technique: This can be effected by the use of

sleeves or cages often carried out in conjunction with some pesticides. An example is the fumigation of some citrus branches covered with sleeves by South and De Bach (1942). The branches were later reinfested with the black scale (Saissetia oleae) and five of these sleeves were opened to let in parasites. Population trends of black scale between closed and unclosed sleeves were then compared. Other investigators Franz (1958), Way and Banks (1958), have conducted similar experiments. The main limitations here include among others, the prevention of dispersal by the prey and also changes in micro-climate which might occur as a result of the cages.

1.4. Insecticidal Check Method: This involves a procedure where some experimental plots are treated with a selective insecticide which apparently kills the predator but leaves the prey population unaffected. The sprayed plots are then compared with the unsprayed ones and the difference in numbers is taken as an index of the effectiveness of the predator in population regulation. De Bach (1946) demonstrated this principle in his biological control of Pseudococcus adonidum and Icerya purchasi by the selective killing of the natural enemies with DDT. De Bach et al (1950), De Bach (1955), Fleschner et al (1955), Kiritani et al (1971), have carried out similar experiments on a number of pest populations. Since no insecticide is truly selective, one of the major drawbacks of this method is that prey populations are liable to be killed thus giving an underestimate of the effectiveness of the predator.

1.5. Hand Removal Technique: This technique, as its name implies would appear to be the most reliable of all exclusion techniques, which has the advantage of not interfering with the environment when it is properly carried out. It has been successfully used by a number of workers on predator/prey studies especially on Homopteran pests (Fleschner et al.,

1955; Way and Banks, 1958; Pollard, 1969, 1971). One of its serious limitation is that it is extremely time-consuming and laborious. Other methods like the biological check method and the insecticidal trap method have been used (Kiritani and Dempster, 1973).

1.6. Radioactive Marking of Prey: Of the analytical methods used, radio-active marking of prey has been popular with some investigators (Jenkins and Hasset, 1950; Baldwin et al., 1955). When a predator develops radio-activity, this usually is indicative of its having fed upon a prey which has been marked with radio-active material. The level of radioactivity in a predator could be an indication of the number of prey eaten, if it were possible to give each prey individual a similar dose of radio isotope. However, in practise this technique appears hard to quantify for several reasons.

(a) The level of radio activity of individuals seem very variable.

(b) Since quite a proportion of predators eat only part of their prey, the rate of excretion of radio active material would appear to depend upon the amount of food subsequently eaten.

Baldwin et al (1955), showed that the same level of radioactivity could result from the consumption of different numbers of prey. As explained by Southwood (1966), this could be due to the variable radio-active content of the prey and the different rates of assimilation of the predator.

Radioactive tagging looks good in a restricted environment, but as observed by Loughton et al (1963), it tends to tamper with field conditions before the evaluation of predation.

2. Serological Methods

One of the ways of studying predator-prey relationships is by serological methods. This technique was originally developed by vertebrate zoologists (Southwood, 1966), but has lately been applied to an aspect of entomological research, namely the study of insect predator/prey relationships. Early observations by Nuttal (1904) establishing a correlation between the antigenic relationships of animal serum proteins and the zoological classification of species (Weitz, 1956), led to the development of serological techniques which have formed the basis for several studies. The technique has a series of advantages, the most important being that it enables a critical and objective assessment to be made on the intensity of predation occurring in the field under natural conditions (Service, 1973), with minimum interference after biotope.

2.1. The Precipitin Test:

The test is based on the interaction of prey protein in the gut of predators with antibodies directed against the prey prepared in rabbits by immunizing them with an extract (antigen) of the prey.

The method has been widely used in the study of predator-prey relationships (Brook and Proske, 1946; Hall et al., 1953; Fox and MacClellan, 1956; Dempster, 1960, 1963, 1964 & 1966; Rothschild, 1966; Frank, 1967; Service, 1973, 1974; Ashby, 1974) among others. The tests rely upon the preparation of high-titred specific antisera and have been adapted from bloodmeal identifications of haematophagous arthropods (Weitz, 1956, 1960; Boreham, 1972; Tempelis, 1974).

2.1.1. Preparation of Antigen: An antigen is defined as a substance whose injection into an animal induces the formation of antibodies which specifically combine with that antigen. The majority of antigens are

proteins although some may be polysaccharides or even lipids.

The procedure for the preparation of antigens have been described by Leon (1947), Dempster (1960), Service (1976). It essentially involves the preparation of a cell-free extract of the prey in saline solution. The prey is ground up in a pestle and mortar in normal saline. The mixture is stored for 24 hours at $4^{o}C$ with consistent stirring. Dempster (1960), when preparing an extract of Phytodecta olivacea, added $^{M}/1000$ potassium cyanide to the prey homogenate to inhibit the deposition of melanin. Silverman (1963), Service (1976), noted that protein (antigen) extraction, carried out for more than 24 hours at $4^{o}C$ could produce non-specific effects which could interfere with the serological testing. The supernatant is then filtered through a Seitz E.K.S. sterilizing filter pad and either used in this form or transferred to 5 - 10 ml sterilized ampoules, with the liquid removed by freeze drying for storage. The antigen is stored for future use by sealing the ampoules in air-free condition.

2.1.2. Preparation of Antisera: Serological tests rely on the preparation of a high-titred specific antisera (Boreham, 1975). Several basic methods have been adopted by different investigators in the preparation of antisera (Proom, 1943; Weitz, 1952, 1956; Dempster, 1960, 1964, 1966; Rothschild, 1966; Davies, 1969; Reynoldson, 1970; Murray, 1970; Boreham and Gill, 1973; Service and Lyle, 1974). Quite a number of workers have used Alum precipitated serum (A.P.S.) injected intramuscularly to immunize rabbits (Leon, 1947; Dempster, 1960). This consists of the addition of 10% potash alum to an equal volume of antigen and after adjusting the pH to 6.8 by the addition of 1N sodium hydroxide solution, the mixture was centrifuged and the resulting precipitate washed several times with saline, resuspended in a known quantity of saline before injection into the rabbit. The alum

acts as an adjuvant to antibody production (Crowle, 1961; Kabat and Mayer, 1967). Several routes of injection have been used by investigators namely

(a) Intramuscular injection into the hind legs of the rabbit (Weitz, 1952; Dempster, 1960; Rothschild, 1966).

(b) Intravenous injection of the antigen into the marginal vein of the ear of the rabbit without adjuvant (Leon, 1947; Murray, 1970; Titova, 1970; Solomon, 1973).

(c) Lymph node injection, which involves injecting the antigen in the form of an emulsion with Freunds complete adjuvant; into the axillary and unguinal lymph nodes of the rabbit (Boreham and Gill, 1973).

2.1.3. Specificity of Antisera: The specificity of the antisera prepared need to be tested against homologous and heterologous sera. This is necessary because, antisera prepared for a particular species tend to reach with near relatives of the species for which it is prepared - a process known as 'cross-reaction'. Cross-reactions can be removed by the process of adsorption (Weitz, 1956; Dempster, 1960; Titova, 1970; Boreham, 1975; Service, 1976). Adsorption is a process whereby tissue extracts of the related species are added to the antisera to precipitate out the interfering proteins. Description of the method for absorbing antibodies responsible for cross reactions have been given by Weitz (1956), Service (1976). In doing this, a series of tubes containing equal volumes of antisera is prepared and to each of these tubes is added a known quantity of the volume of serially diluted heterologous sera. The tubes are well mixed and stored at $4^{o}C$ for 24 hours. They are then centrifuged to remove all the precipitate. The resulting supernatant fluid is tested for the presence or absence of heterologous antibodies by the precipitin test.

The different routes of injection tend to influence the sensitivity of the antisera subsequently produced. Solomon (1973) carried out sensitivity tests of anti-locust sera prepared through intravenous, intramuscular and lymph node injections. He found that 13 intravenous injections with quantities of antigen varying from 0.1 ml - 0.9 ml produced antisera with low titre of mean 1:600, while seven intramuscular injection with 2.5 ml antigen at each occasion, and five lymph node injections with 1 ml of antigen, produced antisera of similar sensitivities, with mean titres of 1:3500 and 1:3333 respectively. However, intramuscular immunization requires about 3-4 injections to reach maximum titre (Service, 1976), while the lymph node method requires only 2 injections (Boreham and Gill, 1973), as well as utilizing only about $^{1}/_{10}{}^{th}$ of the amount of antigen needed for intramuscular injection. Whatever method is used immunization is repeated after 7 days. Ten to 14 days after the second injection, the rabbit is test bled from the marginal ear vein and the resulting antiserum is titrated against the prey antigen to determine if a satisfactory titre has been obtained. The injection regimen can be repeated if necessary to improve the titre but there is a risk of decreasing the specificity.

When the titre is satisfactory, 50 mls of blood are taken from the marginal vein of the rabbits ear. The serum is separated by allowing the blood to stand in a water bath at 37°C for 2-3 hours, to accelerate the clotting of the blood. Thereafter, the serum is pipetted off, centrifuged and filtered through a Seitz E.K.S. filter pad. In certain situations, it might be necessary to defat the antiserum, in which case, the lipoids could be removed by mixing the antiserum with $\frac{1}{2}$ volume of ether and freezing the mixture below -25°C (Macfarlane, 1942). When thawed, the ether and fat layer are siphoned off and the antiserum refiltered to remove the precipitated lipoids. The antiserum so treated, is then ready for sensitivity testing against the antigen, or if required to be used at

a future date, could be freeze-dried and stored at room temperature. Antisera dried from the frozen state have been known to store for 5 years without deterioration (Weitz, 1956).

2.1.4. The Precipitin Test: The methods for actually performing the precipitin test has been reviewed by Weitz (1956, 1960); Tempelis (1975) and Boreham (1975). The methods that are adopted depend on the number of tests required and on what apparatus that is available. The first method is known as the capillary ring test (Weitz, 1956; Dempster, 1960, 1966; Boreham, 1972). The multiple serological automatic dispenser (Plate 2), specially designed for carrying out this test on a large scale, has been described by Weitz (1957). The machine is constructed in such a way that twelve tests could be performed at the same time. Capillary tubes each 2-3 mm in diameter are connected automatically to a suction unit of the machine. About 0.05 ml of the antigen extract is drawn up into each tube, followed by an equal volume of the antiserum contained in a metal trough. Since the antiserum is denser than the antigen extract, there is no mixing and an interface is seen between the two liquids. Air is drawn into the capillary tube bringing the liquids into the middle of the tubes. The tubes are then forced down into a strip of plasticine held in a wooden holder, and released from the suction unit. The tubes are left standing at room temperature (about 20°C) for 2 hours. The precipitate formed from the incubation of the antigen and antibody is shown as a white ring at the junction of the liquids and read by an indirect light against a black background.

A simpler apparatus devised by Hole for carrying out a similar test, but only when a relatively small number of tests is required, is described by Weitz (1956) and Service (1976). This consists of a 2-2.5 mm bore precipitin tube fitted with a small rubber tubing at the end of which is

attached a 1 ml hypodermic syringe held vertically in a clamp with its nozzle pointing downwards. The plunger of the syringe is fitted with an extension spring and above it a wheel bearing an eccentric cam. This enables small quantities of antigen and antiserum to be drawn up and the test similarly carried out as previously described.

2.2. The Agar-Gel Double Diffusion Method:

An alternative to the capillary ring test, which has been applied in prey-predator studies is the agar-gel double diffusion method (Ouchterlony, 1948). This is also a precipitin test, the principle of which is based on the fact that when antigen and antibody are placed in holes cut in agar, they diffuse towards each other and precipitate to form an opaque line in the region where they meet in optimal proportions.

The method consists of pouring an even layer, 1% agar in glycine buffer pH 7.2 on microscopic slides or petri-dishes and after cooling, the slides or dishes are transferred to a moist chamber for 4-5 hours to set hard. A number of equidistant holes are punched in the agar film consisting of a central well and five - seven wells in a circle around it. The antiserum is placed in the central well and the antigen extract placed in the outer wells. Alternatively, the antigen may be placed in the central well and several antisera placed in the surrounding wells. The slides or dishes are examined after 48 hours for lines of precipitate. At times the detection of these lines are difficult but can be improved by staining in a protein stain (0.2% Naphthalene black for 3-5 minutes, Becker, 1975) or thiazine red (Pickavance, 1970). Excess stain could be removed in 2% acetic acid in methyl alcohol.

2.3. Immunoelectrosmophoresis (Crossover electrophoresis, Counter current electrophoresis)

This technique was developed for use in forensic medicine but has

recently been adapted and applied to the serological investigation of invertebrate predators of the sheep tick - Ixodes ricinus (L.) (Healey and Cross, 1975).

The test is basically similar to the Ouchterlony technique and the principle underlying this test involves the migration of antigens (protein) and antibody in the serum towards the anode and cathode respectively during electrophoresis. When therefore homologous antigen and antiserum placed in wells in agar-gel are appropriately positioned in the electrophoresis tank, the reactants are rapidly brought together with formation of a sharp white precipitate. The experimental details of performing this test has been described in Healey and Cross (1975). The technique is said to be more sensitive than other techniques in common use and results could be obtained in under 30 minutes.

Other methods of carrying out the precipitin test which have been used in bloodmeal identification have not as yet been applied in prey-predator studies, for example, Flocculation test in capillary tubes (Tempelis and Lofy, 1963), the gel-surface preciptation (Eliason, 1971), and the precipitation in microplate method (Tesh et al., 1971).

2.4. The Precipitin Test and its Limitations:

The precipitin test, inspite of its popularity and use in both bloodmeal identification and in prey-predator studies has a series of limitations.

