A SEROLOGICAL METHOD USED IN THE INVESTIGATION OF THE PREDATORS OF THE PUPAL STAGE OF THE WINTER MOTH, *OPEROPHTERA BRUMATA* (L.) (HYDRIOMENIDAE)

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A method is described for producing antibodies to winter moth pupae antigens in experimental rabbits. This method proved useful in estimating the extent of feeding by certain predatory beetles on winter moth pupae when more direct methods were impracticable.

The populations of oak-defoliating Lepidoptera in a study area at Wytham Wood, Berkshire, England have been under investigation for a number of years by Varley and Gradwell (1960, 1963a, 1963b). One of the most abundant of these species is the winter moth, which is in the pupal stage underground between the end of May and the end of November. These pupae suffer a high mortality. Varley and Gradwell (1963a) suggested that the principal causes of this mortality might be moles, mice, and predatory beetles, particularly species of the carabid genera *Pterostichus* and *Abax* and the staphylinid *Philonthus decorus* (Gr.). Laboratory feeding tests (Frank 1967) showed that several species of *Pterostichus*, *Abax parallelepipedus* (Pill. and Mitt.) and *Philonthus decorus* were able to penetrate the cocoon and integument of the winter moth pupae and eat the contents. In order to evaluate the number of pupae taken by each species of predator a serological technique has been used, based on that of Dempster (1960) but with several differences.

This is not an exhaustive study of serological methods applied to ecology, but indicates techniques which may be of use to those wishing to study predator-prey relationships, and is a sequel to West (1950). Crowle (1961) and Wieme (1965) are useful reference works.

Successive injections of a foreign protein into a mammalian blood stream lead to the formation of antibody as described by Nossall (1964) and Speirs (1964). These antibodies may be highly specific to individual antigens. Usually a given antigen induces maximal antibody formation when it is pure. If immunisation is done with a mixture of several antigens it is less likely that an antibody will be formed against any given antigen.

Experimental animals injected with winter moth proteins might therefore be expected to produce antibodies specific to the proteins present. Winter moth proteins derived from predator guts, if unaltered by enzymatic action, should give an immune reaction with the antibody. A white precipitate, suitable precautions having been taken, would be proof of this reaction.

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PREPARATION OF MATERIALS

The account given here relates the steps used in the preparation of an antiserum in experimental rabbits to winter moth pupal antigen. The extraction of predator gut contents and the testing of these against the prepared antiserum are recounted. After the results of this experiment, I have explained the reasons for some of the processes and mentioned some of the alternative methods available.

Collection of Predator Meals

Overnight catches from pitfall traps in the study area were made twice a week in the summer and autumn of 1964 and 1965. Possible predators were removed from the traps and killed with ethyl acetate vapour.

Immediately upon return to the laboratory the crops of these possible predators were removed and smeared onto filter papers. The filter papers were labelled with date, species and sex of the animals, and stored in a desiccator over phosphorus pentoxide. It is stated that such smears have been stored for over 2 years without deterioration (Hall *et al.* 1953).

The Experimental Mammals

Three adult male rabbits were obtained as experimental animals, and a series of injections given. Mature animals are usually better antibody producers than young ones. Prolonged immunisation should be avoided because of increase in the chance of antibody formation against minor impurities, and because of the risk of production of sera with less specificity.

Preparation of Antigen

Winter moth larvae were allowed to pupate in peat in rearing cages in the laboratory. Within a few weeks of pupation, healthy pupae were removed from their cocoons and weighed.

One g of pupae at a time was crushed by pestle and mortar in 25 ml of 0.9% sodium chloride solution. The contents of the mortar were washed into a flat-bottomed flask, and a few drops of M / 1000 potassium cyanide were added to precipitate melanins (Dempster 1960). The flask was left for 24 hr in a refrigerator at 5 C. The contents of the flask were then divided equally between 2 centrifuge tubes and centrifuged for 1 hr at 2500 rpm, and approximately 1400 *g*. The clear liquid was then passed through a Seitz EK sterilising filter pad and collected in a thick-walled flask with a side arm to which a filter pump was fitted. The total volume of saline used in crushing the pupae and washing the mortar, flat-bottomed flask, and centrifuge tubes was 50 ml.

