AN EVALUATION OF THE POTENTIAL OF SEROLOGICAL METHODS FOR THE RAPID DETECTION OF NUCLEAR POLYHEDROSIS VIRUS IN WHITE-MARKED TUSSOCK MOTH LARVAE

by

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Abstract

A study was made of the feasibility of using serological methods for early diagnosis of nuclear polyhedrosis virus in tussock moth larvae. It was possible to distinguish healthy and infected larvae in haemagglutination tests. Antisera against hemolymph from healthy and infected larvae were used, with dissolved inclusion body proteins as an antigen. Acrylamide electrophoresis alone or combined with double diffusion also showed specific bands or precipitin arcs distinguishing the two types of hemolymph. Immunoelectrophoresis in agarose and crossed immunoelectrophoresis displayed precipitin arcs and peaks characteristic for infected hemolymph. The above methods are, however, too time-consuming to routinely use them for the mass diagnosis of the tussock moth larvae. Radial diffusion, electrophoretic and chromatographic separations did not provide a clear distinction between hemolymph samples taken from healthy and infected larvae.

Great individual variability, together with changes in blood protein composition due to the growth of the larvae, preclude the use of methods such as double diffusion, electrophoresis and radial diffusion.

An evaluation of the potential of serological methods for the rapid detection of nuclear polyhedrosis virus in white-marked tussock moth larvae.

Introduction

Nuclear polyhedrosis virus has been used to control tussock moth outbreaks (Cunningham, 1975). Larvae collected after spraying of the infested areas are usually examined microscopically to establish the level of infection. This procedure is time-consuming, tiring and inappropriate for field use. A study was made of the practicability of using simpler diagnostic procedures.

The protein composition of the hemolymph of lepidopterous larvae infected with nuclear polyhedrosis viruses undergoes changes with the progress of the disease (Martignoni and Milstead, 1964, Watanabe, 1967, Young and Scott, 1970 and Weiser and Lysenko, 1972). It also changes with the development and growth of the larvae (Hudson, 1966, Whitemore and Gilbert, 1974). Therefore any feasible method would have to distinguish both types of changes since field-collected larvae would be in different instars and would be infected to different degrees.

Materials and Methods

Insects. White-marked tussock moth, Orgyia leucostigma larvae were reared on artificial diet in 1 1/4 oz plastic cups. The larvae were infected by covering the surface of the diet with a suspension of polyhedra at a concentration of about 10^7 polyhedra/ml; 100% mortality occurred in 10 to 11 days. Larval length and head capsule width were recorded.

Antigens. A suspension of virions, and a solution of inclusion body protein of the nuclear polyhedrosis of white-marked tussock moth were prepared as described by Bergold (1958).

Hemolymph samples from healthy (HH) and infected larvae (IH) were taken from larvae at different instars on the 7th day after infection unless otherwise specified.

Antibody. Antisera against hemolymph from healthy and infected larvae were prepared by injecting rabbits with progressive doses of hemolymph incorporated in complete Freund's adjuvants. Starting with 0.5 ml, three injections were given at weekly intervals. The first and third injections were administered intracutaneously; the second injection was given intraperitoneally. Four intravenous injections were then given ten days after the last injection in adjuvants. The total volume of injected hemolymph was 4.0 ml. The rabbits were exsanguinated four days after the final intravenous injection.

The schedules for the preparation of the antisera against the virions and inclusion body proteins were similar to that for the preparation of the antisera against the hemolymph. In the first three injections (given in adjuvants) the animals received totally 200 mg of proteins and 100 mg were divided in four subsequent intravenous injections. In the first three injections a total of 7.5 mg of proteins were used and 6.5 mg were divided in five subsequent intravenous injections.

Electrophoresis. Electrophoresis in agarose was performed using several media:

1. Commercially prepared agarose film in a special container. This Black Box* method was used as recommended by the manufacturers. Hemolymph from small larvae (10-12 mm length) middle sized larvae

*Analytical Chemists Incorporated, Palo Alto, Calif.

(12-18 mm) and large larvae (over 18 mm of length) were used. Infected hemolymph samples were taken on the seventh day of infection.

2. Agarose glass plates. The hemolymph samples were the same as in above method. Veronal buffer, pH 8.6, molarity 0.05 was used and 18V per cm were applied for one hour.

3. Agarose mixed with polyvinylpyrrolidone av. mol. wt. 10,000 and 360,000. Electrophoresis was performed as described by Watanabe (1967).

4. Acrylamide gel electrophoresis. The method was essentially that described by Davis (1964), but neither the intermediate gel nor the sample gels were used. The samples, containing 10% sucrose, were layered directly on the gels. Healthy and infected hemolymph from the small, medium and large larvae were compared.

5. Combined method. The procedure was described previously (Krywienczyk and Hayashi, 1972). Acrylamide gels containing electrophoretically separated proteins of the hemolymph from large insects only, were embedded in agarose and allowed to cross-react with the appropriate antiserum contained in the troughs cut in the agarose.

6. Immunoelectrophoresis. The commercial method (Analytical Chemists Incorporated, Palo Alto, Calif.) was applied according to the recommendations of the manufacturer. Infected hemolymph from small and large larvae obtained on the seventh day after application polyhedra to the diet were used. The larvae were also examined microscopically for the presence of polyhedra. Immunoelectrophoresis on agarose

was performed as described previously (Krywienczyk 1962).