(a) Cross-reaction: The precipitin test cannot be satisfactorily used in analysing preys or species which are closely related. This is because antisera prepared from a species may not be specific to that prey species, but may cross-react with related species. Loughton et al (1963) found that the budworm antisera produced, cross-reacted with extracts of closely

related Acleris variana (Fem) and with Zeiphera sp. Dempster (1960) produced anti-phytodecta serum which reacted with other chrysomelidae, Cossididae and Coccinellidae. Hall et al (1953) noted that serum obtained against the forest tent caterpillar - Malacosoma disstria, reacted positively with the eastern tent caterpillar - M. americanum. Fox and MacClellan (1956) in their study of the predators of a wire worm - Agriotes sputator, found that the antiserum prepared reacted with all other Elateridae tested. Titova (1970) found that antisera prepared against Eurygaster intergriceps a pest of grain in the USSR, reacted quite strongly with antigens of a near relative - Eurygaster austriacus (Shrank). As already indicated the cross reaction spectrum can be narrowed by a process known as absorption. However, the process of absorption may lower the titre of the antiserum if not carried out with care (Weitz, 1952; Dempster, 1960, 1964; Loughton et al., 1963; Service and Lyle, 1974; Service, 1976). Weitz (1956) noticed no appreciable drop in homologous titre following absorption, by adding just the minimum of the heterologous antibodies.

(b) A second limitation concerned with specificity of the antiserum is that the antisera produced may not be specific to all stages of an insect, e.g. larvae, pupae and adults. This could be due to differences between the antigenic properties of the different stages of the insects (Smith and Silverman, 1966; Telfer and Williams, 1953; Spencer, 1969; and Service, 1976). Spencer (1969) found variation in sensitivity and specificity between instars of Locusta migratoria. The anti-locust serum made up for adult locusta, gave a titre of 6,400 for the antigen extract used for preparing the antiserum, and for 3rd, 4th and 5th instars, the titre was 3,200. However for 1st and 2nd instars, the titres were very much lower 400, and for eggs, a low titre of 50 was obtained. Similar instar variation was found for the spruce budworm (Choristoneura fumiferana (Clem),

Loughton et al (1963)). Ashby, (1974) working with Pieris rapae found that antiserum prepared from the pupae was more sensitive to antigen response, than that prepared from larvae. For reliable results therefore, the production of an antiserum that will react with more than one stage of the insect is necessary.

(c) Precipitin tests cannot detect when more than one individual prey has been eaten by a predator. This is a major reason why most of the tests carried out have been largely qualitative rather than quantitative. In most cases what has been achieved with the tests has been to show what predators are feeding on a prey. There is lack of knowledge regarding the number of prey taken by a predator in a meal (Rothschild, 1966; Service, 1971). However, under certain circumstances, quantitative estimates can be obtained (Dempster, 1960, 1966, 1967; Rothschild, 1966; Davies and Reynoldson, 1971; Ashby, 1974). Dempster was able to establish that a positive reaction in the precipitin test represented a single feed, and from the estimate of the number of predators in the population, the number of prey consumed was estimated. For example if (X) represents the number of predators present in a population, (a) the proportion of those tested which gave positives, (t) the length of time that a meal remains detectable and (T) the total time that the prey are available for predation, then the number of prey consumed is given by

$$\frac{X\ a\ T}{t}$$

Dempster's estimate based on this method corresponded closely with the predation estimate from field samples and estimates made by Richards and Waloff (1961). In most prey-predator systems however, involving gregarious preys and voracious predators, it would appear that Dempsters assumption would be unjustified and inapplicable.

(d) As already indicated in the above formula, the length of the digestion period influences the value of the precipitin test. It is therefore important to determine the period of time after feeding during which a given prey can remain detectable in the predator's gut. This is so because as digestion proceeds, the prey antigens become altered and may no longer react with homologous antibodies. West and Eligh (1952) note that the prey can elude detection even when partially digested food is still present in the gut of the predator. Information is fairly scanty on detection periods of meals in the gut of predators. Dempster (1960) found that Phytodecta meal remained detectable in the gut of its main predator (Orthotylus adenocarpi) (Miridae) for 24 hours. Loughton et al (1953), found that 1st and 2nd instar larvae were detectable in the gut of spiders for only 24 hours after feeding while larger larvae remained detectable for 4-6 days. Davies (1969) found that Triclads could be detected in the guts of most predators for 24 hours. Table I shows the digestion periods of a number of predators as shown by various investigators.

The period of detectability of prey in predators gut is not only influenced by the rate of digestion but also on other factors such as size of the meal and climatic factors especially temperature (Dempster, 1960, 1966, 1967; Hall et al., 1953; Loughton et al., 1963).

(e) The results obtained from the precipitin test depend on the state of the antigen and antiserum. For reliable results, especially with the capillary ring test, it is essential that both antigen and antiserum are completely clear. Cloudy reagents are likely to render visibility of the ring difficult. However, for the Ouchterlony method, clearness of the reagents is not essential, an advantage over the ring test.

(f) Sutton (1970), Tod (1973) and Service (1976) noted that one of the

Table I. The digestion time of different predators following feeding on a number of preys as determined by the precipitin test.

Predators	Species fed on (prey)	Digestion time (hours)	Authority
Orthotylus adenocarpi	Phytodecta olivacea	24	Dempster, 1960
Earwig	Phytodecta egg	27-30	Dempster, 1960
Heterocordylus	Phytodecta egg	18-22	Dempster, 1960
Nabis apterus	Phytodecta 4th instar larva	48	Dempster, 1960
Anthocoris	Psyllid or Aphid	5-7	Dempster, 1963
Carabid sp.	Cabbage root fly egg	24	Coaker & Williams, 1963
Spider spp.	Spruce budworm 4th instar larva	96-120	Loughton & West, 1963
Spider spp.	Spruce budworm 1st & 2nd larva	24-72	Loughton & West, 1963
Mites	Spruce budworm eggs	24	Loughton & West, 1963
Pterostichus madidus	Sitona regenst-einensis	36-48	Danthanarayana, 1965
Linyphia triangularis	Orthotylus	48	Dempster, 1966
Tytthus	Conomelus eggs	12	Rothschild, 1966
Oligolophus spp.	Conomelus adult	60	Rothschild, 1966
Linyphia triangularis	Conomelus 2nd & 3rd instar	38	Rothschild, 1966
Coccinella - 7 punctata	Conomelus 5th & adult	17	Rothschild, 1966
Anthocoris	Conomelus 5th & adult	22	Rothschild, 1966
Carabidae	Abax parallelep-ipedus pupae	96	Frank, 1967
Pyrrhosoma nyphula	Triclad	48	Davies, 1969
Dytiscid	Aedes cantans larva	24	Service, 1973a
Dytiscid	Aedes cantans pupae	20-22	Service, 1973a
Phalanguim opilio	Pieris rapae	72	Ashby, 1974

limitations of the precipitin test was that antigens from living and dead bodies could not be distinguished and as a result was unsuitable for distinguishing actual predation which caused population loss and carrion feeders which had no direct effect on prey population.

2.5. Value of the Precipitin Test:

Inspite of these limitations, there are a number of useful information which could accrue from precipitin tests.

(a) The evaluation of prey-predator relationships has been difficult and some investigators consider the influence of natural enemy on their prey population as one of the most difficult aspects of population ecology to study (Kiritani and Dempster, 1973). However, the progress so far made with the precipitin test method has considerably eased the situation. The assessment of the number of prey eaten by a predator is often difficult if not impossible, because, either the prey is consumed entirely, or even when not, partially eaten preys are difficult to find in the field. In this situation, the value of this method cannot be underrated. It clearly offers a means of not only demonstrating predator species of a prey but also offers a means of finding out what proportion of a population of a predator species has fed on a particular host.

(b) A reliable quantiative estimate of predator action can only be based on an intensive study by the most direct techniques. In view of this, in studying prey-predator relationships, it is considered that the identification of prey in the gut contents of the predator is a good, if not a better alternative to direct observation of predation. If certain basic assumptions as indicated earlier are made as Dempster did, quantitative evaluation of predation could be made. This is of interest to both ecologists and biological control specialists, who are not only interested

in knowing what predator has preyed on a prey species, in itself, a useful information, but are equally interested in a fairly accurate estimate of the effect of a given predator on a given prey population. From this sort of information, it is possible to examine the density dependency of predation on the abundance of the prey (Titova, 1970; Kiritani and Dempster, 1973).

(c) On account of the sensitivity of this method, it is a useful tool in detecting insignificant amounts of prey antigen in the gut of a predator, even as little as 10^{-6} g in 1 ml of fluid (Titova, 1970).

2.6. Other Potential Serological Test for Prey-Predator Identification:

As a result of the limitations of the precipitin test, it seems appropriate to suggest that investigations of other potential serological methods which do not suffer such limitations and have been applied in other situations, for example in bloodmeal identification and other immunological research should be explored for prey/predator studies. A brief outline of one such method is given below.

2.6.1. The Haemagglutination Inhibition Test: The haemagglutination inhibition test, a supplement to the precipitin test, has added a new dimension to the study of the host ranges of arthropods. The test has been described by Weitz and Jackson (1955), Weitz (1956, 1970) and Boreham (1972). The test involves the sensitization of normal rabbit cells with either tannic acid, Chromium chloride or Bisdiazotized benzidine (BOB). The sensitized cells are then coated with a serum protein of a given species and can be agglutinated by an antiserum prepared against the proteins. In this way, an unknown serum can be identified by adding a saline extract of it to a series of agglutinating antisera and testing the mixture with a series of tanned red cells suspension previously coated with

the serum of the respective series. This method has been applied in many immunological laboratories, especially in bloodmeal identification, when it is essential to differentiate the individual species of wild animals on which insects of tsetse fly may have fed (Boreham, 1972). The limitation of this process is that it tends to be difficult and elaborate compared with the precipitin test. The success of this method again depends on the careful selection of the right concentration of antiserum, since specific inhibition can only occur under this condition (Weitz, 1956). There is a decrease in sensitivity when a higher concentration of antiserum than the optimum is used, while a lower concentration of antiserum than optimum results in an increase in sensitivity, but then specificity decreases. The haemagglutination inhibition test could be applied to prey predator systems, especially to differentiate individual species, since as already indicated, cross-reactions do occur in species which are closely related.

A number of other immunological methods could perhaps be used for identification of predators of insects. These include the fluorescent antibody method (Coons and Kaplan, 1950) and the complement fixation test (Roitt, 1971). The use of such tests have not so far been evaluated for this purpose.

2.7. Application:

Serological tests have been most widely used in the identification of arthropod bloodmeals and have been reviewed by Weitz (1956, 1960, 1966), Boreham (1972, 1975) and Tempelis (1975). As applied to field pests, very little is known about the activity of predators during the period of life of pests in the field, consequently, the quantitative assessment of the effectiveness of a predator in the field is made difficult. The only conclusive proof that a prey has been consumed by a predator is by seeing it being eaten, or by identifying prey material in the gut of the predator.

This later approach which may involve serological tests, (e.g. the precipitin test), is now gaining popularity as a means of identifying invertebrate insect predators. The application of serological methods for demonstrating predator species of a prey can be divided into a number of headings for convenience of comprehension.

2.7.1. Prey/Predator Identification - Terrestrial: A number of research workers have applied serological tests, especially the precipitin test, to identify predators of important pests in various localities. Hall et al (1953) used the test in studying the predators of the eggs of Tortricids and Oleuthreutids and of the larvae of the forest tent caterpillar - Malacosoma disstria. Downe and West (1954), applied the test in studying the association between a reduviid predator Zelus exsanguis (Stahl) and the forest tent caterpillar - Malacosoma disstria. From the results of their study, they concluded that the reduviid was an occasional feeder on the tent caterpillar at low population levels but fed more regularly when the caterpillars were abundant. They therefore suggested that predation on the tent caterpillar was a matter of chance encounter by Z. exsanguis and therefore could not be regarded as an important controlling agent. Fox and MacClellan (1956) similarly studied the Carabids and Staphylinids preying on the wire worm, Agriotes sputator (L). Although these studies were essentially qualitative, they however unequivocally revealed the species which were predatory in the field. Evidence was thus no longer only circumstantial.

Quantitative effects of predators could also be estimated although this is rarely done and requires a programme of a population study of prey and regular estimates of numbers of predators in the field. Dempster (1960) studied the predators of the young stages of Phytodecta olivaceae on broom in the field by means of the precipitin test. About 11286 gut contents

of the various predators were tested and of these, five species of Miridae, two Anthocoridae, one Nabid, one earwig and a red mite were all identified as having regularly fed on the beetle. This study was quantified by making certain assumptions indicated earlier, and in this way, the effect of each predator on the beetle population was estimated. The close agreement between population estimates of Phytodecta killed by predators (145341, Richards and Waloff, 1961), and an estimate of 111581, obtained by Dempster from the precipitin test, gave credence to this technique as an effective tool for evaluating predators.

From the results, it was shown that predation of the eggs and larval stages was the key factor in altering the levels of the population of the beetle, a useful information necessary for the understanding of the population dynamics of the broom beetle.

A study of the natural prey of three species of Anthocoris was also studied by Dempster (1963) in Silwood Park. The results showed that psyllids were the predominant prey of two species of Anthocoris namely A. nemoralis and A. sarothamni. A third species A. nemorum, was non-specific in its attack and made use of prey on many different plants. Similar precipitin test studies were made by Dempster (1964) on the natural food of five species of the Miridae. Dempster (1966) equally studied the arthropod predators of some species of Miridae on broom by means of the precipitin test. About 20 species of different arthropod predators especially members of the Heteroptera and some spiders were said to have caused considerable nymphal mortality in some years. By adopting his original method he estimated the number of Orthotylus adults destroyed by spiders especially by Linyphia triangularis as equivalent to 424 Orthotylus/day, or 1 per 16.3 webs. The precipitin test method was also used by Dempster (1967) to show the full effect of arthropod predators on Pieris rapae, since several

other studies by Pimentel (1961) and Harcourt (1966) only stressed the importance of parasites and disease as the major factors affecting its population. As P. rapae is nocturnal in habit and cannot be seen on plants during the day, the precipitin test appeared to be the only reliable method of studying the influence of arthropod predators on the population of this insect.