Prior to filtration the filter-pad holder, thick-walled flask, and a freeze-drying flask had been sterilised by autoclaving. The liquid in the thick-walled flask was freeze-dried using liquid air. The freeze-dried material was stored in a sterile, stoppered tube in a refrigerator until needed. When sufficient antigen had been freeze-dried it was pooled in order to minimise variation and re-stored.

Care in the preparation of the antigen is necessary to avoid denatur-

ing the proteins present. Normal methods of drying may cause a change in the protein structure. Sterile equipment is necessary to prevent bacterial attack and accidental introduction of foreign protein.

Production of Antiserum

Approximately 0.2 g of the freeze-dried antigen was reconstituted with 10 ml of distilled water, and the pH was buffered at 6.8 by the addition of disodium hydrogen phosphate and dihydrogen sodium phosphate. To the solution was added an equal volume of a mixture of the emulsifying agent Arlacel 'A' and a light oil, Bayol 'F', in the ratio 3 : 17. The liquids were thoroughly emulsified by drawing into and expelling from a fine glass pipette. The emulsion was divided into three portions, as accurately as possible, and injected subcutaneously into the experimental animals at four sites: over each scapula and over each flank. All the equipment was autoclaved before use, including the hypodermic syringes and '20 gauge' needles.

An interval of a week was left before the second series of injections, when a bacto-adjuvant was used. This antigen had the same composition as that previously injected but contained, in addition, a suspension of killed, dried *Mycobacterium butyricum*. This enhances the immune response (Freund *et al.* 1948). A third and a fourth series of injections was given at intervals of one week and using incomplete adjuvant, i.e. without *Mycobacterium butyricum*.

After a further week rabbit no. 1 was starved for a day in an attempt to eliminate some lipid material from the blood stream, and was then bled from the ear. The reason for attempting to get rid of dissolved lipids in the blood is that these might separate on standing and thus obscure the reaction. A small cut was made in the posterior edge of the ear under sterile conditions and 5 ml of blood were run into a 'Lusteroid' plastic centrifuge tube. Lusteroid is a non-wettable commercial plastic to which blood will not readily adhere. The blood was allowed to clot at room temperature for one hour, and then the serum was centrifuged for one hour at 2500 rpm to precipitate fibrin and blood corpuscles. The clear serum was then decanted into a stoppered plastic tube.

The following day all three rabbits were given a fifth series of injections. On the sixth day after the injections all the rabbits were starved for 24 hours, and on the seventh day all three were bled from the ear. The serum was treated as previously, decanted into labelled plastic tubes and stored in a refrigerator.

Further injections were given at intervals of 6 weeks to keep up the level of immunity. Exsanguination was done every 3 weeks because it was found that the serum tended to deteriorate after being removed from and replaced in the refrigerator several times.

METHODS OF TEST

Preliminary Test

A very small volume of the clear serum was allowed to run up short lengths of glass capillary tubing of 1 mm internal diameter and about 3

cm long, and the lower ends of the tubes were pushed into plasticine. A small quantity of freeze-dried antigen was reconstituted with distilled water, taken up in a micropipette, and layered carefully on top of the serum in the capillary tubes. The tops of the tubes were then sealed with plasticine and the tubes were allowed to stand at room temperature. In less than 10 min a white precipitate had formed at the serum antigen interface. This was taken to indicate that the rabbit had formed antibodies against the winter moth antigen.

The Precipitin Test

Gamma globulins

Various methods have been used by different workers to test the immune reaction between antigen and antibody and some of these are mentioned in the discussion. Basically, the dried predator gut smears are reconstituted with distilled water and reacted with either whole antiserum or a gamma globulin extract of the serum. All immunoglobulins are antibodies, but gamma globulins constitute more than 90% of immunoglobulins, at least in human serum (Wieme 1965). Because several antibodies are likely to be produced to the several components of the water soluble protein from winter moth pupae, it is most probable that some of these will be gamma globulins. By using a solution of the gamma globulins alone from the immune serum, a clearer liquid is produced in which immune reactions are less likely to be obscured.