7. Crossed Electrophoresis. The method described by Laurell (1965) was performed with healthy and infected hemolymph from small, medium and large larvae. The first separation was accomplished on 1% agarose, using veronal buffer pH 8.6, molarity 0.05 and timed according to the migration of the phenol-blue indicator dye. The plate used for the secondary electrophoresis was covered with 1% agarose containing antiserum diluted 1:3 with the same buffer. In some instances the area for the secondary electrophoresis was subdivided into two regions containing two different antisera as suggested by Svendsen and Axelsen (1972). The secondary electrophoresis was allowed to proceed for one hour. In both dimensions of separation a current of 18 V per cm was applied. The plates were then washed, dried and stained with buffalo black as usual.

8. Radial diffusion in agarose. The method was based on that described by Feinberg (1956). Glass plates were covered with 2 mm of agarose containing antiserum against inclusion body proteins diluted 1:3. Samples of hemolymphs from the larvae obtained in ten consecutive days after infection were introduced into pre-punched wells and left overnight. The controls consisted of hemolymph from healthy larvae and dissolved inclusion body proteins.

9. Haemagglutination. The method applied was similar to that described by Stavitsky (1954). Sheep erythrocytes were coated with dissolved polyhedron inclusion body proteins (0.01 mg/ml), centrifuged healthy hemolymph or centrifuged infected hemolymph. The antisera prepared against the following antigens were used: virions, polyhedron inclusion body protein, healthy hemolymph, and infected hemolymph.

10. Chromatographic separations. Cellulose acetate membranes* with a plastic support were cut into pieces 70x35 mm. Samples of hemolymph were applied 6 mm above the lower border of the membrane. In one series of experiments drops of antisera were superimposed on the samples of hemolymph and allowed to react for 15 minutes. Then the strips were put into an appropriate buffer to a depth of about 3 mm and the buffer was allowed to ascend the strip. When it reached the top, the strips were removed, dried, stained in buffalo black and differentiated in 3% acetic acid. The same procedure was repeated with the following buffers: barbiturate buffer pH 8.6 0.05 molar undiluted, diluted 1:3 and double concentrated, barbiturate buffer pH 9.2 and barbituate buffer with an addition of 0.5 M urea and 1% sodium desoxycholate.

Results and Discussion

Larvae collected in the forest are of different instars and sizes, therefore, some easily measurable body dimension had to be taken as a basis of classification into small, medium and large larvae. The easiest dimensions to establish are body length and head capsule width. The width measurement would involve use of a microscope which we wished to avoid. As an alternative, body weight (W) was plotted against the third power of body length (L^3); the results are presented in Fig. 1. Head capsule widths and L^3 are compared in Fig. 2. The inclination of the plot line in Fig. 1 is approximately 45 degrees indicating proportionality between body length and weight. Thus the use of body length alone for grouping larvae into the small, medium or large categories is justified. The proportionality between the body length and the

* Millipore, Bedford, Mass.

body weight is in agreement with the findings of Kittel (1941) and of Sattel (1956) who investigated metabolic patterns of insects as related to the type of growth.

The widths of head capsules as measured in 100 insects represented all the sizes between 0.96 mm and 2.50 mm (Fig. 2). The data show that there is no good correlation between the values.

It has been established by several investigators (e.g., Hudson, 1966; whitmore and Gilbert, 1974), that the protein content of growing lepidopterous larvae changes with the stages of development, from low toward higher concentrations. It was therefore important to be able to distinguish in the hemolymph composition those differences due to the age of the larvae, from those caused by the onset of nuclear polyhedrosis.

The results obtained in passive haemagglutination when tanned sheep erythrocytes were coated with inclusion body proteins and allowed to react with the antiserum against the infected hemolymph were positive. The titer 1:200 showed 1+ agglutination, and the $\pm$ reaction was still distinguishable at a titer of 1:1600. There was no agglutination visible with the antiserum against the healthy hemolymph. Although the results were positive, this method cannot be used for routine diagnosis because the method is much too elaborate.

It also proved impossible to perform a passive haemagglutination test by direct application of tussock moth haemolymph to tanned sheep erythrocytes coated with inclusion body proteins since nonspecific agglutination took place under conditions required for the method.

Immunoelectrophoresis performed by the standard method and

using the "Black Box" method gave identical results. There are differences present between samples of hemolymph. In Fig. 3 the arrow "a" indicates a fraction of the hemolymph which migrates toward the cathode and appears to be present only in the large larvae (both healthy and infected). The arrow "b" points toward a fraction present only in the small larvae. The arrow "c" marks the crossing over of 2 precipitin arcs which appear to be absent in small, infected larvae but present in large healthy larvae and infected larvae. The differences between healthy and infected hemolymphs seem to be restricted only to the minor components, e.g. the arrow "d" points toward a component present only in infected hemolymph in small as well as large larvae.

The cross-electrophoresis was performed for more detailed analysis of the differences between the hemolymphs. In Fig. 4 and 5 the most obvious differences are shown in the peaks representing the chromoproteins in the hemolymph from small larvae. In infected hemolymph, the chromoproteins are broken up into several components still cross-reacting with the antiserum against healthy hemolymph. The antiserum against infected hemolymph (Fig. 6 and 7) seems to produce a lesser number of peaks than the antiserum against healthy hemolymph. The small larvae used for this particular electrophoretic run, might have been less heavily infected than those used in the run illustrated in Fig. 5 although, as judged by microscopical examination, the numbers of polyhedra present in the tissues were comparable. When the antiserum against infected hemolymph was incorporated in the agarose (Figs. 6 and 7) the difference between the two types of hemolymph was less pronounced.

More definite patterns were obtained in the larger insects. In some instances (Fig. 8, 9 and 11) and also to some extent in Fig. 13-15 smudging is seen probably due to the loss of charges in the chromoproteins. The phenomenon was consistent and reproducible.