A number of other workers have applied the technique in studying various prey-predator relationships. Loughton et al (1963), studied the impact of spiders (predator), on the spruce budworm (prey) population and suggested incidence of a functional response to fluctuations in budworm population, as well as the existence of a distinct pattern of predation throughout the season of study. Insect predators of the pupal stage of the winter moth Operophtera brumata (L) were identified by Frank (1967). The tests showed Pterostichus madidus, Abax parallelepidus and Philonthus decorus as the most important predators of the winter moth pupa. Danthanarayana (1965, 1969), identified Staphylinids and Carabids as the major predators of Sitona regensteinensis - a curculonid beetle inhabiting the broom (Sarothamus scorporius) in Silwood Park. Predators of the immature stages of the cabbage root fly (Erioischia brassicae) were identified using the precipitin test by Coaker and Williams (1963). This study indicated that Carabid and Staphylinid predators constituted the major predators of this insect.

Rothschild (1966) followed Dempsters method in quantitatively evaluating predation in the delphacid Conomelus anceps. He showed that immature phalangids, mostly Oligolophus and Leiobunum accounted for 60% of the total predation on this insect. Equally Rothschild (1970, 1971), studied the different predators of rice-earbug and rice stem borers in Sarawak (Malaysia Borneo) by the application of this technique. He demon-

strated the effectiveness of this method by identifying the presence of a pyralid-borer antigen in the gut of the phytophagus grasshopper (Oxya sp), commonly found in rice fields in this area. This apparent anomaly was explained by the fact that the eggs or larvae of the borer could have been accidentally picked up with the rice leaves, the main item of diet of this species.

Sutton (1970), serologically examined predation on the terrestrial isopod - woodlice (Trichoniscus pusillus pusillus and Philoscia muscorum). He demonstrated that the common shrew, Sorex araneus, Lycosid and Dysderid spiders, as well as the centipede - Littobius variagatus all preyed on woodlice and constituted a primary factor in limiting their numbers. Titova (1970), similarly used this test in clarifying the role of predatory arthropods in the population dynamics of Eurygaster intergriceps, considered to be the most important grain crop pest in the U.S.S.R. Laboratory feeding trials were conducted with eggs, nymphs and adults of E. intergriceps offered to suspected predators. From the precipitin test (Method of Ouchterlony, 1948), Carabids and spiders were identified as having fed on the various stages of the insect.

Tay (1972) used the precipitin test to confirm the results of feeding trials carried out in the laboratory and was able to establish that spiders and four species of Nabidae were the main predators of the larvae and adults of Cicadella viridis (L). Ashby (1974) similarly looked at the role of Syrphid predators on Pieris rapae. In his results, he quantitatively estimated that the larvae of Syrphids (Melenostoma pasciatum) and Syrphus novae-zealandiae were responsible for 58.3% and harvestermen (Phalangium opilio), 31.5% of the total arthropod predation on Pieris larvae. Solomon (1973) used the test to detect predation on Psammotettix confinis. The most important predators were said to be Nabids, Phalangium opilio and

Thomisids, each of which contributed to the mortality of larvae and adults of the cicadellid. Tod (1973), applied the precipitin test in investigating the presence of molluscan tissues in the gut contents of carabids and other arthropod predators. Her results indicated that 14 species of Carabids (Table II) and a few other arthropods such as Staphylinidae and Silphidae had all eaten molluscan materials. Through feeding trials, Becker (1975) showed that two Nabid species, N. rugosus and N. ferus were the most effective predators of Macrosteles sexnotatus in oatfields in Silwood Park. The laboratory feeding trials were confirmed through the use of the precipitin test carried out in agar-gel by the method of Ouchterlony. Percentage positive reactions for predators was estimated at 5.2, with the two Nabid species being the most important predators. Sunderland (1975), used the precipitin test in detecting cereal aphid predation by Tachyporus larvae, Syrphid larvae and adult Cantharidae as well as Clubionidae. He however noted that this test was unsuitable for the detection of aphid predation by the small Linyphid species. Table III shows a summary of arthropod preys by orders in which serological investigations have been done.

The precipitin test has been applied in studying the digestion rates in arthropods (Dempster, 1960, 1963, 1966, 1967; Loughton et al., 1963; Sutton, 1970; Ashby, 1974; Service, 1976); a point of considerable interest from the general biological point of view; data of which are lacking from a majority of insect physiology literature.

2.7.2. Prey/Predator Identification - Aquatic: Brooke and Proske (1946), were the first workers to demonstrate the use of the precipitin test in the identification of the predators of mosquito larvae. Service (1973) studied the predators of Aedes cantans in England using this test. During this study, the gut contents of 2893 predators of this insect were analysed.

Table II. Carabid predators of molluscs showing numbers tested and % positive reactions.
(Data after Tod, 1973)

Predator species	Number Tested	% Positive reactions
Carabus violaceus (Linneaus)	9	100
Carabus catenulatus (Fabricius)	16	67
Carabus nemoralis (Mueller)	7	71
Feronia nigra (Schaller)	34	43
Feronia vulgaris (Schaum)	23	35
Cychrus caraboides (Linnaeus)	23	73
Feronia madida (Fabricius)	509	20
Calathus fuscipes (Goeze)	5	60
Nebria brevicollis (Fabricius)	427	2
Feronia anthracina (Illiger)	6	17
Calathus piceus (Marsham)	25	12
Nebria gyllenhalli (Schoenherr)	37	8
Calathus micropterus (Duftschmid)	54	7
Calathus melancephalus (Linneaus)	53	2

Although no egg predators were identified, it was shown that the larval stage of the mosquito was preyed upon by larval Dytiscidae but caused no apparent reduction in the population of pre-adults. However, the emerging adults were preyed upon by some members of the Diptera, resulting in the destruction of about 13.5% of the total emergent population in 2 years. Results conclusively showed that predation on emerging adults caused greater reduction in population size of A. cantans than larval predation. Service (1973a, b), adopted the same method in identifying the predators of both the larvae and adults of Anopheles gambiae complex. Various spiders, Diptera, Coleoptera and Amphibia were all implicated as predators in the regulation of mosquito. The importance of these predators in the regulation of A. gambiae population even in small water collections was acknowledged.

The precipitin test has been applied in the identification of the predators of Simulium damnosum complex in the Ivory Coast (Service and Lyle, 1974). The damnosum - antisera, prepared from extracts of larvae of S. damnosum complex was used to identify the natural predators of the fly. Various Trichopteran larvae especially members of the genera, Hydropsyche and Cheumatopschye were reported as important predators of Simulium damnosum. Odonatan nymphs of Zygonyx flavicosta fed on immature S. damnosum, while adult dragonfly Metacnemis singularis was presumed to have fed on adults in the Bandame river of the Ivory Coast.

2.7.3. Identification of Food of Invertebrates other than Insects: In invertebrate studies, serological techniques have been applied in the analysis of the diets of small carnivorous invertebrates. The diet of the Triclad - Dendrocoelum sp. and three other planariid species on Ascellus was investigated by Young et al (1964) by the precipitin tests. It was found that Ascellus was much more important in the diet of Dendrocoelum sp., than it was in the diet of the other three species.

Table III. Serological studies on prey-predator 'terrestrial' are summarized according to orders of insects and other arthropods.

Order	Insect prey studied	Investigator(s)
Lepidoptera	Forest tent caterpillar (*Malacosoma disstria*)	Hall *et al.*, 1953
	Spruce budworm (*Choristoneura fumiferana*)	Loughton *et al.*, 1963
	Winter moth (*Operophtera brumata*)	Frank, 1967
	Pieris rapae	Dempster, 1967 Ashby, 1974
	Rice stem borers	Rothschild, 1971
Homoptera -	*Conomelus anceps*	Rothschild, 1970
Hemiptera	*Cicadella viridis*	Tay, 1972
	Psammotetix confinis	Solomon, 1972
	Macrosteles sexnotatus	Becker, 1972
	Cereal Aphid	Sunderland, 1975
Heteroptera -	*Miridae*	Dempster, 1964
Coleoptera	Broom beetle (*Phytodecta olivaceae*)	Dempster, 1960
	Wire worm (*Agriotes sputator*)	Fox & McClellan, 1956
	Sitona regensteinensis	Danthanarayana, 1965
	Eurygaster integriceps	Titova, 1970
Diptera	Cabbage root fly (*Erioischia brassicae*)	Coaker & Williams, 1963
Other Arthropods		
Isopoda	*Woodlice* (*Trichoniscus pusillus pusillus*)	Sutton, 1970
Mollusca	Molluscs	Tod, 1973

From the results obtained, as well as data from previous studies, Young et al were able to provide a general comparison of the food of these four species of triclad.

Pickavance (1970, 1971) adapted the agar-gel Ouchterlony method in analysing the gut contents of the invertebrate planarian Dugesia trigrina (Girard) for the presence of prey species. By utilizing this method, he was able to identify simultaneously a number of different preys in the gut of a single predator. Davies and Reynoldson (1971), studied the influence of predation on the distribution and abundance of four lake-dwelling triclads in Great Britain by serological methods. Of the 75 predatory species studied 15 were identified as predators of Triclads (Table IV). These included among others, leech (Erpobdella octoculata) (L), Odonata larvae, Trichoptera larvae, Newts and some species of fish. They were able to establish that on stony shores, the predation on triclads was below the level at which intraspecific competition for food regulated the population, but on weedy habitats, which maintained fish population and Odonata larvae, predation was capable of eliminating triclad population to a level at which competition for food was unlikely. Lock and Reynoldson (1976) applied this test in detecting the food requirements of two triclads - Crenobia alpina and Polycelis felina in the spring fed shores of North Wales.

They established that the food requirements of these two triclads were similar and this provided the basis for the interspecific competition which occurred between the two species. By analysing the diets of various carnivorous invertebrates through serological methods, it is possible to attempt to explain the distribution and abundance of animals and the degree of intra and interspecific competition for food in their habitats - a subject of considerable interest in ecological research.

Table IV. Predators of triclads in the field showing numbers tested and % positive reactions.

(Data after Davies and Reynoldson, 1971)

Predator	Number tested	% Positive reactions
Erpobdella octoculata (L)	1009	10.7
Polycentropus flavomaculatus (F. J. Pictet)	69	2.9
Rhyacophila sp.	43	4.6
Enallagma cyathigerum (Charpentier)	238	17.6
Ischnura elegans (Van der Linden)	142	17.6
Coenagrion puella (L)	74	45.6
C. pulchellum (Van der Linden)	10	60.0
Sympetrum striolatum (Charpentier)	111	15.3
Triturus helveticus (L) adult	21	42.9
larvae	280	65.0
T. cristatus (L) larvae	15	6.7
Scardinius erythrophthalmus (L)	146	17.9
Rutilus rutilus (L)	234	25.2
Perca fluviatilis (L)	49	10.2
Pygosteus pungitius (L)	148	8.8
Gastrosteus aculeatus (L)	146	20.5

2.7.4. Application to Taxonomic Investigations: There have been other practical applications of serological techniques in the field of taxonomy. It has been used by taxonomists to investigate phylogenetic relationships (Martin and Cotner, 1934; Noland, 1959), and has been acknowledged as a useful taxonomic method especially when correlated with morphology. The work of Martin and Cotner (1934) was perhaps the most extensive in the use of serological techniques in insect taxonomy. They demonstrated the value of the precipitin test in determining the phylogenetic relationships within the genera and subfamilies of moths in the family Phalaenidae. Cumley (1937) applied the precipitin ring test among others, in the study of the interrelationship of a series of species of Drosophila. He arrived at the conclusion that results obtained from serological ranking were more specific than those based on morphological characteristics. Leon (1947) utilized the precipitin test in the study of the systematic relationships of members of the Orthoptera, principally the family Blattidae. He made a serological comparison of whole insect extracts and found that in general, the precipitin reactions ran parallel with the systematic position of the species compared. For instance, it was demonstrated that the Oriental roach - Blatta orientalis and the American roach, Periplanata americana were more closely related to each other than the German roach - Blatella gemanica. Also using the ring test, it was possible to obtain reactions to four different families in the order Othoptera. Of these the Gryllidae and the Tettigonidae appeared to be more closely related to the Blattidae, than did the Acrididae and Mantidea.

Thomas and Kryweinczyk (1966), adopted serological techniques in carrying out preliminary studies on the relation between the immature stages of the families Curculionidae and Scolytidae. The preliminary tests showed no distinct separation between the two families, and they suggested

that more extensive testing would be required to evaluate precisely the current constitution of these two families. Landsteiner (1947) refers to reviews by Chester (1937) on investigations using serological reactions in the field of systematic botany. Results of such investigations were however said to be controversial, in view of the fact that plant extracts comprised of different sorts of proteins along with other substances such as lipids and tannins, which caused errors through the formation of precipitates.

2.7.5. Other Applications: In forensic investigations, the precipitin test has been used in the identification of blood stains (Weitz, 1952; Landsteiner, 1962; Gray, 1970). Sensitive serological protein reactions have been used in detecting particular proteins even in very minute amounts. Adulterated food stuffs of protein origin (meat, milk and egg powders), as well as contaminated flour have been detected through serological methods.