Method used

The method used in the following experiments was modelled on that used by R.A. Webb and G.P. Gladstone at the Sir William Dunn School of Pathology, Oxford. An agar of the following composition was made up: 'Bacto Agar' 3.0 g, sodium chloride 1.7 g, barbitone 1.48 g, glycine 4.5 g, distilled water 180 ml. The chemicals were weighed out into a large beaker, and the distilled water was added and stirred well. Difficulty was experienced in getting the barbitone to dissolve, so the beaker and contents were autoclaved for about 10 min, which proved effective in dissolving the barbitone. Twenty ml of a 10^{-4} M solution of merthiolate (thiomersal) were added as an antibiotic, and the agar was then filtered through sterile cotton wool and stored in sterile screw-topped jars kept in an oven at 50 C. It was found that the agar tended to deteriorate if kept for more than 10 days.

An apparatus was made up consisting of a levelling table onto which was fitted a perspex tray. This tray held eight glass microscope slides side by side, and eight holes were punched through the tray, one under the position of each slide and slightly towards one end. The sides of the tray were of such height that when the microscope slides were in position 1 mm of the rim was exposed, and thus a layer of agar of depth 1 mm could be poured over the slides.

Two rosettes, each containing a central and six peripheral well-cutters were fixed into another piece of perspex which was of such construction that it would run along the longitudinal rims of the tray, and could be pressed down over each slide to cut wells in the agar. The peripheral cutters of each rosette were each at a distance of 7.5 mm

from the central one, and each cut a circular well of diameter 2 mm. The agar remaining in the wells was sucked out by a glass pipette attached to a filter pump.

In operation the agar is poured over 8 slides of standard thickness, allowed to solidify, and then the wells are cut and their centres sucked out. Antibody is put into the central well of each rosette, and antigen into some or all of the outer wells by means of glass micropipettes. The tray of slides is allowed to stand in a water-saturated atmosphere for the precipitates to develop. After this, cuts are made carefully through the agar at the edges of each slide, and the slides are removed from the tray. The slides and agar layers on them are placed in petri dishes containing 0.9% sodium chloride solution for 24 hr. Staining is done for about 30 sec in a 5% solution of amido black in 60% alcohol, and the stain is differentiated for about 24 hr in a 2% solution of acetic acid in distilled water. The slides are then washed in tap water to remove the acid. A photographic record of the precipitate lines may be made at this stage, and a visual assessment of the strength and position of the lines is made.

The agar is now dried *in situ* on the microscope slides in an oven at 50 C. Alternatively the layer of agar may be separated from the microscope slide and dried on a lantern slide for direct projection, or dried on a stiff, glossy card for filing. During the staining process the slides must be handled carefully in order not to loosen or damage the agar. There is some danger of splitting of the agar during the drying process. Incorporation of glycerol into the acetic acid solution used for destaining will prevent this but makes the agar somewhat sticky. Some very faint precipitin lines are liable to disappear during the drying process.

Reconstitution of predator meals and testing

Strips of filter paper carrying the predator meals were cut out and put into 2 ml centrifuge tubes, and 1 ml of 0.9% sodium chloride was added. The tubes were allowed to stand for several hours at room temperature and then placed in a refrigerator overnight to allow solution of the material. Before testing they were allowed to stand at room temperature for 30 min.

The peripheral wells of each rosette were filled with antigen by glass micropipettes and the central wells were filled with antiserum by the same method. The micropipettes and tubes were sterilised for re-use by boiling in dilute hydrochloric acid and washing in distilled water.

Control tests

Reconstituted antigens as well as fresh material from winter moth pupae reacted with the prepared antiserum at full strength. In addition, fresh material from pupae of the species *Erannis aurantiaria* (Esper) and *E. defoliaria* (Clerck) reacted with the prepared antiserum, but much less strongly. Antiserum from rabbit 3 gave much the strongest reactions.