The results of cross-immunoelectrophoresis employing plates which contain 2 antisera (Fig. 12-15) show also some characteristic peaks.

In Fig. 12 the arrows "a" and "b" indicate the presence of 2 antigens characteristic of the infected hemolymph, since the curves indicated by both arrows are present only in that arch of agarose containing the antiserum against infected hemolymph. In Fig. 13 the arrows "d" and "e" mark the presence of antigens specific for the healthy hemolymph. Those findings are in agreement with the results of Young and Scott (1970) who also observed a reduction of protein content in infected hemolymph, loss of two protein components and formation of new antigens serologically unrelated to the virions or inclusion body proteins.

Although Young and Scott did not observe any cross-reactions between inclusion body protein and infected hemolymph, some positive results were obtained in the present investigations. One precipitin line was formed in double diffusion tests when infected hemolymph was taken from the larvae on the tenth day after infection. In a second instance, sheep erythrocytes coated with inclusion body proteins were hemagglutinated by the antiserum prepared against infected hemolymph. Unfortunately these reactions have no value

for practical use for diagnosis of NPV infection. The double diffusion gave positive results at a time when the infection could be recognized visually due to the milky nature of the infected hemolymph. The positive results of the haemagglutination has only a theoretical value because it would be impractical to inject each sample into a laboratory animal to obtain antibody for the diagnostic purpose.

The results of acrylamide gel electrophoresis of healthy and infected hemolymph from the medium sized large larvae are depicted in Fig. 16 A and B. In both size ranges, infected hemolymph shows a reduction in the quantity of slow-migrating chromoproteins. Hemolymph from the middle size larvae (Fig. 16B), in addition to having a reduced quantity of chromoproteins, appears to form an additional band (arrow a) specific to the infected hemolymph, and shows some differences in the mobility of the faster proteins. In the large larvae (Fig. 16 A) healthy hemolymph shows a strong band (arrow c) which is only weakly present in infected hemolymph. The strong band indicated by the arrow "b" in infected hemolymph is only faintly represented in healthy hemolymph. The position and presence of the fast moving bands (arrows "d" in Fig. 16 A and B) is inconsistent.

The acrylamide gel electrophoresis combined with double diffusion (Fig. 17 A & B) showed differences in the numbers and position of the precipitin arcs. Generally, the precipitin arcs formed by the gels containing products of separation of healthy hemolymph and either antisera to healthy or infected hemolymph were stronger and more numerous than those formed by the gels containing separation products

of infected hemolymph. In Fig. 17B the arrow "a" indicates an arc which is present only in the infected hemolymph, but as it has been formed with the antibodies against healthy hemolymph, this factor must also exist in a small quantity in the healthy hemolymph. The arc "b" formed with the healthy hemolymph and homologous antiserum appears to be specific for the healthy hemolymph. With the development of the disease, progressive disintegration of the components of the hemolymph occurs with the development of the disease, or alternatively the fat body tissues affected by the virus fail to synthesize fully the components of the hemolymph. In all the precipitin patterns obtained by all the methods the healthy hemolymph displays more and stronger bands or arcs.

Negative results in combined electrophoresis were obtained when the troughs between two embedded acrylamide gels containing products of electrophoretic separation of healthy and infected hemolymphs were filled with the antisera against the polyhedron proteins or against the virions.

As an additional attempt to visualize differences between healthy and infected hemolymph with the possibility of diagnostic application, spots of hemolymphs were applied on cellulose acetate and separated chromatographically in several buffers. No easily distinguishable differences between healthy and infected hemolymph within the size ranges were observed (Fig. 18). Neither of the buffers used showed any clear cut advantage.

Watanabe (1967) reported a good differentiation of the hemolymph proteins from healthy larvae of Hyphantria cunea and larvae infected with nuclear polyhedrosis by electrophoresis on agarose mixed with polyvinyl-pyrrolidone dissolved in phosphate buffer pH 6.8, ionic strength 0.05μ. As the molecular weight of polyvinyl-pyrrolidone used was not specified by this author, we applied two preparations of two different molecular weights, 10,000 and 360,000. The larvae from which the samples of hemolymph were drawn were examined microscopically to determine the level of virus infection. After the glass plate was stained, a person unaware of the order in which the healthy and infected samples had been placed attempted a diagnosis (Fig. 19 A,B). However, the margin of error was too wide to accept this method for routine use.

In the course of this investigation great individual variability between larvae and inconsistency of hemolymph patterns was observed; this was also reported by Whitmore and Gilbert (1974).

The general conclusion is that, in spite of some differences in the composition of healthy and infected hemolymph, they cannot be used as the basis of a method to replace the microscopical examination as a method of detecting nuclear polyhedrosis virus infection.

Bergold, G. H. 1958. Viruses of insects. In Handbuch fuer Virusforschung, Bd. IV (III Erg. Bd.), pp. 60-142. Springer, Vienna.

Davis, B. J. 1964. Disc Electrophoresis II. Method and application to human serum proteins. Am. N.Y. Acad. Sci. 121, 404-427.

Feinberg, J. G. 1956. A new quantitative method for antigen-antibody titration in gels. Nature, 177: 530-531.

Hudson, Anne. 1966. Proteins in the hemolymph and other tissues of the developing tomato hornworm Protoparce quinquemaculata Haworth. Can. J. Zoology 44: 541-555.

Kittel, A. 1941. Sauerstoffverbrauch der Insekten in Abhaengigkeit von der Koerpergroesse. Z. Vergl. Physiol. 28: 533-539.