The practical applications of the precipitin test in prey/predator systems can therefore be summarized as follows

(a) Qualitative determination of predator species of important pests and the relative importance of individual predator species.

(b) Quantitatively, in evaluating individual predator species and examining the density dependence of predation on prey populations, an essential and useful information in formulating pest control strategies.

(c) By its successful use in analysing the food of certain invertebrates, it has been possible to understand the distribution, abundance and the degree of intra and interspecific competition of these animals in their various habitats.

(d) Its application and successful use in the area of taxonomy is bound to stimulate greater interest in this branch of science.

Section IIA

The determination of the minimum concentration of insect protein (antigen) necessary to produce a high-titred antiserum

Introduction

When foreign proteins (antigen) are injected into a mammal, antibodies specific to those proteins are produced. Nosall (1964) and Spiers (1964) describe the processes involved. The titre of an antiserum, which partly depends on the concentration of the prey protein used for immunization and partly on the animal, is defined as the greatest dilution of protein material (antigen) which shows a positive reaction against the antiserum. There is little data on the actual protein concentration of a prey extract necessary to produce a high titred antiserum. Early investigators have used varying quantities of sera with unspecified protein concentrations (Weitz, 1952, 1956; Dempster, 1960, 1963, 1964, 1967; Tempelis and Lofy, 1963; Murray, 1970; Service and Lyle, 1974; Vickerman and Sunderland, 1975). The problem of not determining quantitatively the protein content of the extract used, is that inoculation of an animal with excess amounts could induce a state of immunological tolerance and a poor antibody response. Insufficient antigen will not stimulate the hosts immune system sufficiently to produce a good antibody response. It is not always easy to collect insect antigen and it is therefore important to conserve this material.

The purpose of this study was therefore to determine quantitatively the minimum amount of protein (antigen) necessary to produce a high titred specific antisera, by injecting known amounts of protein extract into the axillary and inguinal lymph nodes of rabbits (Boreham and Gill, 1973).

3. Materials and Methods

3.1. Preparation of Antigen:

Preparation of the antigen was carried out using Locusta migratoria (L), since this was readily available in large numbers in the insectaries at Silwood Park. Fifty adult locusts were cut in small pieces with scissors, after being killed by deep-freezing at -20°C. These were crushed in a pestle and mortar with 0.9% saline solution. The mixture was transferred to a Buckner flask and mechanically stirred for 24 hours at 4°C. The material was then centrifuged at 2500 R.P.M. for 20 min., filtered through sintered glass and passed through a Seitz E.K. sterilizing filter pad. The filtration process was repeated several times and the supernatant pooled and dialysed against saline solution for 24 h to remove excess salt. The clear sterile antigen was freeze-dried for 24 h. The freeze-dried extract was reconstituted with the smallest amount of distilled water possible and dispensed in 1 ml aliquots in small vials and kept in the deep-freeze at -40°C until required for use.

3.2. Protein Measurement of Locust Extract using the Folin Phenol Method:

The modification of the method described by Lowry et al (1951) was used in the measurement of the protein content of the antigen. The reagents used were as follows:

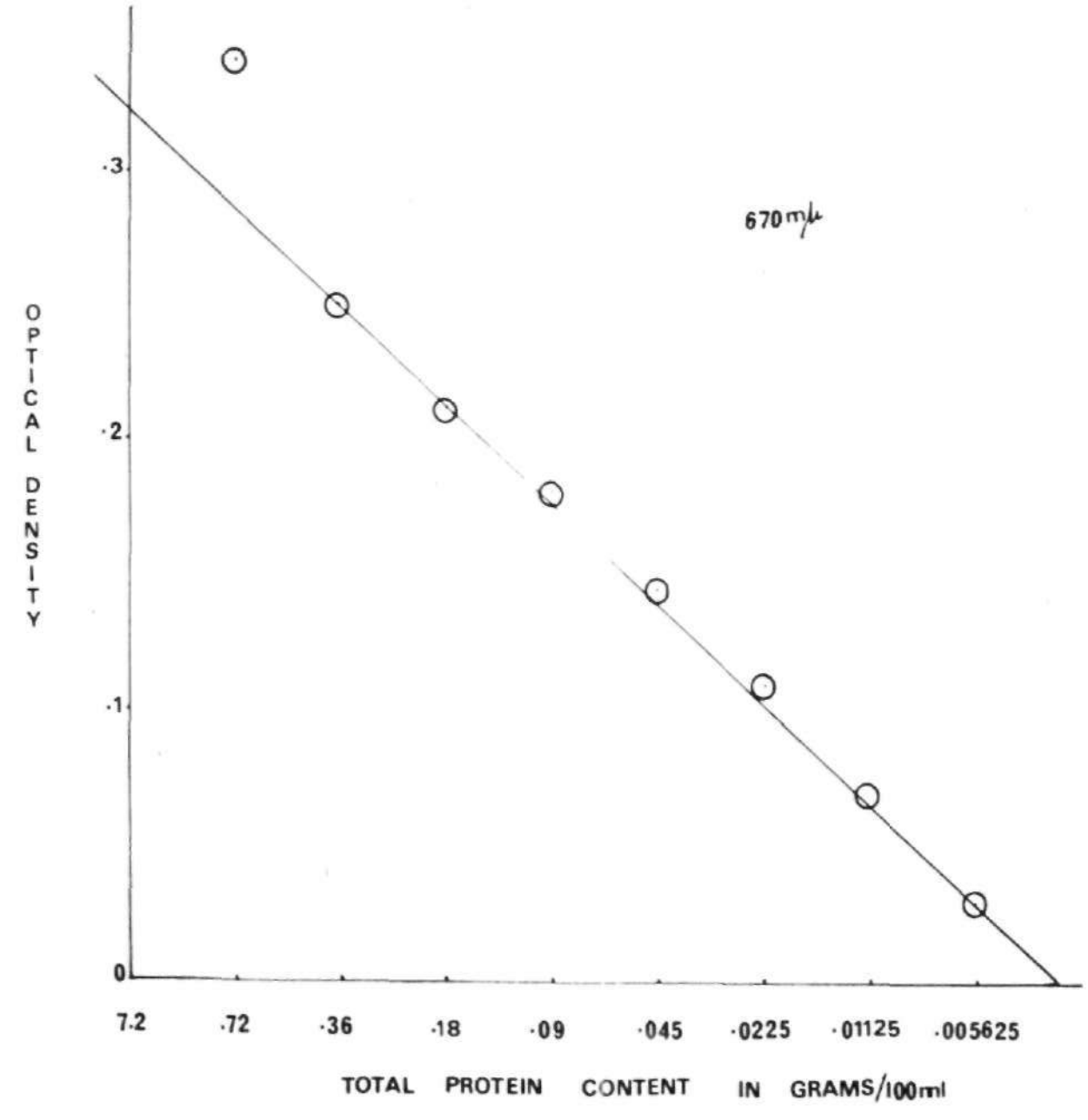
A - 2% Sodium carbonate in 0.1 N NaOH.

B - 0.5% Copper sulphate in 1% Potassium tartrate.

C - Alkaline copper solution prepared by adding 1 ml of B to 50 mls of A and discarded after standing for 24 hours.

D - Dilute folin reagent (BDH Chemicals) kept at 4°C.

Fig. 1. Graph of optical density and total protein content determined from serial doubling dilutions of Versatol.



2 ml of mixture C was added to 0.4 ml of the locust extract. The solution was mixed and allowed to stand for 20 min. 0.2 ml of folin reagent D, was added rapidly, mixed thoroughly and allowed to stand for 30 mins. A control consisting of 0.4 mls distilled water and equal quantities of reagents C and D was set up. After the 30 min. standing period, the optical density was read in a calormeter (Gallenkamp) at 670 um. A standard line was drawn by determining the optical density of serial doubling dilutions of Versatol (General diagnostics, W. R. Warner and Co. Ltd., Hampshire), containing 7.2 g/100 ml. The protein concentration of the extract was subsequently determined by reading from the standard line (Fig 1).

3.3. Preparation of Antisera with Three Different Protein Concentrations of Locust Antigen:

The freeze-dried antigen with a total protein content of 115.2 mg/100 ml was reconstituted with 11.5 ml of sterile distilled water, thus giving a concentration of 10 mg of protein/ml of antigen. Three different dilutions of the reconstituted material were made giving the following protein concentrations of antigen used for the tests.

(i) 10 mg/ml

(ii) 1 mg/ml

(iii) 0.1 mg/ml

Five New Zealand rabbits numbered 589, 590, 591, 597 and 598, each weighing 2-4 kg were used. Rabbit (number 589), was injected with a dose of 10 mg/ml. Rabbits 590 and 591 were each injected with 1 mg/ml, while 597 and 598, each had a dose of 0.1 mg/ml of antigen extract.

The rabbits were normally held firmly on their backs with their legs fully extended during each injection. 1 ml of each protein extract was

thoroughly emulsified with 1 ml of Freund's complete adjuvant (Difco laboratories, Detroit, Michigan). Aliquots were injected into each of the two axillary and two inguinal lymph nodes of the rabbits. In some cases, it was difficult to locate the lymph nodes and in such circumstances, the injection was given in the general area of the nodes. A second injection was administered seven days later. Ten days after the second injection, the rabbits were test bled (Boreham and Gill, 1973).

In order to remove a blood sample for the sensitivity test the rabbit was placed in a retaining box. 5 ml blood samples were taken from the marginal ear vein of the rabbits.

The serum containing antibodies was separated by placing blood samples in a water bath at 37°C for 1 h to accelerate the clotting of the blood. The separated serum was transferred to a centrifuge tube and centrifuged at 3000 R.P.M. for 10 min. The clear serum was pipetted off and filtered through a Seitz E.K.S. pad in a Hemmings filter. This was then ready for use in the sensitivity test to locust antigen.

The antiserum obtained from each rabbit was titrated against serial doubling dilutions of the antigen to determine if a high enough titre has been obtained. A third injection was given after the preliminary test if necessary. When the antisera were sensitive enough, 50 ml of blood was taken from each rabbit and the serum containing the antibodies was separated in the manner previously described. These were placed separately in labelled bottles and stored in a deep-freeze at -40°C until required for use.

3.4. The Precipitin Test:

Determination of the titre of the antisera was performed by using the precipitin ring test. Twelve doubling dilutions (Table IV) of the locust

antigen were prepared ranging from 1:10 to 1:20480. These dilutions were prepared in a rack of 12, 10 x 45 mm plastic tubes. Saline was pipetted into each of the tubes as indicated in table V. 0.1 ml of antigen was pipetted into tube A and mixed with the saline by drawing the mixture in and out of the pipette. 0.5 ml of this diluted antigen was then put into tube B, containing 0.5 ml of saline and mixed together. Similarly all the remaining dilutions were prepared. Approximately 0.04 mls of antigen dilution was sucked into small glass capillary tubes using the multiple dispenser described by Weitz (1957). This was followed by an equal volume of the test antiserum. If the antiserum was drawn up carefully, mixing did not occur due to its greater density. The capillary tubes were sealed in plâsticine and allowed to stand for 2 h. Preliminary readings were taken after 30 min and 1 h. A positive result was shown by a ring of precipitate at the interface between the antigen and antibody. The titre of the anti-serum was taken as the reciprocal of the greatest dilution giving a positive result.

3.5. Results:

Determination of the protein content of locust antigen using the folin phenol method:

Table VI shows the optical densities of the standard versatol preparation (containing 7.2 gm/100 ml), and the locust antigen extract. The graph showing the relationship between optical density and the protein concentration of various versatol dilutions is shown in Fig. 1.

From this standard line, the protein concentration of the locust antigen extract was determined and was estimated at 180 mg of protein/100 ml of extract. The total volume of the extract was measured at 64 mls. This was equivalent to (64 x 1.8 mg), 115.2 mg of protein per 50 adult locusts or 2.3 mg protein per locust. Leone (1947) estimated a protein

Table V. Method of serial double dilution of locust antigen.

Tube	Dilution	Antigen		Saline solution
A	1/10	0.1 ml	+	0.9 ml
B	1/20	0.5 ml	+	0.5 ml
C	1/40	0.5 ml	+	0.5 ml
D	1/80	0.5 ml	+	0.5 ml
E	1/160	0.5 ml	+	0.5 ml
F	1/320	0.5 ml	+	0.5 ml
G	1/640	0.5 ml	+	0.5 ml
H	1/1280	0.5 ml	+	0.5 ml
I	1/2560	0.5 ml	+	0.5 ml
J	1/5120	0.5 ml	+	0.5 ml
K	1/10240	0.5 ml	+	0.5 ml
L	1/20480	0.5 ml	+	0.5 ml

content of 4 mg/ml for the straight-laced grasshopper and for the Florida lubber grasshopper at 8 mg/ml.

Table VI. Optical densities of versatol dilutions and locust extract.

Dilutions	Optical density at 670mμ
1:10	0.30
1:20	0.25
1:40	0.21
1:80	0.18
1:160	0.14
1:320	0.11
1:640	0.07
1:1280	0.03
Control (0.4 ml distilled water + 2.0 ml Reagent C + 0.2 ml reagent D)	-
Locust Extract	0.22

Determination of the best standing time for the detection of antisera titre:

The results of the titre readings from the precipitin test at different time intervals are shown in Table VII. At lower dilutions of the various protein extracts, the precipitate developed within a short time of 30 min but there were no traces in higher dilutions of the antigen; thus, the greater the dilutions, the longer it took for the 'ring' and precipitate to develop at the interface of antigen and antibody.