It was then necessary to ascertain whether the antiserum would react with protein recovered from the guts of predators, and to discover how long it took for the enzymatic action in the (predator) guts to denature the protein so that it would not give the precipitin response.

In order to test this, a number of *Abax parallelepipedus* which had been

starved for a week, were confined in plastic containers, each with a winter moth pupa. Feeding was observed, and when the greater part of the pupa had been destroyed, the remains of the pupa were removed from the container and a note was made of the date and time. Two specimens of *Abax* were killed every 24 hr after feeding, and their guts were removed, smeared onto filter paper, dried and stored. For testing, after allowing time for the soluble gut contents to dissolve, drops of this liquid were tested against winter moth pupal antiserum. It was found that the guts of those *Abax* which had been killed 24 hr after feeding gave the strongest precipitin reaction, and the reaction diminished rapidly with time, so that it was only just discernible for those individuals killed 96 hr after feeding.

Specimens of *Pterostichus melanarius* (Ill.) gave results identical to those obtained from *Abax* specimens and it was assumed that specimens of *Pterostichus madidus* (F.) did not differ in this respect.

Specimens of both *Abax* and *Philonthus decorus* fed on larvae and pupae of *Phormia regina* (Meigen) gave no precipitin reaction with winter moth pupal antiserum, showing both the dissimilarity of this dipterous and winter moth protein, and that it is not merely the presence of food in the predator gut which causes the reaction, but the presence of winter moth protein which is of importance.

The precipitin reaction of winter moth antiserum with winter moth antigen, the latter either fresh or freeze-dried and reconstituted, gave 2 strong lines of precipitate, and occasionally a third, weak one, probably indicating that antibody had been formed against more than one protein. This formation of more than one line may have been an artifact (Crowle 1961) due to change in temperature during the reaction or to high antibody concentration but, since it occurred many times during the course of the experiments the former explanation seems likely. The quantity of antibody formed against any one of these proteins would probably be less than if a single pure protein had been injected, but there were obvious difficulties in attempting to separate the various winter moth proteins and it was unnecessary to do this for the purposes of this study. In actual tests of predator gut contents, of course all lines were found to be weaker than when using pure antigen, it was often found that there was one strong line and a second barely discernible.

The antigen prepared from pupae of *Erannis* species gave only one line which was in the same position as the strongest line formed by winter moth antigen, but weaker than this, when tested against winter moth pupal antiserum. The apparent similarity of these proteins shown by the similar position of the precipitin lines seems to stress the affinity of the genera *Operophtera* and *Erannis*. It should be possible to distinguish representatives of the two genera by the difference in pattern of the lines in the precipitin reaction if their antigens were concentrated enough, but the antigens recovered from predator guts were dilute. A large amount of *Erannis* protein taken from a predator gut could be confused with a much smaller amount of winter moth protein from a similar source. In this experiment it was necessary to consider that a small proportion of the positive reactions was due to genera related to *Operophtera*. The smallness of this proportion was partly because of the great numerical

superiority of winter moth larvae over those of related genera in 1964 and 1965 at Wytham, and partly because *Operophtera* antigens gave much stronger reactions with *Operophtera* antiserum than did those of related genera. These latter reactions, in many cases, were probably not detected.

A fact emerging is that *Philonthus decorus*, *Quedius lateralis* (F.) and *Nebria brevicollis* (F.) fed on adult winter moth in November 1964, and subsequently tested against winter moth pupal antiserum did not give any indication of a precipitin reaction. Similarly, an extract of adult winter moth prepared in December 1965 did not react with pupal antiserum, whilst fresh pupal antigen prepared on dates up to the middle of November continued to give a strong reaction. It must be concluded that there is a rapid change in protein structure just prior to adult emergence, or that the proteins to which antibodies are formed lie in the pupal integument. The former seems to be the more likely because the antiserum was prepared against the water-soluble proteins of the winter moth pupa, and the proteins of the pupal wall must be highly insoluble.

The criticism that antibody may be formed against plant material in the gut of the winter moth larva, and present in the pupa, and which may also be present in the guts of suspected predators, thus giving an immune response, can be dismissed. The winter moth larva evacuates its gut before pupation (Gradwell, personal communication). If this were not the case then various plant materials would have to be tested against the pupal antiserum.