Krywienczyk, J. 1962. Immunoelectrophoretic studies of inclusion body proteins. J. Insect Pathol. 4: 185-191.

Krywienczyk, J. and Y. Hayashi. 1972. Serological investigations of subcellular fractions from Malacosoma disstria larvae infected with cytoplasmic polyhedrosis. J. Invertebr. Pathol. 20: 150-156.

Laurell, C. B. 1965. Antigen-antibody crossed electrophoresis. Ann. Biochem. 10: 358-361.

Lowry, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Martignoni, M. E. and J. E. Milnstead. 1964. Hypoproteinemia in a Noctuid larva during the course of nucleopolyhedrosis. J. Insect Pathol. 6: 517-531.

Sattel, M. 1956. Die Groesse der Tracheeninnenflaechen von Seidensspinnerraupen in Abhaengigkeit von der Koerpergroesse. Z. Vergl. Physiol. 39: 89-101.

Stavitsky, A. B. 1954. Micromethods for the study of proteins and antibodies. I. Procedure and general application of hemagglutination and hemagglutination inhibition reactions with tannic acid and protein treated red blood cells. J. Immunol. 72: 360-364.

Svendsen, P. J. and N. H. Axelsen. 1972. A modified antigen-antibody crossed electrophoresis characterizing the specificity and titre of human precipitins against Candida albicans. J. Immunol. Methods 1: 169-176.

Watanabe, H. 1967. Electrophoretic separation of the hemolymph proteins in the fall webworm, Hyphantria cunea, infected with a nuclear-polyhedrosis virus. J. Invertebr. Pathol. 9: 570-571.

Whitmore, E. and L. I. Gilbert. 1974. Hemolymph proteins and lipoproteins in Lepidoptera - a comparative electrophoretic study. Comp. Biochem. Physiol. 47B: 63-78.

Weiser, J. and O. Lysenko. 1972. Protein changes in the hemolymph of Galleria mellonella (L.) larvae infected with virus and protozoan pathogens. Acta Entomologica Bohemoslovaka 69: 97-100.

Young, S. Y. and Scott, H. A. 1970. Immunoelectrophoresis of the cabbage looper, Trichoplusia ni, during the course of a nuclear polyhedrosis virus infection. J. Invertebr. Pathol. 16: 57-62.

Fig. 1. Relationship between body length (L°) and body weight in white-marked tussock moth larvae.

▼▲ maximum and minimum lengths measured.

● mean length.

Fig. 2. Relationship between body length (L^{3}) and head capsule width in white-marked tussock moth larvae.

○ maximum and minimum width measured.

● mean width.

Fig. 3. Immunoelectrophoresis in agarose (commercial method). The antigens in the wells, the antibodies in the troughs.

H - healthy hemolymph

I - infected hemolymph

The letters "L" and "S" refer to large (L) or small (S) larvae.

Figs. 4-11. Crossed electrophoresis. In the first dimension the antigens were run in barbiturate buffer pH 8.6, 0.05 molarity for 1 hour at 18V/cm. Second dimension: agarose contained antiserum diluted 1:3; other conditions as for the first dimension.

Fig. No.	Antigen		Antibody against
	Length of larvae in mm	H or I	
4	10-12	H	H
5	10-12	I	H
6	10-12	H	I
7	10-12	I	I
8	20-30	H	H
9	20-25	H	I
10	20-30	I	H
11	20-30	I	I

Fig. 12-15. Crossed electrophoresis. The second dimension : agarose is divided in 2 parts. One part contains antiserum against H the second part contains antiserum against I. Other conditions as above.

Fig. No.	ANTIGEN (hemolymph)		Antibody	
	Length of larvae in mm	H or I	lower part	upper part
12	10-12	I	H	I
13	20-25	H	I	H
14	20-25	I	I	H
15	10-12	H	I	H

Fig. 16. Acrylamide gel electrophoresis, 5mA per gel, glycine buffer pH 8.3 diluted 1:5.

A : Healthy and infected hemolymph from middle-sized insects.

B : Healthy and infected hemolymphs from large insects.

Arrows: see text.

Fig. 17 A and B. Acrylamide gel electrophoresis combined with double diffusion. Troughs containing antibody against the infected (A) and healthy hemolymph (B) are cut between embedded acrylamide gels. Arrows: see text.

Fig. 18. Chromatographic separation of hemolymph from healthy and infected insects. Size range: from 10 to 30 mm. Phosphate buffer pH 6.8 diluted 1:3.

Fig. 19. Electrophoresis of healthy and infected hemolymph on agarose with addition of:
A: polyvinyl-pyrrolidone mol. wt. 360,000.
B: polyvinyl-pyrrolidone mol. wt. 10,000.
Veronal-sodium acetate buffer, pH 8.6, 150V, 0.05 molarity.