The highest dilution at which the precipitate was visible was $^{1}/_{320}$, and this was in rabbit 591 with an injection of 1 mg/ml protein extract. Others were visible at a lower dilution of $^{1}/_{160}$ at $\frac{1}{2}$ h standing period. Of interest, however was the fact that similar titres were obtained ($^{1}/_{160}$) in rabbits 589 with an injection of 10 mg/ml as well as in rabbits 597 and

598 with a much lower protein concentration (0.1 mg/ml). This might be attributable to the response of the animals to the injection of foreign proteins.

Higher titre readings were obtained in three out of the five cases after 1 h standing period. The period of 2 hrs seemed to have provided the maximum time at which precipitates were detectable in the higher antigen dilutions. With the exception of rabbit 590 which had similar titres at 1 h and 2 hrs respectively, all others showed an increase after 2 hours. Injection of 10 mg/ml produced a titre reading of $^{1}/10240$ after 2 hours, four times greater than the reading after 1 hour ($^{1}/2560$). It would therefore appear that the period of 2 hours was the best standing time at which insect antisera titre could be detected.

Table VII. Readings of antisera titre at different time intervals.

Rabbit No.	Protein concentration injected	* Readings after		
		$\frac{1}{2}$ hr	1 hr	2 hrs
589	10 mg/ml	$^{1}/160$	$^{1}/2560$	$^{1}/10240$
590	1 mg/ml	$^{1}/160$	$^{1}/1280$	$^{1}/1280$
591	1 mg/ml	$^{1}/320$	$^{1}/320$	$^{1}/640$
597	0.1 mg/ml	$^{1}/160$	$^{1}/160$	$^{1}/320$
598	0.1 mg/ml	$^{1}/160$	$^{1}/320$	$^{1}/640$

* All readings taken after the 3rd injection of rabbits.

Titre of antisera using different protein concentrations of locust antigen:

Tables VIII and IX show the titres of the antisera, read after the different injection regimes and the degree of the reactions resulting from the injection of different antigen concentrations. Each rabbit received a

total of four injections. From table VIII the rabbits injected with 10 mg/ml antigen, showed similar responses after the second and third injections respectively with a titre of $^{1}/10240$, but after a fourth injection the titre had decreased quite considerably by a factor of four.

Rabbit 590, with 1 mg/ml produced an antiserum with a titre of $^{1}/1280$ after the second injection and died 3 days after the third injection. On the other hand rabbit 591 with a similar protein concentration had a low titre even after the third injection and this low reading of ($^{1}/640$) was remarkably constant after the 2nd, 3rd and 4th injections. There was an increase in titres by a factor of 2 in rabbits 597 and 598 after the 3rd injection ($^{1}/320$) but a decline was shown ($^{1}/160$) as in the case of 597 after the fourth injection while 598 remained constant at the same period. Antibody production is known to diminish with repeated injections of foreign proteins. In order to prepare an insect antisera, 2 or 3 injections of the insect antigen are required for the production of a high titred antiserum.

In table VIII the titres obtained by injecting varying amounts of protein extract (antigen) into rabbits and the degree of the reactions obtained are shown. The reactions were categorised into six, ranging from very strongly positive, represented by (3^{+}) to (-) indicating no reaction. The final titres were obtained after the third injection, except for rabbit 590, whose titre was obtained after the second injection, having died 3 days after the third injection.

In the precipitin test performed, the antisera with 10 mg/ml detected clearly antigen dilutions of $^{1}/10240$ with very strong positive (3^{+}) reactions occurring at dilutions of $^{1}/640$. The antisera produced in the two rabbits each with an injection of 1 mg/ml, showed difference in titres.

Rabbit 590 had a titre of $^{1}/1280$ after the second injection greater than that in 591 where the titre was constant at $^{1}/640$ at the various injection regimen. Antisera produced with 0.1 mg/ml in rabbits 597 and 598 detected low antigen dilutions of 1:320 with a trace at 1:640 in rabbit 598. These results indicated that 10 mg/ml of protein extract, gave the highest titre of antiserum. However, considering the fact that this concentration represented a substantial quantity of protein, probably easily obtained from large insects like locusts and large beetles, a much lower protein concentration of 1 mg/ml as shown from the results, capable of producing antisera with titres of about 1:1280 might be adequate for smaller insects such as aphids.

Table VIII. Titre readings after 2nd, 3rd and 4th injection of different protein concentrations.

Rabbit No.	Protein Concentration	Titre readings after 2nd	3rd	4th (Injections)
589	10 mg/ml	$^{1}/10240$	$^{1}/10240$	$^{1}/2560$
* 590	1 mg/ml	$^{1}/1280$	-	-
591	1 mg/ml	$^{1}/640$	$^{1}/640$	$^{1}/640$
597	0.1 mg/ml	$^{1}/160$	$^{1}/320$	$^{1}/160$
598	0.1 mg/ml	$^{1}/320$	$^{1}/640$	$^{1}/640$

* Rabbit died 3 days after 3rd injection.

Specificity of locust antiserum:

The specificity of the anti-locust serum was tested against a range of dilutions of insect extracts. All the extracts, except locust antigen, were already available in the Immunology laboratory, some prepared as far

Table IX. Degree of reaction of antisera produced from the injection of different protein concentrations and serial double dilutions of locust antigen. (Based on readings after 3rd injection of rabbits except rabbit 590 tested after 2nd injection).

Rabbit No.	Protein Concentrations injected	Serial double dilutions of antigen										
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240
589	10 mg/ml	3+	3+	3+	3+	3+	3+	3+	2+	2+	+	+
590	1 mg/ml	3+	3+	3+	3+	2+	2+	+	±			
591	1 mg/ml	3+	3+	3+	3+	2+	+	+	-	-	-	-
597	0.1 mg/ml	3+	3+	2+	2+	+	+	-	-	-	-	-
598	0.1 mg/ml	3+	3+	2+	2+	+	+	tra	-	-	-	-

3+ Very strongly positive 2+ Strongly positive + Positive ± Weakly positive

tra Trace - Negative reaction

back as 1972 by other research students. The extracts were standardized to a concentration of 1 mg of protein/ml of extract. The results of the tests are shown in Table X. Positive reactions were obtained for the Aphididae tested with titres of 1:20 for Nettle aphid and 1:40 for A. fabae. The titre of the leafhopper extract was 1:80. There was no cross-reaction of the anti-locust serum with extracts of the cabbage moth, Delphacidae and D. melonagaster respectively. The results of these specificity tests would suggest that the locust antiserum was not entirely specific. This apparent non-specificity of the antiserum is due to cross-reacting antibodies in the antiserum. However, the sensitivity of the antigen to these insects was much lower than its sensitivity to locust extract. The specificity of an antiserum could be increased by absorption (Weitz, 1952). It could have been possible to prevent the anti-locust serum from reacting to aphid and leafhopper extracts by adding aphid or leafhopper material to the antiserum to precipitate the respective antibodies. This was not done since it would have resulted in the lowering of the sensitivity of the antiserum (Weitz, 1952; Dempster, 1960), however, the degree of cross-reaction seen here is unlikely to affect its use in precipitin reactions.

Table X. Specificity of anti-locust serum to homologous and heterologous antigens.

Antigen from	Titre
Orthoptera (Locusta migratoria)	1:10240
Lepidoptera (Pieris brassica)	0
Diptera (Drosophila melonagaster)	0
Cicadellidae (Leafhopper)	1:80
Delphacidae (Several sp.)	0
Aphididae (Aphis fabae)	1:40
Aphididae (Nettle aphid)	1:20

3.6. Discussion:

The primary objective of this section was to determine the concentration of protein extract (antigen) necessary to produce maximum specific titres, by immunizing rabbits with different protein concentrations. Arbitrary amounts of antigen (protein), but not quantified, have been used by various investigators. Without quantitative data, there would always be the tendency for investigators to use too little or excess amounts of protein with the consequent decline of the antibody titre obtained and/or tolerance of the sensitized animal. The different protein concentrations used in the tests for inoculating the different rabbits, showed differences in the final titres, but 10 mg/ml protein concentration was shown to give the maximum titre of antiserum at an incubation time of 2 h. In the two rabbits immunized with 1 mg/ml, there appeared to be a difference in antibody response. Similar results were obtained with rabbits immunized with 0.1 mg/ml protein extract. These differences which did not appear to be very significant, could be explained from the fact that different rabbits, differ in their capacity to produce antibodies (Brand, 1965; Dempster, 1960; Downe, 1962; Pickavance, 1970). The specificity and avidity of an antiserum is characteristic of the individual rabbit in which the antiserum is produced.

Titova (1970) immunized rabbits intravenously through the marginal ear vein with Eurygaster integriceps extracts of protein content of 22-26 mg/ml. The precipitin titre of the serum obtained against E. integriceps extract by the ring test was said to be 1:1000, while in agar-gel precipitin test, it was 1:400. Thus, the maximum dilution of antigen yielding a reaction in her experiment was estimated at 0.025 mg protein/ml of extract calculating on the basis of 25 mg protein/ml of extract injected. Similarly Ashby, (1974) sensitized rabbits subcutaneously by injecting

34.7 mg of Pieris rapae protein in 2ml of saline at 14 days intervals. This was followed by a larger dose of 69.5 mg protein/ml of saline mixed with 2 mls of Freunds complete adjuvant. Antiserum titre was said to be greater than 1:1600. Calculations based on similar lines as those of Titova's showed that the maximum dilution of Pieris rapae antigen giving a reaction ranged approximately from 0.01 - 0.04 mg protein/ml of extract.

On the basis of these results, it was calculated that the maximum dilution of locust antigen capable of giving a reaction in this experiment was 0.001 mg/ml based on 10 mg of locust protein/ml injected into the rabbit through the lymph nodes. This figure represents a significantly lower figure than those of Titova or Ashby. This difference might in fact be due to different routes of immunization employed. Lymph node injection as applied in this test has been found to produce an antiserum of higher titre than antisera obtained either through intravenous or subcutaneous injection (Solomon, 1973). This could be attributed to the fact that lymph node injection ensures that all the antigen (protein) gets to the site of antibody production, while other routes do not. Subcutaneous and intramuscular injections require more antigen material to produce the same titre as that produced through lymph node injection, the latter method requires approximately 10% of the antigen material used by the former methods. It could also be speculated that the low titre figures obtained from the quoted experiments could have resulted from high concentrations of protein extract used for immunizing rabbits, a situation likely to depress antibody formation in the animals.

Section IIB

A latex agglutination test for evaluating insect prey-predator systems

Introduction

The objective of this study was to develop a simple technique applicable under field conditions for the identification of insect predators. The feasibility of adapting the latex agglutination method which has been developed for use in forensic science, for the identification of the origin of blood stains and for other diagnostic purposes already enumerated, was investigated. The test essentially involves the sensitization of biologically inert particles such as polystyrene latex or Bentonite with immuno-globulins directed against the antigen to be studied. The investigation as to the suitability of the method was initially done using, Locusta migratoria, as the test insect. The latex particles were coated with immuno-globulins (IgG), separated from the locust antiserum and this sensitized latex was then tested against serial doubling dilutions of the locust antigen. The method of actually performing the test is described later.

Further investigations were carried out using an aphid/coccinellid system, whereby the presence of the aphid (Acyrthosiphon pisum) - pea aphid, fed to (Coccinella septempunctata) (Coccinellidae) in the laboratory was detected in the guts of these coccinellids. The latex agglutination test appeared not only suitable for identifying predators, but was equally satisfactory as a means of detecting how long the meal of aphids remained detectable in the gut of the coccinellids.

The schematic representation of the process involved is shown in fig. 2, as well as the method of coating the latex particles with the separated IgG which is shown in fig. 3.

4. Materials and Methods

4.1. Preparation of Locust Antiserum:

The preparation of locust antiserum has already been described in Section IIA.

4.2. Separation of Immuno-Globulin (IgG) from Locust Antiserum by the Caprylic Acid Method:

The method used for separating IgG from the locust antiserum was a modification of the method described by Steinbuch and Audran (1969).

The reagents used consist of the following:

(i) 0.1 M Glycine-saline buffer pH 8.2. This was prepared by weighing out 7.5 g Glycine, 8.5 g sodium chloride and 1.0 g sodium azide. These were dissolved in distilled water and made up to 1 litre. The pH was adjusted to 8.2 with 0.1 N Sodium hydroxide.

(ii) Acetate buffer pH 4.0. This was made up in the ratio of 8 volumes of 0.06 M acetic acid and 2 volumes of 0.06 M sodium acetate.

Twenty ml of acetate buffer pH 4.0 was added to 10 ml of anti-locust serum at 20°C. 0.74 ml Caprylic acid (Octanoic acid) (Sigma Chemical Company) was added dropwise and vigorously stirred for 30 min. The resulting mixture was centrifuged at 2000 r.p.m. for 20 min, the supernatent being removed and dialysed against 0.1 M glycine-saline buffer pH 8.2 for 24 h at 4°C.

4.3. Protein Measurement of Separated IgG by the Folin Phenol Method:

The method adopted was similar to that used in determining protein concentration of locust extract already described (Section IIA, 3.2). The protein concentration of the separated IgG was estimated at 1.575 mg/ml.

4.4. Sensitization of Latex with Separated IgG:

A 5% suspension of latex (BDH Chemicals) was prepared by diluting the 40% suspension of polystyrene latex with 0.1 M glycine saline buffer; pH 8.2. The fixing of the IgG to the latex particles was done by adding 0.2 ml, 5% latex to 0.8 ml of IgG solution, whose optimum dilution has been determined in glycine-saline buffer. The mixture was heated in a water bath at 56°C for 30 min. To this mixture was added 2 drops (0.06 ml) of 2% Bovine serum albumin (B.S.A.) to stabilize the latex particles, and the solution thoroughly mixed in a whirl mixer, and allowed to cool before testing against doubling dilutions of locust antigen.