It was found on testing the predator gut contents with undiluted antiserum, that other components of the serum in addition to gamma globulins tended to be deposited around the antibody well, and these to some extent obscured the precipitate lines. Because of this, a solution of gamma globulins was prepared by adding a saturated solution of ammonium sulphate to whole serum (giving a concentration of about 50%) to precipitate the gamma globulins, which were then centrifuged down and redissolved in 0.9% sodium chloride. In all experiments whole serum was put into the central well of one rosette of each slide, and gamma globulins into the central well of the other rosette. This would indicate whether there were any antibodies present which were not gamma globulins and it also helped in orientating the slide after the staining process.

A plan of the distribution of the reagents was drawn at the beginning of each test, and the precipitin lines were drawn on this when they became apparent. I found it preferable to use only three of the outer wells of each rosette for the precipitin lines to be at their clearest.

Absorption techniques

An absorption technique was used by Dempster (1960) to make his antiserum more specific to the protein of his prey species. He found that the precipitin reaction of his prey species, *Phytodecta olivacea* Forst. was about 400 times as strong as the reaction to the *Phytodecta* antiserum of *Coccinella septempunctata* L., which also occurred in his study area. He therefore added just sufficient *Coccinella* material to precipitate the antibodies to it, and then used the serum for his precipitin reactions. A slight excess of *Coccinella* would greatly reduce the sensitivity of the ser-

um to *Phytodecta*.

This method would have presented problems in the present experiment because of the several related genera present. Adequate sensitivity was obtained without it. After precipitation from the serum of antibody material common to the winter moth and to the various species of *Erannis* and other related genera present in the study area, the antibodies remaining in the serum would probably produce very weak reactions with winter moth pupal antigens. There would be practical difficulties in performing accurately a series of absorption reactions. The population of *Erannis* in the study area was much smaller than that of the winter moth so that its effects could be largely ignored.

RESULTS

Of 122 *Abax parallelepipedus* taken during the time the winter moth was in the pupal state during 1964 and 1965, 21 gave a positive precipitin reaction, indicating that they had fed on winter moth pupae not more than 4 days before they were captured. Six of 226 *Philonthus decorus* taken under similar conditions had fed not more than 3 days before they were captured. The period after which winter moth pupal remains were no longer detectable in *Philonthus decorus* was found to be 3 days, a shorter time than for the carabid species tested. This is probably correlated with the fact that the food of *Philonthus decorus* is ingested in liquid form and is more easily digested. Three of 18 *Pterostichus madidus* and 1 of 5 *Pterostichus melanarius* had fed within 4 days on winter moth pupae. No other species of beetle and no beetle larvae gave positive results, but a male and a female of the vole *Clethrionomys glareolus* Schr. taken in May 1965 were both shown to have fed on winter moth. Using these figures and the population sizes of the beetles it was possible to estimate the number of winter moth pupae taken in a unit area by each species of predator, the number of winter moth pupae per unit area having already been estimated (Frank 1967).

DISCUSSION

The winter moth protein giving the precipitin reaction seems to be a complex of several proteins because, in well-defined reactions, three distinct lines of precipitate could be seen. There are techniques for the separation of proteins in such systems. Moving boundary electrophoresis and zone electrophoresis can be used only when fairly large quantities of the reagents are available. They allow proteins to be distinguished by the rate at which these migrate towards an electrode in a conducting solution.

The method of immunoelectrophoresis (Williams 1960) separates the precipitin lines of the various proteins present. An agar plate is used with a central, longitudinal trough filled with antibody. The antigen is placed in two wells, one on either side of the trough, and the various proteins in the antigen diffuse in a longitudinal direction because of elec-

trodes placed at either end of the plate. The antibody diffuses outwards towards the antigen, and precipitin arcs are formed in various positions on the plate depending upon the proteins present. By comparing the positions of the arcs, it is possible to determine whether two antigens are of the same composition. This method would be of value in comparing the proteins present in closely-related lepidopterous pupae, such as those of *Erannis* and *Operophtera*, in an initial study. It would be too time consuming to use in routine sampling such as was done by simple diffusion.