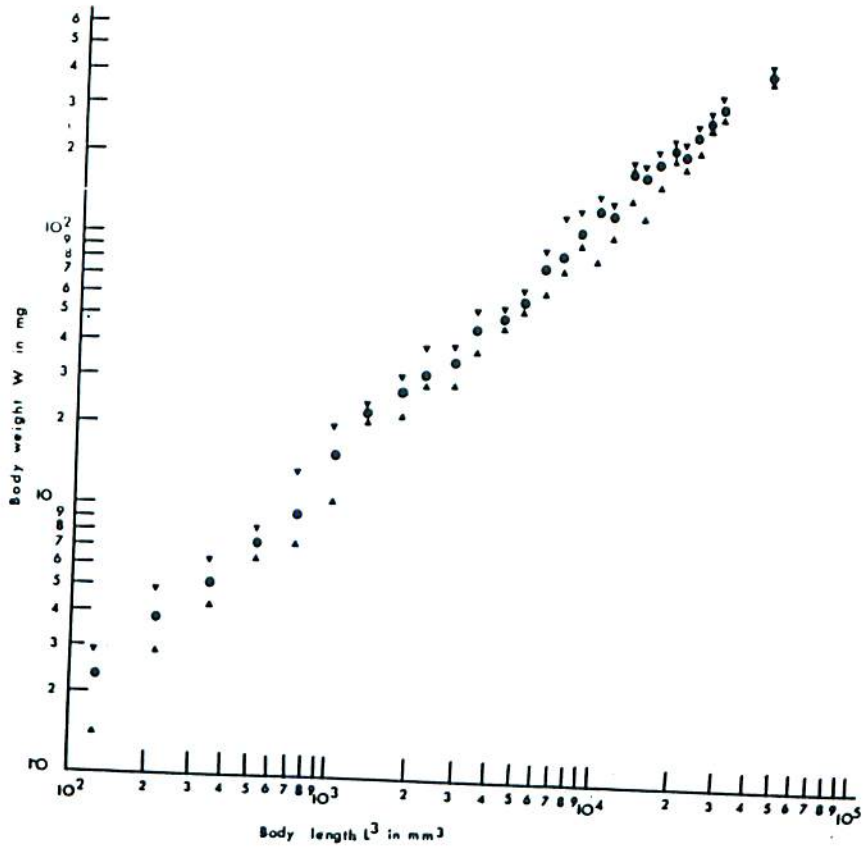
Body weight W in mg
Body length L³ in mm³

Fig 1

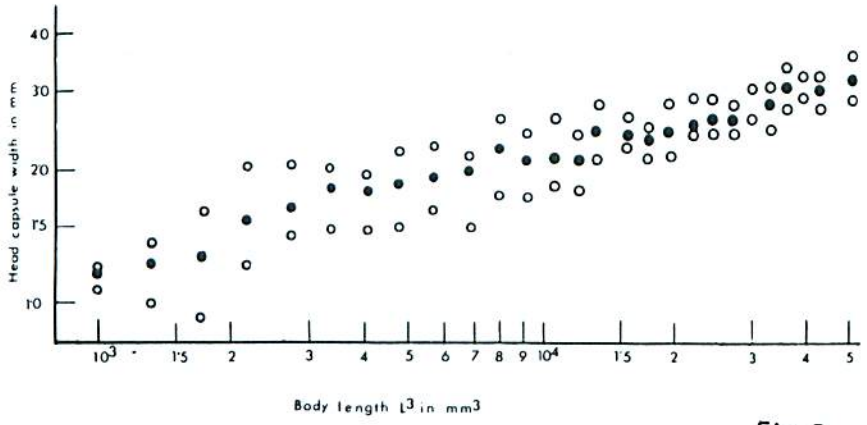
Head capsule width in mm
Body length L³ in mm³