4.5. Determination of the Optimum Concentration of IgG for Latex Fixation:

Serial doubling dilutions of the IgG solution was prepared ranging from neat IgG to dilutions of $^{1}/_{32}$. These various IgG dilutions were fixed to 5% polystyrene latex in the manner previously described. The sensitized latexes and control were then tested against serial doubling dilutions of the locust antigen to determine which dilution gave the best agglutination, and this dilution was subsequently used in all tests.

4.6. Testing Procedure:

The apparatus for testing consisted of a glass slide 7 cm x 7 cm and a small pasteur pipette. Reagents were transferred to the glass slide by means of the pipette. One drop of the sensitized latex approximately (0.03 ml) was placed onto the clean slide and a drop of the approximate antigen dilution was added to it. The drops on the slide were mixed with a clean glass rod and the slide gently rocked from side to side to obtain maximum mixing. The slide was then left on top of some black paper to provide a black background for the easy detection of the agglutinated material. Positive reaction occurred within 1-2 min. If no reaction occurred within this time, the test was regarded as negative. The reactions

were observed and graded depending on the degree of agglutination that had taken place. The reactions were graded negative if no visible clumping was observed and the fluid was definitely milky. (1^+) represented small clumping, (2^+) fairly large clumping with an opague background; while (3^+) represented large clumping with clear background. In all cases controls using sensitized latex and 0.1 M glycine saline buffer pH 8.2 were set up (Plate 1).

4.7. Specificity of Sensitized Latex to Homologous and Heterologous Sera:

The sensitized latex was tested against a range of insect antigens already available in the Immunology laboratory. Most of these antigens have been in storage for over 4 years. Serial doubling dilutions of the heterologous antigens were made and these were tested against the IgG sensitized latex. A comparison of the results with those of the precipitin test was made.

4.8. Effect of Storage on Sensitized Latex:

The latex particles were sensitized with the IgG as earlier described and initially tested against locust antigen; with serial doubling dilutions ranging from $^1/_{10}$ to $^1/_{20480}$. The sensitized latex was then split into 4 portions and stored in small bottles at different temperatures viz 4^oC, room temperature, -20^oC and in an incubator regulated at 37^oC. Storage was done over a period of 5 months and tests carried out at time intervals indicated in Table XIII to detect the best storage temperature.

4.9. Results:

Determination of the optimum dilution for latex fixation:

Table XI shows the reaction of the different dilutions of the separated IgG against serial doubling dilutions of the locust antigen, ranging from Neat IgG to 1:32, to indicate the optimum dilution which would

result in the best agglutination. The neat IgG had a negative reaction at 1:10 dilution of antigen probably due to prozone effect (Gray, 1970). A positive (2^+) to strongly positive (3^+) reaction was however obtained at dilutions ranging from 1:20 to 1:160 with only a weak reaction ($\pm$) occurring at 1:1280. Other dilutions of the IgG showed different results, but it would appear that a dilution of 1:2 of the IgG resulted in the best agglutination as indicated in (Table XI). This dilution was therefore subsequently used in all tests. The protein concentration of this concentration of IgG was calculated at 0.8 mg/ml.

A weak positive ($\pm$) reaction was obtained with the homologous locust antigen at a dilution of 1:10240, similar to the results obtained by using the ring test, an indication that this method is as sensitive as the precipitin test. With the heterologous antigens some weak cross-reactions occurred with the leafhopper (Cicadellidae), at a dilution of 1:40, cereal aphid (Aphididae), 1:10, nettle aphid (Aphididae), 1:10 and Aphis fabae (Aphididae) at 1:20.

These results are consistent with those obtained with the ring test, although the later test showed slightly higher heterologous reaction according to Table X, p 52. From the results it would appear that IgG separated from the locust antiserum was not completely specific. Since the cross-reactions observed were weak, no attempt was made to remove them by absorption, as this may have resulted in the lowering of the sensitivity of the antiserum.

Specificity of IgG-fixed latex:

The specificity of the separated IgG was tested against homologous and heterologous antigens and the results are shown in Table XII.

Table XI. Optimum dilution of IgG* required for latex agglutination.

IgG	Dilutions									
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	Control
Neat	-	2^+	3^+	3^+	3^+	1^+	1^+	±		-
1:2	3^+	3^+	3^+	3^+	2^+	2^+	2^+	±		-
1:4	1^+	1^+	1^+	1^+	1^+	1^+	±	±		-
1:8	±	±	-	-	-	-	-	-		-
1:16	±	±	±	-	-	-	-	-		-
1:32	±	-	-	-	-	-	-	-		-

* IgG was separated from the antiserum obtained with rabbit immunized with with 10 mg/ml of locust antigen.

Table XII. Testing the specificity of IgG fixed latex to homologous and heterologous antigens by the latex agglutination method.

Extracts from	Serial doubling dilutions of Antigen												
	C	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10T	1/20T
Lepidoptera (Pieris brassica)	-	-	-	-	-	-	-	-	-	-	-	-	-
Orthoptera (Locusta migratoria)	-	2+	3+	3+	3+	2+	2+	2+	2+	+	+	±	-
Aphididae (Cereal Aphid)	-	±	-	-	-	-	-	-	-	-	-	-	-
Aphididae (Nettle Aphid)	-	±	-	-	-	-	-	-	-	-	-	-	-
Aphididae (Aphis fabae)	-	±	±	-	-	-	-	-	-	-	-	-	-
Aphididae (Microlophium evansi)	-	-	-	-	-	-	-	-	-	-	-	-	-
Diptera (Drosophila melonagaster)	-	-	-	-	-	-	-	-	-	-	-	-	-
Cicadellidae (Leafhopper)	-	-	-	-	-	-	-	-	-	-	-	-	-
Cicadellidae (Graphocephala coccinea)	-	-	-	-	-	-	-	-	-	-	-	-	-

C = Control (Sensitized latex + glycine buffer pH 8.2)

Dilution of IgG = 1:2

Effect of storage at different temperatures on sensitized latex:

The result of the effect of storing sensitized latex at different temperatures is shown in Appendix I and summarized in Table XIII. An initial test of the anti-locust latex with serial doubling dilutions of homologous antigen gave weak positive reaction at a dilution of 1:10240. The IgG used was that derived from the antiserum obtained through the immunization of a rabbit with 10 mg/ml of locust extract. The storage extended for about 5 months. During this period room temperature varied from 20°C to 31°C with the highest room temperatures (25-31°C) occurring in the month of July.

During each test negative and positive controls were set up consisting of sensitized latex and glycine buffer pH 8.2 (negative control), and sensitized latex and locust antigen (positive control). For the first five days of storage, there appeared to be no significant change in the sensitivity of the latex at the different temperatures, but after the 12th day, there was a remarkable decrease in sensitivity especially at 37°C (1:640) and -20°C (1:320) respectively. Latex stored at room temperature and 4°C were less drastically affected. After a month storage period, the sensitized latex except that stored at 4°C showed considerable decline in sensitivity. Loss in sensitivity was detected in the sensitized latex at 4°C after 2 and 3 months (1:1280) and after 5 months storage, the sensitivity declined to (1:640) more than 10 times its original sensitivity. However for the first three months of storage, there was no indication of complete loss of sensitivity in the sensitized latex stored at the different temperatures.

Table XIII. Length of storage and sensitivity of latex at different temperatures.

Temp. °C	Sensitivity of Latex after								
	1	5	12	19	31	61	91	150	DAYS
4°	1:10240	1:10240	1:5120	1:5120	1:5120	1:1280	1:1280	1:640	
Room Temp.	1:5120	1:5120	1:1280	1:640	1:320	1:80	1:80	1:40	
37°	1:5120	1:1280	1:640	1:320	1:80	1:20	1:20	-	
-20°	1:5120	1:1280	1:320	1:80	1:20	1:10	1:10	-	

The identification of antibody-antigen reaction by the latex agglutination method using the larvae of the Lepidopterous insect (Vanessa io) - Peacock butterfly

In order to test the reliability of the results obtained in Section IIA, further tests were performed with extracts from the larvae of the Peacock butterfly (Vanessa io).

The extract was standardized to a protein concentration of 10 mg/ml and the titre of the resulting antiserum tested by both the latex agglutination test and the precipitin test.

5. Materials and Methods

5.1. Preparation of Antigen:

About 160 last instar larvae of the Peacock butterfly (Vanessa io) were collected from the common stinging nettle on the 6th July 1976, outside the Immunology laboratory at Silwood Park. These were placed in a plastic container and starved for 48 hours, to remove most of the food from the guts, which might affect the test.

They were then transferred into a bottle and killed by deep-freezing at -20°C. The caterpillars were cut up with scissors and carefully ground with saline in a pestle and mortar.

An extract was prepared as described in Section IIA. The total volume of extract obtained was 60 mls. This extract was freeze-dried and stored in a deep freezℓ(-40°C) until required for use. The protein concentration of the antigen was determined by the folin-phenol method.

5.2. Antiserum Preparation:

The protein concentration of the extract was estimated at 2.25 mg/ml, thus a total antigen extract of 60 mls will contain 135 mg (60 x 2.25 mg) of Vanessa protein. The freeze-dried material was reconstituted with 13.5 ml of distilled water to give a protein concentration of 10 mg/ml, which was used for the preparation of the antiserum. Two rabbits labelled (616, 617) each weighing 3-4 kg were used for the antisera production.

Two injections of 1 ml each were given at 7 day intervals by the lymph node route (Section IIA). The sensitivity of the anti-Vanessa serum was tested initially by the ring precipitin test. The titre obtained was 1:10240 for each of the rabbits used. As the titre was considered satisfactory, no further injections were administered. 50 ml of blood was taken from each rabbit and the sera containing antibodies were separated as already described. As the titres were similar the anti-sera were pooled together and used for the various tests carried out.

IgG was separated from the Vanessa antiserum by the Caprylic acid method (page 56) and the sensitization of the polystyrene latex with the separated IgG carried out in the manner already described. An IgG dilution of 1:2 was used to sensitise the latex.

The sensitivity of the anti-Vanessa latex was tested against serial doubling dilutions of Vanessa antigen, ranging from 1:10 to 1:20480. The procedure for carrying out this test has already been described (page 57). Results obtained were compared with those from the precipitin test.

5.3. Results:

Sensitivity of anti-Vanessa serum:

The degree of the reaction between the anti-Vanessa serum and twelve doubling dilutions of the antigen ranging from 1:10 to 1:20480 is shown in

Table XIV. Testing of the sensitivity by the latex agglutination method was carried out after the separation of the immunoglobulin from the anti-Vanessa serum and the sensitisation of the latex with the separated IgG. A negative control consisting of sensitized latex and glycine saline buffer pH 8.2 was also set up.

Positive reactions were detected in each case up to a dilution of 1:10240. The weak reaction obtained at 1:10 dilution of the antigen is due to prozone action (Gray, 1970).

Specificity of anti-Vanessa latex:

The specificity of the anti-Vanessa latex and anti-Vanessa serum was tested against a range of insect antigens as indicated in Table XVIII, by the two methods. All extracts were standardized to a protein concentration of 1 mg/ml.

There was no cross reaction with extracts of Locusta migratoria, members of the Aphididae, as well as D. melonagaster and leafhopper (Cicadellidae) used. There was however a slight cross reaction with the cabbage moth (Pieris brassica) extract, and titres of 1:40 and 1:80 were detected by the latex and ring capillary test respectively. This cross-reactivity may be attributed to the phylogenetic proximity of Pieris brassica to this butterfly (Vanessa io).

Table XIV. Anti-Vanessa titre as determined by the precipitin and latex agglutination tests.

Rabbit No.	Serial doubling dilutions of antigen											
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240	1/20480
616	±	3+	3+	3+	3+	3+	3+	3+	2+	+	±	-
617	±	3+	3+	3+	3+	3+	3+	3+	+	+	±	-
By latex agglutination test	±	2+	3+	3+	3+	3+	3+	2+	2+	+	±	-
Control (Sensitized latex & glycine-saline buffer pH 8.2)	-	-	-	-	-	-	-	-	-	-	-	-

Optimum Dilution of IgG 1:2

Table XV. Specificity to homologous and heterologous antigens as determined by the latex test and ring capillary test.

Antigens from	* L.A.T.	Ring test
Lepidoptera (Vanessa io)	1:10240	1:10240
Lepidoptera (Pieris brassica)	1:40	1:80
Orthoptera (Locusta migratoria)	0	0
Diptera (Drosophila melonagaster)	0	0
Cicadellidae (Leafhopper)	0	0
Aphididae (Aphis fabae)	0	0
Aphididae (Microlophium evansi)	0	0
Aphididae (Acyrthosiphon pisum)	0	0
Aphididae (Ceral aphid)	0	0

* L.A.T. = Latex agglutination test.

Discussion

The sensitization of latex with IgG at a dilution of 1:2 appeared to give the maximum agglutination with locust antigen. The protein concentration of the separated IgG estimated at 1.575 mg/ml corroborates with a figure of 1-2 mg/ml given by Cayzar and Whitehead (1973), as the optimum concentration of globulin. This protein concentration of the IgG would probably depend on the quality of the antiserum used for the separation and also the purity of the separated IgG. The sensitivity of the anti-locust latex (1:10240) as determined by the latex test was similar to that obtained by the precipitin ring test. Weak cross reactions were observed between the anti-locust latex and various unrelated extracts. These results were equally confirmed by the capillary ring test, but the extent of the cross reactivity with the heterologous antigens was better shown with the latter method. This could be due to the difficulty in interpreting results of the latex test especially at high dilutions of extracts (antigen) rather than to the difference in the effectiveness of the two methods. It is therefore suggested that the capillary ring method may not necessarily be more sensitive than the latex agglutination method.