In the preparation of the antiserum the incorporation of the antigen in a water-in-oil emulsion prepared from paraffin oil (e.g. Bayol 'F') and Arlacel (mannide monooleate) enhances and sustains antibody formation and the alteration of sensitisation may occur concomitantly, but they appear to be distinct (Freund *et al.* 1948). The immune response is not potentiated when vegetable oil is substituted for paraffin oil, nor when the antigen is administered in an oil-in-water emulsion.

Dempster (1960) used 0.4% potassium alum to precipitate the soluble proteins so that they should diffuse more slowly into the blood stream of the rabbit and thus sustain antibody formation, a method described by Proom (1943).

The factors in promoting and sustaining the immune response are probably prolonged absorption as well as the protection of antigens against destruction and elimination, favourable cellular reactions about the antigenic depots, and the production of multiple foci of antibody formation in lymph nodes (Freund *et al.* 1948).

The test method used by Dempster (1960) was to draw a small volume of the smear extract into a capillary tube, followed by an equal volume of the antiserum. Because of density differences an interface is produced where a white precipitate of antibody combined with antigen forms and is viewed by indirect light against a black background. Clarity of the liquids is essential for satisfactory results. The method has several disadvantages. The precipitate formed is not permanent, and for recording purposes it is necessary to photograph the capillary tubes and their contents. The layering of the antisera must be done with extreme care to obtain sharp interfacial division. Jarring of the tubes should be avoided; very fine precipitates easily become diffuse and may be lost due to carelessness.

Oudin used a similar method, but coated the interior of the tubes with a layer of agar (Williams 1960). A layer of precipitate forms in the agar and is less ephemeral than in the first method described. When several antigen - antibody systems are present, several layers of precipitate form at different levels in the tube depending upon the rates at which the antigens diffuse into the agar. The drawbacks are the difficulties inherent in the layering of the liquids and in the placing of the agar coating, and also in obtaining a permanent record.

Leone (1947) followed Boyden and Defalco (1943) in using a more elaborate method which has some of the drawbacks of the method used by Dempster (1960), but gives a quantitative measurement. He used the Libby photronreflectometer to measure the turbidity of the precipitin reaction (Libby 1938). Here it is necessary to dilute the antisera by an appropriate factor because of the weakness of the insect antigens. A

disadvantage was that the exact protein concentration had to be known in all antigen solutions in order for the reaction titres to be comparable.

The technique developed by Ouchterlony (Williams 1960) allows antigen and antibody to diffuse into an agar-filled petri dish which originally contains neither reagent. Small wells are cut in the agar and a few drops of the antigen and antibody solutions are placed in these. The antigen and antibody diffuse outwards towards each other at rates in proportion to their concentration and diffusion coefficients. Where the antigen encounters its specific antibody a line or precipitate forms. The lines for different antigens are distinctly separated because of differences in diffusion rate. This clear separation of lines makes it possible to distinguish more reactions by this technique than with the Oudin tubes.

Various modifications of this basic Ouchterlony concept have been used. Wadsworth (1957) devised a micro-immunodiffusion technique, which was further modified by Crowle (1958), based on the use of a template for cutting wells in the agar. Feinberg (1964) further modified this by floating a pattern-perforated disc of thin, rigid, transparent plastic onto molten agar, and then allowing the agar to solidify in contact with the disc. The disc is not subsequently removed from the agar, and the reagents are applied at the apertures of the disc where agar is exposed. The difference between this method and that of Wadsworth and Crowle is that no holes are cut in the agar, and the disc does not need to be of such thickness as to supply wells to contain the reagents. Antigens and antibodies lie on the exposed agar and will not under-run the disc because of the effective seal formed.

Another method uses a similar technique to that of Wadsworth and Crowle, but with cellulose acetate instead of agar. The cellulose acetate strips used can be handled with less risk of damage during the staining process than can agar (Johnson *et al.* 1964).

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