Fig 2

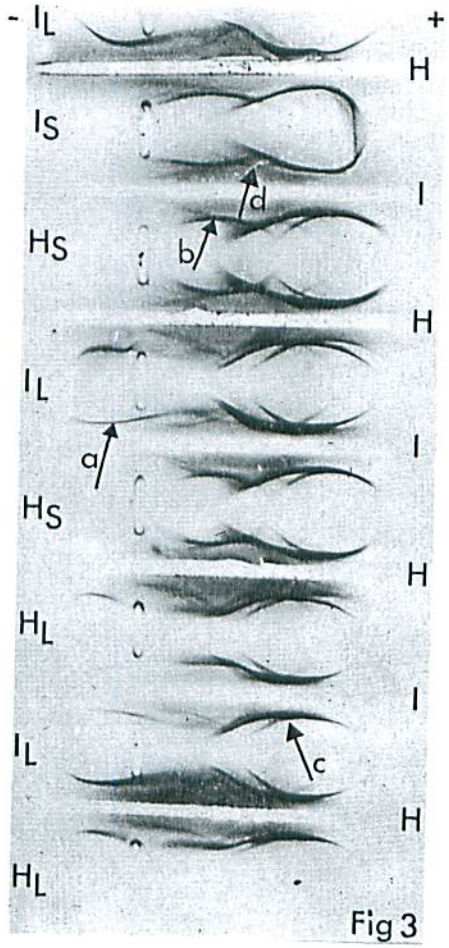

Fig 3

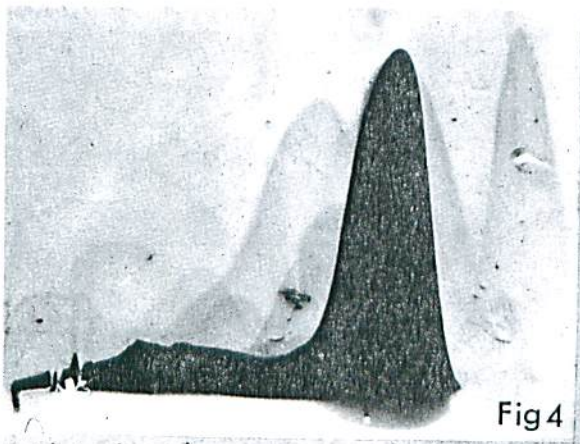

Fig 4

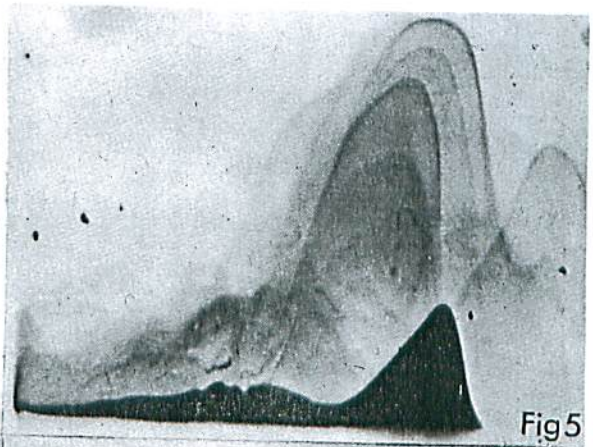

Fig 5

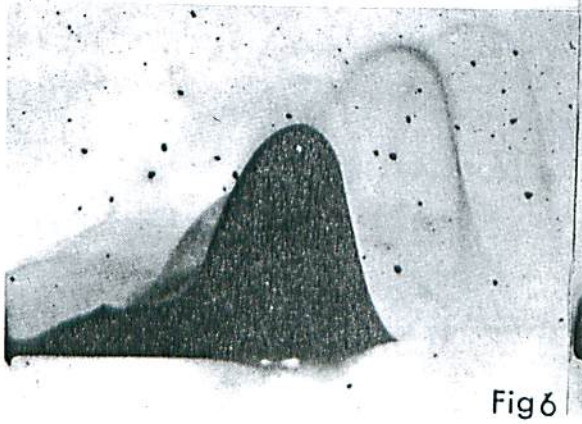

Fig 6

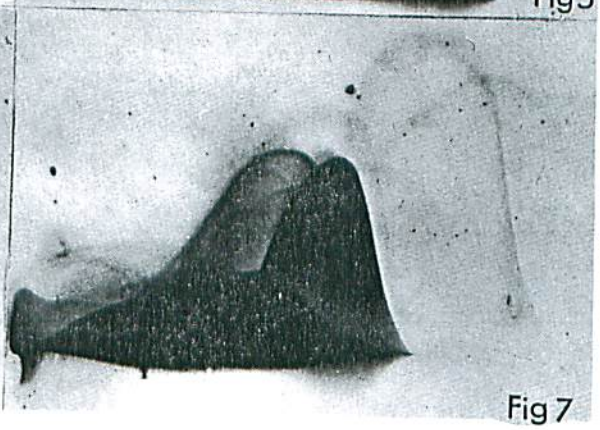

Fig 7

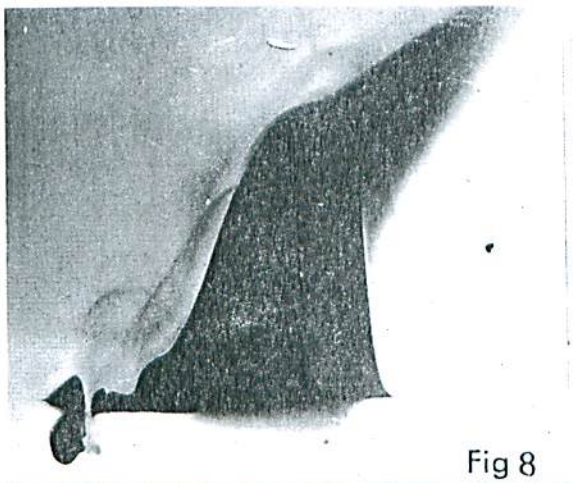

Fig 8