By innoculating rabbits with extracts of Vanessa larvae at a concentration of 10 mg/ml, an anti-Vanessa serum with a titre of 1:10240 was detected by both the latex agglutination and the precipitin tests. The antiserum and anti-Vanessa latex were equally specific. The results of the tests are not only consistent with earlier results obtained by using similar protein concentrations of Locusta migratoria extract in producing a high-titred specific locust antiserum but also clearly showed the latex agglutination method as an effective test for identifying antigen-antibody reactions.

Results of storage tests carried out showed that even though there was some loss in sensitivity at 4°C after 5 months of storage, it was clearly the best storage temperature for sensitized latex compared with storage at room temperature, 37°C and -20°C respectively. Room temperature could be used for short periods if a referigerator were not available but the reagents should be kept as cool as possible and not in direct sunlight. It is recommended that new preparation be made every month and stored at 4°C but not frozen, since the results indicate some measure of deterioration even at 4°C.

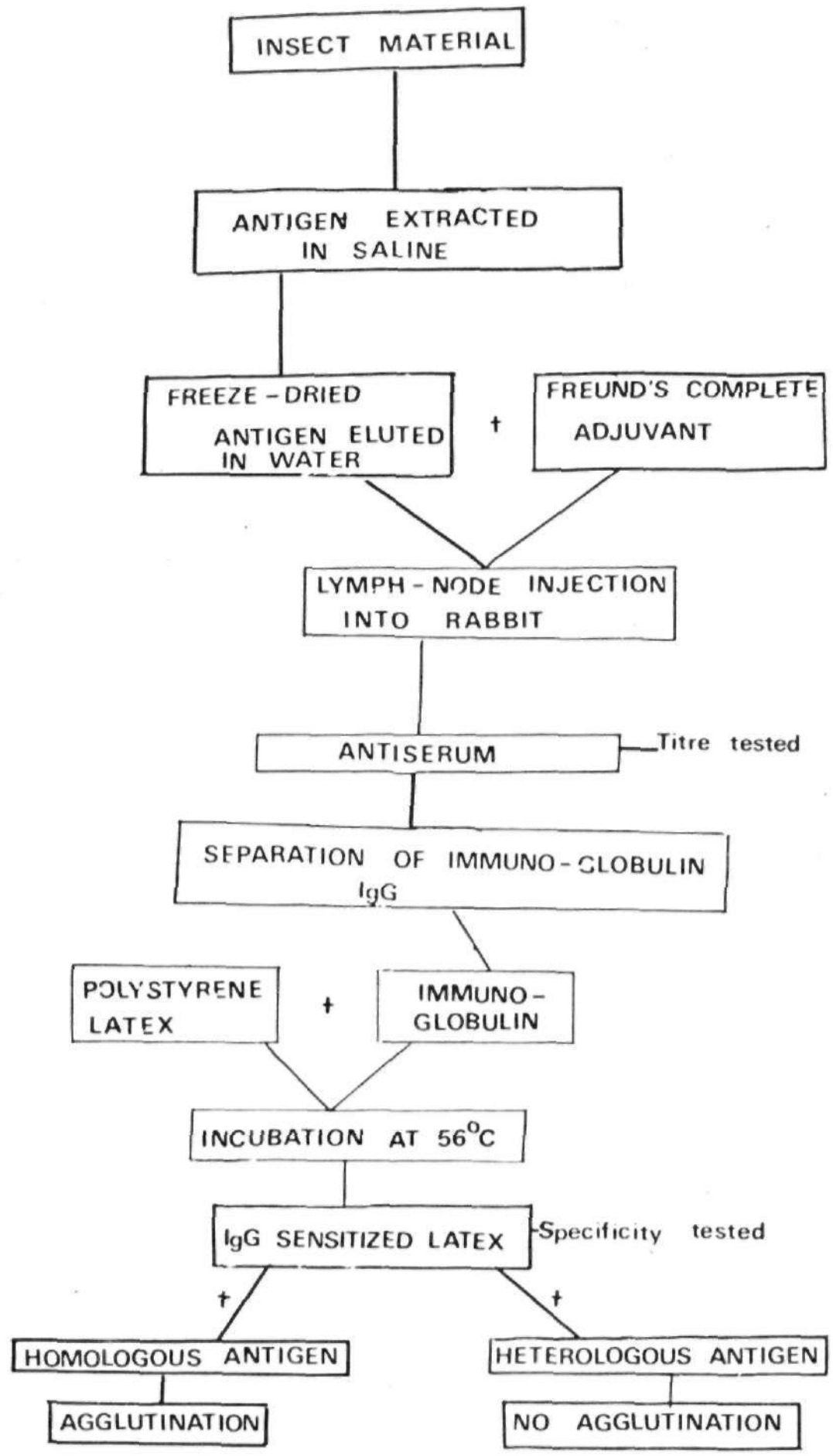


Fig.2 SCHEMATIC REPRESENTATION OF THE LATEX AGGLUTINATION METHOD FOR PREY-PREDATOR STUDIES

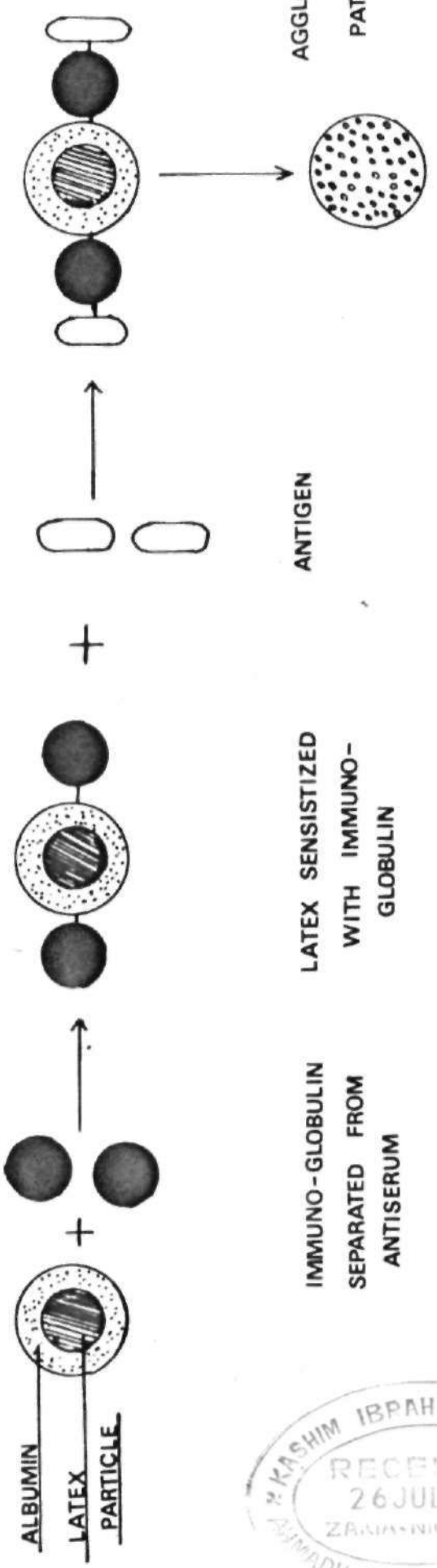


Fig. 3. DIAGRAM OF THE LATEX AGGLUTINATION TEST

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Section IIC

Application of the latex agglutination technique in detecting the presence of the meal of the pea aphid (Acyrthosiphon pisum) in the gut of the predator - Coccinella septempunctata

6. Materials and Methods

6.1. Establishment of A. pisum Culture and Preparation of Antigen:

A. pisum adults were obtained from laboratory cultures in the Insect Behaviour Laboratory in Ashurst Lodge of the Field Station, and introduced into five porcelain pots containing bean seedlings. These potted plants were kept in muslin cages in the greenhouse and were watered three times a week. More porcelain pots were sown with beans and infested by transfering some adult aphids from infested plants by means of a camel hair brush. In all, a total of 40 pots used in culturing a large number of aphids required for the preparation of the antigen.

As each pot became heavily populated with aphids, all the bean plants in it were severed from the base with scissors and all the aphids were shaken free from the plants into a plastic container. The aphids were then trasferred into glass tubes, killed by deep freezing and stored. All the aphids so collected, were pooled, crushed in pestle and mortar with normal saline and transferred to a Buckner flask where it was mechanically stirred for 24 h at 4°C. The mixture was centrifuged and filtered through a Seitz E.K. sterilizing filter pad.

The clear sterile antigen was then dispensed in 1 ml vials and stored in a deep freeze at -40°C until needed for use. The protein content of the A. pisum antigen was determined by the folin phenol method as described

previously, and this was estimated at 0.9 mg of protein/ml.

6.2. Preparation and Testing of the Specificity of A. pisum Antiserum:

The antiserum was prepared as described in Section IIA, by injecting 1 ml of antigen (0.9 mg/ml protein) into the lymph nodes of rabbits with Freunds complete adjuvant.

The titre after two injections was unsatisfactory, and so two further injections were administered during a period of 49 days. The sensitivity of the antiserum was finally tested after the 4th injection by the capillary ring test in the first instance, and later, after the separation of the IgG from the antiserum, by the latex agglutination method. The specificity of the antiserum was tested against a range of dilutions of homologous and heterologous antigens by the capillary ring test.

6.3. Separation of IgG from Aphid Antiserum, Sensitization of Latex and Testing Specificity of the Sensitized Latex:

IgG was separated from the aphid antiserum by the Caprylic acid method (Section IIB). The latex particles were sensitized with the separated IgG, using the 'neat' IgG for this purpose, as a result of the weak titre of the antiserum indicated by the ring capillary test. The procedure for sensitization of latex particles was similar to that used for anti-locust latex fixation. The specificity of the sensitized latex was finally tested against a range of insect extracts.

6.4. Testing the Sensitivity of the Antiaphid Latex:

Serial doubling dilutions of the A. pisum antigen were made ranging from 1:10 to 1:1280 (Table V). One drop of the sensitized latex (0.03 ml) was placed on a clean slide followed by a drop of the antigen dilution. This was mixed thoroughly and the slide rocked from side to side to ensure thorough mixing of the reagents. Results of the reactions were graded and

recorded after 1-2 minutes incubation period.

6.5. Collection and Feeding of Coccinellids with A. pisum and Preparation of Smears:

A collection was made of the 7-spotted coccinellids (Coccinella septempunctata) from several sites on the field station, particularly from the experimental plots located at the 'Silwood Bottoms'. A total of about 200 adult coccinellids were collected and starved for 24 h. These were kept in batches of 25 in tubes covered at the open end with cheese cloth. The potted plants with A. pisum colonies were taken into the laboratory and enclosed in a muslin cage measuring 30 cm x 30 x 30 cm. One porcelain pot with aphid colonies was used to feed a batch of 25 coccinellids in order to ensure that all the predators had a chance of coming in contact with an aphid. The feeding time allowed for the purpose of this experiment was 1 h. The activity of the released predators was closely observed and as soon as an aphid was consumed, the coccinellid was removed from the cage and bottled. No attempt was made to separate the aphids into different instars and as such different instars of the aphid were eaten by the coccinellids.

After the expiration of the feeding time allowed, each batch of coccinellids was kept in a closed container and digestion allowed to proceed at room temperature (20-25°C) at predetermined time intervals (2, 4, 6, 8, 16, 24, 48 and 72 hrs) respectively. At the end of this period, the predators were killed by deep freezing at -20°C. The gut contents were removed and smeared onto filter papers. These smears were removed, labelled, dried and stored in a desiccator over silica-gel until needed for use.

6.6. Testing Procedure by the Precipitin and Latex Agglutination Methods:

The smears were cut from the filter papers and placed in 1 ml plastic centrifuge tubes, with 0.2 ml of normal saline solution. The smears were

allowed to soak for 24 h at 4°C and were then centrifuged. The clear supernatant fluid was then used for testing. Digestion time was determined by two methods - the capillary ring test and the latex agglutination test (see Sections IIA and IIB).

6.7. Results:

Determination of the sensitivity of the antiaphid serum by the capillary ring and latex agglutination tests:

The sensitivity of the antiaphid serum and the antiaphid latex was determined after each injection regimen as indicated in table XVI. A low titre was detected in each case (1:160, ring test) and (1:80, latex test). Results indicate no significant difference in titres during the different injection periods, confirming the earlier indication that 2 or 3 injections were sufficient for obtaining maximum titres through the lymph node method.

Table XVI. *Sensitivity after different injection periods.*

Injection Period	Titre determined by	
	Ring test	Latex agglutination test
After 2nd injection	1:80	1:80
After 3rd injection	1:160	1:80
After 4th injection	1:160	1:80

Specificity of antiserum determined by the latex agglutination test and the capillary ring test:

The specificity of the antiserum and the sensitized latex was tested by both methods using a range of dilutions of homologous and heterologous antigens. The results are shown in Table XVII. Weak cross reactions were detected by both methods for some members of the Aphididae namely, Nettle aphid and Cereal Aphid respectively. However, the weak cross reaction that

was detected by the ring test in the case of A. fabae was not shown by the latex test. No cross reaction was detected by both methods in any of the unrelated extracts tested such as Locusta migratoria, leafhopper, Delphacidae extract and D. melonagaster.

Table XVII. Showing reactions to homologous and heterologous antigens.