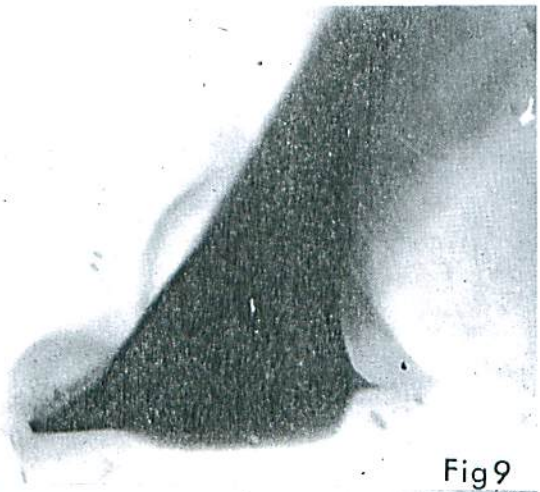

Fig 9

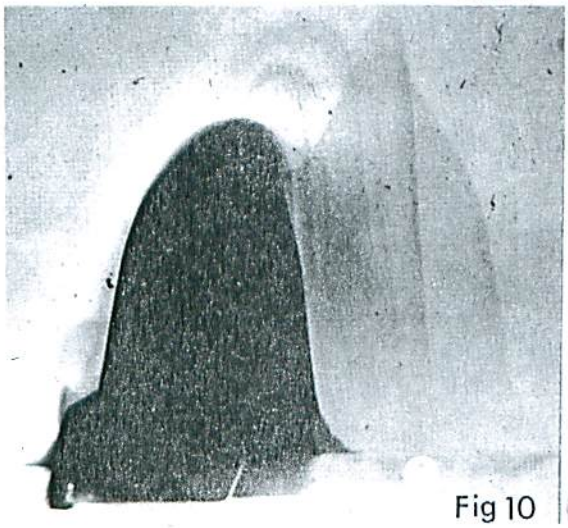

Fig 10

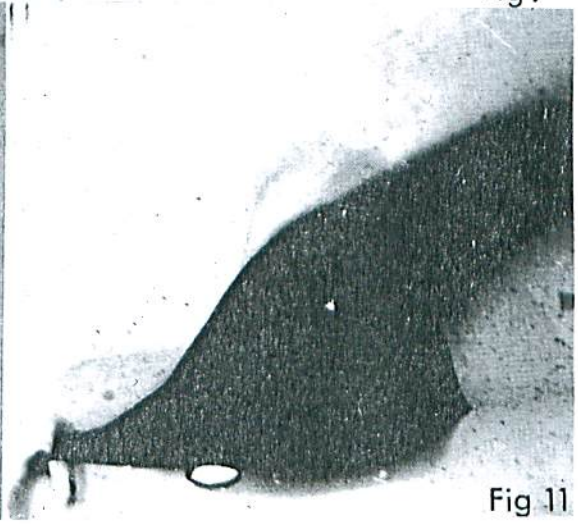

Fig 11

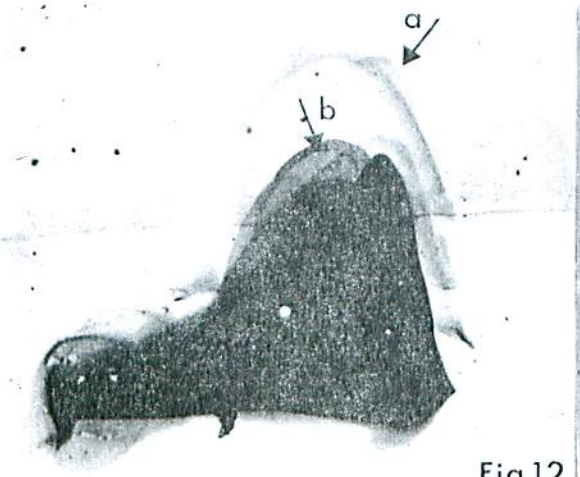

Fig 12

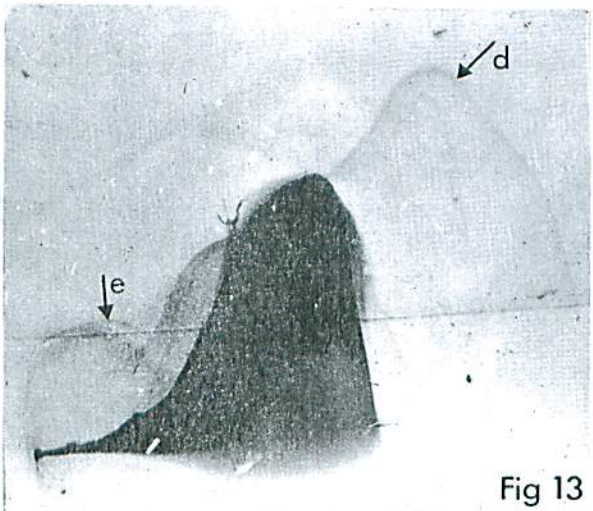

Fig 13

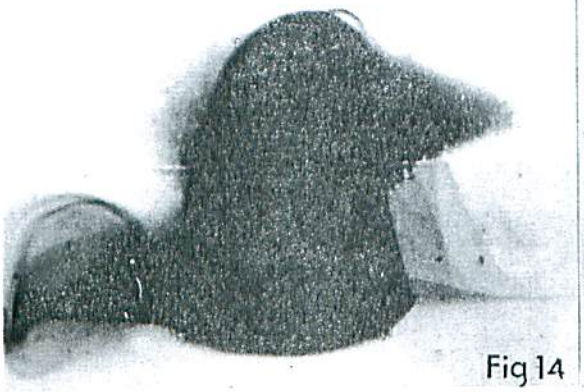

Fig 14

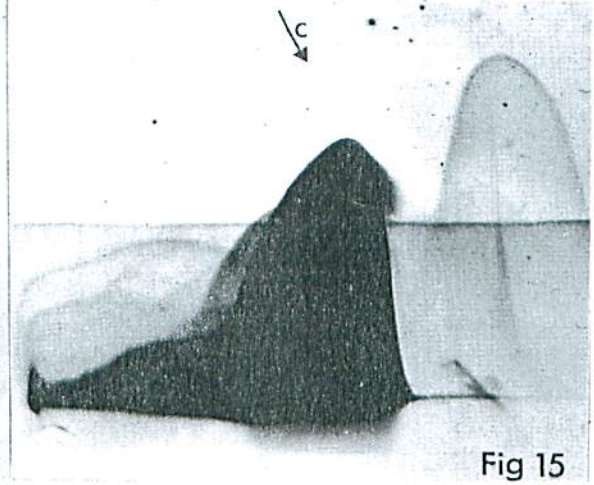

Fig 15

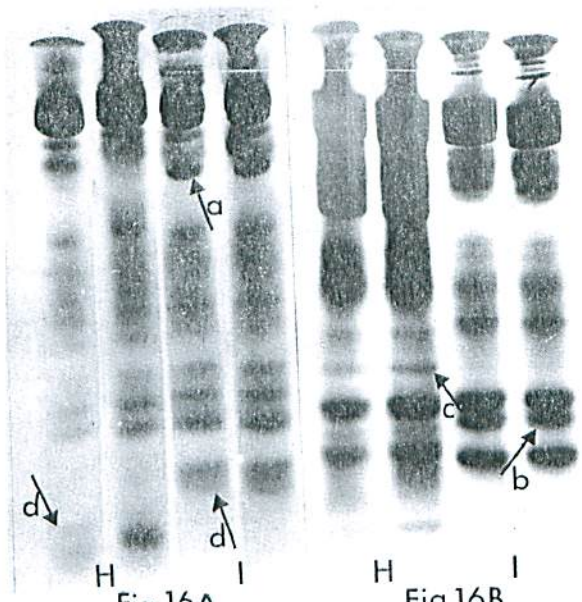

Fig 16A Fig 16B

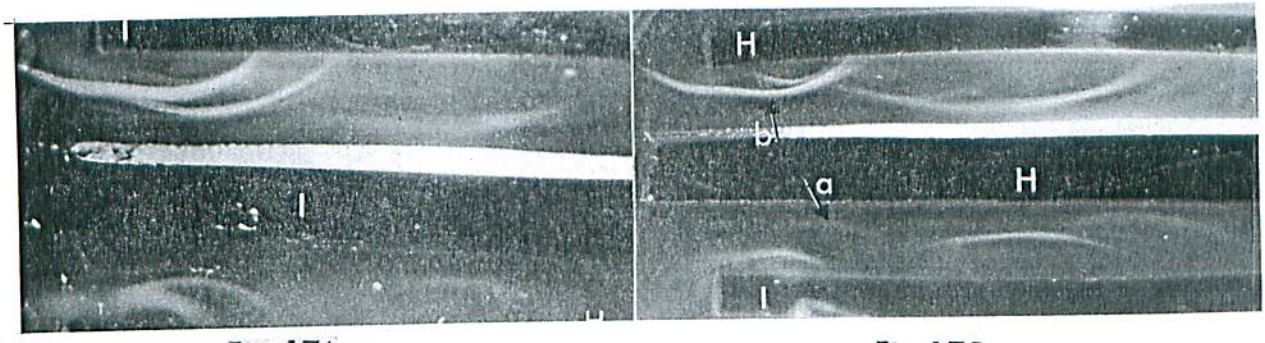

Fig 17A Fig 17B

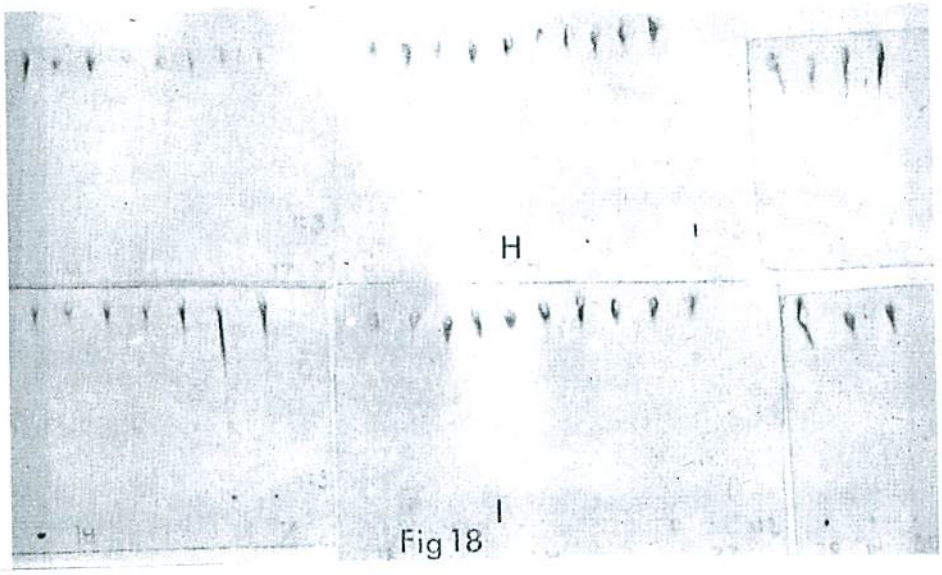

Fig 18

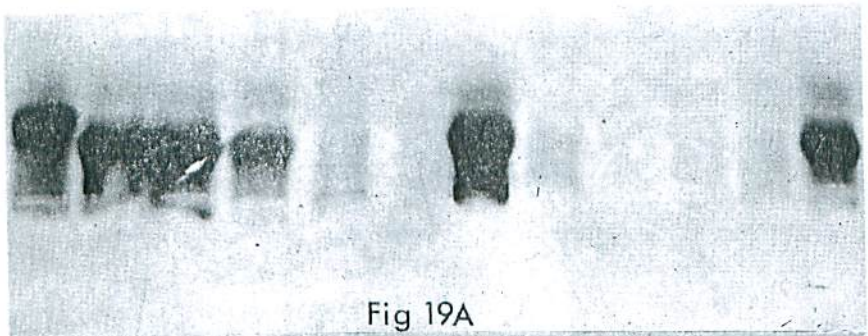

Fig 19A

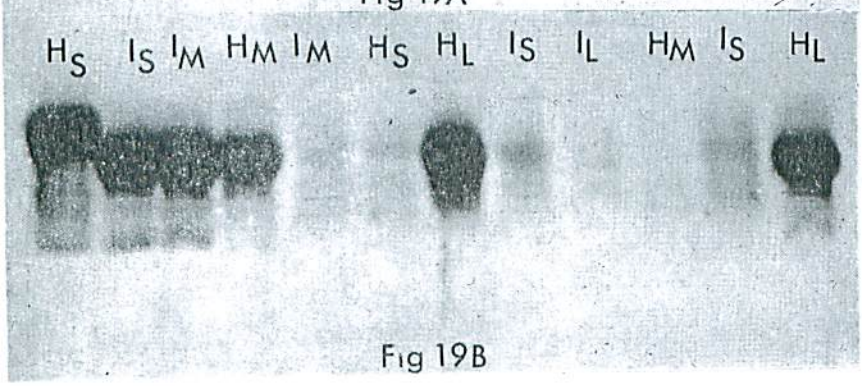

Fig 19B