Antigens	Sensitivity	
	Capillary ring test	Latex agglutination test
Aphididae (Acyrothosiphon pisum)	1:160	1:80
Aphididae (Aphis fabae)	1:10	0
Aphididae (Nettle Aphid)	1:10	1:10
Aphididae (Cereal Aphid)	1:10	1:10
Orthoptera (Locusta migratoria)	0	0
Diptera (Drosophila melonagaster)	0	0
Delphacidae (Several species)	0	0
Cicadellidae (Leafhopper)	0	0

Determination of the digestion time of Coccinellids following feeding on A. pisum as determined by the precipitin and latex agglutination methods:

The number of C. septempunctata tested for the presence of A. pisum meal after a given digestion time, and the percentage positive response obtained by both methods are shown in Table XVIII. From the results, the latex test compared quite favourably with the ring test in detecting the presence of A. pisum in the gut of the predator. The tests indicated that approximately 80% of the prey material remained detectable after 8 hrs but subsequently declined. After 24 hours, less than half of the predators tested reacted positively. However, 25% of the predators tested showed positive results after 48 hours but there was no indication of the presence of the aphid meal after 72 hours. It appears therefore, that a meal of

pea aphids would remain detectable in the gut of the 7-spotted coccinellids for about 48 hours. The results are comparable with those of similar tests carried out on different insect species by various investigators (Table I).

Table XVIII. Number of coccinellids tested, hours of digestion and % positive reactions.

Digestion time (hrs)	2	4	6	8	16	24	48	72
No. of Coccinellids tested	25	25	25	25	25	25	25	25
% Positive by the ring test	88.3	85	82	77	62.5	45.2	25	0
% Positive by the latex test	81.8	82.3	80.8	71.3	60	45	25	0

6.8. Discussion:

The antiserum with a titre of 1:160 used for the tests was weaker than all other antisera titres produced in the course of the investigations. This could be attributed partly to the equally weak extract (0.9 mg/ml) used for inoculation of the rabbits and partly on the immune response of the animal. However, the antiserum and the sensitized latex showed some measure of specificity. The weak cross reactions which occurred with Nettle and Cereal aphids as well as A. fabae were not surprising since all belong to the Aphididae. No attempt was made in removing the cross reactivity, as this would have further depressed the sensitivity of the antiserum. Although the aphid antiserum was weak, it was still useful in detecting the presence of aphid meal in the gut of the coccinellids.

The time that the meal of a prey remains detectable in the gut of a predator depends on the size of the meal, the predator itself, as well as environmental factors especially temperature. The results indicated that 80% of the coccinellids showed positive reactions after 8 hours of digestion. Since the predators were allowed a free choice of different instars of the aphid to feed on, the 23% that showed negative reaction within this period

(8 h) could either have not fed at all, which was most unlikely, or fed on younger instars, which could have eluded detection. Loughton and West (1962) found that the detection time was much shorter in spiders which had fed on younger larvae of the spruce budworm, than those which had eaten later instars and adults. It is therefore suggested that difference in percentage positives obtained from the results at different times, could be a reflection of the low sensitivity of the antiserum and the differences in the rate of feeding of the predators on the individual aphids.

GENERAL DISCUSSION

The thesis is divided into two major parts. The first section deals with a review of the serological techniques which have been applied by several investigators in the study of prey-predator relationships as well as the practical implications of such studies. The second part is the experimental section which is further divided into three sections (see Index pages 4-6). Discussions on individuals experiments have been included in each section but a few points need to be reiterated.

1. One of the most serious problems in serological investigations has been the production of antisera of high sensitivity and specificity, without which identifications of most predatory species would be extremely difficult. Hall et al (1953), showed that for prey-predator studies, antisera titres of at least 1:5000 would be required. Since insect proteins are known to have poor antigenicity (O'Rourke, 1958), it is necessary to investigate and quantify the amount of protein, which when injected into an animal, usually a rabbit, would yield a high-titred antiserum. Such a test was carried out using different concentrations of locust antigen to immunize rabbits (Table VIII).

A test of the sensitivity of the resulting antisera showed that the locust extract with protein content of 10 mg/ml was satisfactory, yielding a homologous titre of 1:10240. Similar titre readings were obtained when the experiment was repeated with the antigen of the Peacock butterfly (Vanessa io) at a concentration of 10 mg of protein/1 ml of extract. The antiserum produced was in addition highly specific.

Insect proteins of higher concentrations than those reported in these experiments have been used by some investigators but the antisera titres obtained were not particularly high. For example, Titova (1970) used

approximately 25 mg/ml of Eurygaster integriceps (Put.) antigen in immunizing rabbits and obtained an antiserum titre of about 1:1000 by the ring test and only 1:400 with the agar-gel precipitin test. Ashby (1974), used an even higher concentration of Pieris rapae antigen (34.7 mg/2 ml in the first injection, increasing to 69.5 mg/ml in the second). The final antiserum titre obtained was under 1:2000. These results are comparable with antiserum titre of (1:1280) obtained in this study with considerably lower protein concentration of 1 mg/ml (Table VIII). However, the production of a high-titred antiserum appears to depend not only on the level of protein used for immunization, but also on the immunological features of the animals used which often vary considerably, as well as on the route of injection.

Indications from results on tests with Acyrothosiphon pisum antiserum are that an antiserum of low titre could be used in serological studies provided such an antiserum is specific. For instance, the aphid antiserum was shown to have a low titre of 1:160 with the ring test and 1:80 with the latex agglutination method. This antiserum was specific. Such a low titred antiserum was useful in detecting the presence of Acyrothosiphon pisum antigen in gut smears of the predator Coccinella septempunctata, and the digestion time was estimated at 48 hours. This point is supported by the evidence of Hall and West (1954), who found that antisera of very low titre but of high avidity (1:100) was capable of detecting mosquito antigen in smears of predators.

2. The use of the latex agglutination method in prey-predator studies has not been previously reported, although this has been used extensively in medicine. The work outlined here indicates that the technique has advantages compared with other methods currently being used in evaluating prey-predator relationships. The technique is simple and once the reagents

have been prepared, a large number of tests can be performed in a day. Results of the storage tests at different temperature regimen indicate that sensitized latex stored at 4°C could retain sensitivity for at least 5 months. This is an added advantage, since the tests could be performed in a laboratory under standard conditions and taken into the field as necessary. Bercks and Querfurth (1969) noted that sensitized latex was stable for at least 3 years when stored in a refrigerator. There was however some deterioration in the sensitivity of the sensitized latex prepared in this experiment after 1 month of storage, probably because the latex particles were not tightly bound. Certainly, this is an area where care has to be taken when preparing the reagents. The sensitivity of the test is comparable with the ring test. There is also the advantage of economy of reagents, cheap and readily available equipment for the performance of the test and especially the speed of reaction.

Evidence from the results of the experiments carried out with this method indicates that the test could be employed as a time-saving field device in which large numbers of predators could be rapidly screened.

SUMMARY

1. A review of the serological techniques used in invertebrate prey-predator studies, especially the precipitin test, and the practical applications of such techniques are discussed. The actual procedure for the performance of the precipitin test including the preparation of antigen and antiserum are described. Other potential serological techniques which have been successfully used in bloodmeal identification, but have not so far been applied in insect prey-predator studies are also mentioned.

2. An experiment was conducted to quantify the amount of insect protein (antigen), which, when injected into an animal, would produce an antiserum with a titre of high sensitivity and specificity, since most serological identifications depend on this factor. This was found to be 10 mg/ml although a satisfactory antiserum could be produced with 1 mg/ml.

3. The technique of the latex agglutination was investigated to assess its potential for detecting invertebrate predators under field conditions. A comparison was made between this method and the standard precipitin test in common use (ring capillary test). Results indicated that this technique was not only as sensitive as the ring test in detecting antigen-antibody reactions but also showed superiority over the ring test in terms of speed of reaction, economy of reagents and simplicity of operation.

4. The sensitized latex used was effective for at least 5 months when stored at $4^{\circ}C$ although some loss of sensitivity was indicated after 1 month storage. It might therefore be advisable to prepare fresh material after each month of storage to ensure that the material being used was still of high sensitivity.

5. The latex agglutination technique was applied in detecting the presence of a meal of pea aphids (Acyrthosiphon pisum), in the gut of the predator - Coccinella septempunctata (Coccinellidae), artificially fed in the laboratory. Results indicated that the meal of pea aphids remained detectable in the guts of these coccinellids for at least 48 hours. This result was confirmed by using the precipitin test.

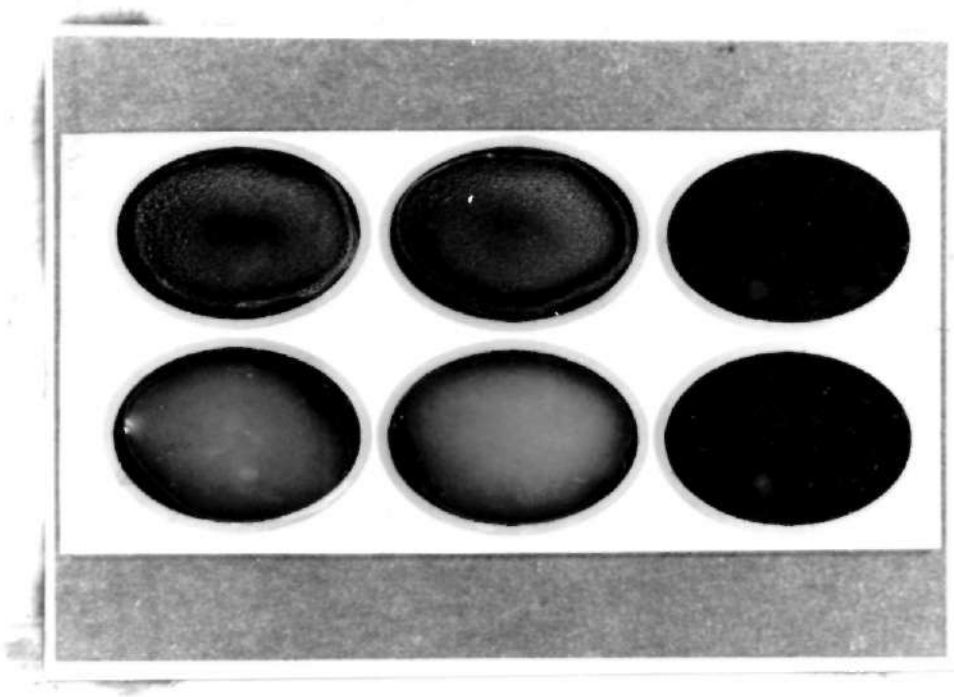


Plate 1. The pattern of agglutination as shown by the latex agglutination test.

1 & 2 - show agglutination (positive reaction)

4 & 5 - control



Plate 2. The automatic serological dispenser for the performance of the precipitin test.

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Appendix 1. Effect of storage at different temperatures on IgG sensitized latex.

Temperatures	Dilutions											
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240	1/20480
Initial test	3+	3+	3+	3+	3+	2+	2+	2+	2+	+	±	-
4°C after 1 day	3+	3+	3+	2+	2+	2+	+	+	+	±	±	-
Room temp. after 1 day (31°C)	3+	3+	2+	2+	2+	2+	+	+	±	±	-	-
37°C after 1 day	2+	2+	2+	2+	+	+	+	±	±	±	-	-
20°C after 1 day	2+	2+	2+	2+	+	+	+	+	±	±	-	-
4°C after 5 days	3+	3+	3+	2+	2+	2+	2+	+	+	±	±	-
Room temp. after 5 days (28°C)	3+	3+	2+	2+	+	+	+	+	±	±	-	-
37°C after 5 days	2+	2+	2+	2+	+	+	+	±	-	-	-	-
-20°C after 5 days	2+	2+	2+	+	+	+	±	±	-	-	-	-

Appendix 1. Continued.

Temperatures	Dilutions											
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240	1/20480
4°C after 12 days	3+	3+	3+	2+	2+	2+	2+	+	+	±	-	-
Room temp. after 12 days (24°C)	3+	3+	2+	2+	2+	+	±	±	-	-	-	-
37°C after 12 days	2+	2+	2+	+	+	±	±	-	-	-	-	-
-20°C after 12 days	2+	2+	+	+	±	±	-	-	-	-	-	-
4°C after 19 days	3+	3+	3+	2+	2+	2+	2+	+	+	±	-	-
Room temp. after 19 days (25°C)	3+	3+	2+	2+	+	+	±	-	-	-	-	-
37°C after 19 days	2+	2+	2+	+	+	±	-	-	-	-	-	-
-20°C after 19 days	2+	2+	+	±	-	-	-	-	-	-	-	-
4°C after 31 days	3+	3+	3+	2+	2+	2+	2+	+	+	±	-	-
Room temp. after 31 days (22°C)	3+	2+	2+	+	+	±	-	-	-	-	-	-
37°C after 31 days	2+	2+	+	±	-	-	-	-	-	-	-	-
-20°C after 31 days	+	±	-	-	-	-	-	-	-	-	-	-

Appendix 1. Continued.

Temperatures	Dilutions											
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240	1/20480
4°C after 61 days	3+	3+	3+	2+	2+	2+	+	±	-	-	-	-
Room temp. after 61 days (21°C)	3+	2+	+	+	-	-	-	-	-	-	-	-
37°C after 61 days	+	±	-	-	-	-	-	-	-	-	-	-
-20°C after 61 days	±	-	-	-	-	-	-	-	-	-	-	-
4°C after 91 days	3+	3+	3+	2+	2+	2+	+	±	-	-	-	-
Room temp after 91 days (21°C)	2+	2+	+	±	-	-	-	-	-	-	-	-
37°C after 91 days	+	±	-	-	-	-	-	-	-	-	-	-
-20°C after 91 days	±	-	-	-	-	-	-	-	-	-	-	-
4°C after 150 days	3+	3+	3+	2+	2+	+	±	-	-	-	-	-
Room temp. after 150 days	3+	+	±	-	-	-	-	-	-	-	-	-
37°C after 150 days	-	-	-	-	-	-	-	-	-	-	-	-
-20°C after 150 days	-	-	-	-	-	-	-	-	-	-	-	-