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THE INTERRELATIONSHIPS OF THREE PATELLA SPECIES ON THE SOUTH COAST OF BRITAIN

A thesis submitted for the degree of Doctor of Philosophy of the University of Southampton.

by

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

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Doctor of Philosophy

THE INTERRELATIONSHIPS OF THREE PATELLA SPECIES ON THE SOUTH COAST OF BRITAIN

by Deborah Jane Hatch

There are three limpet species of the genus Patella found on the south coast of Britain, namely P. vulgata, P. intermedia and P. aspera.

In 1935 Ronald G. Evans, working on the south coast, observed that in western regions such as Cornwall the limpets possessed distinctive specific features and can be easily separated into three species. However as one moves eastwards along the coast the population becomes more variable until, on the Isle Of Wight, one can observe 'transitional' forms possessing characters of two or more species thus making classification very difficult.

The two main theories that have been put forward to explain this phenomenon are firstly that the three species have evolved from a common vulgata-like root-stock and that, for some reason, complete separation has not been achieved in certain areas, and secondly that the 'transitional' forms are in fact the result of hybridisation.

The purpose of this work was to discover which of these two explanations seems the most likely.

The limpet population on the south coast was surveyed in order to confirm the existence of the 'transitional' forms and to estimate their frequency.

A study of the distribution and the breeding cycles of the three species was made to establish whether hybridisation was feasible, and later hybridisation experiments were carried out in the laboratory to discover whether the three species can be successfully crossed to produce viable larvae.

Analyses of the blood serum of the three species and of the 'transitional' forms were made using electrophoretic and immunological methods to provide additional criteria for the separation of the three species and to try and determine the relationship of the 'transitional' forms to the three species.

The survey confirmed that the proportion of 'transitional' forms in the population does increase as one moves from west to east along the south coast. On the Isle of Wight the percentage of the population consisting of 'transitional' forms was estimated at 21%.

Hybridisation between the three species was successfully achieved and it was found that the pattern of development followed that of the species represented by the maternal parent.

It was found not to be possible to distinguish between the blood sera of the 'transitional' forms and of P. vulgata by immunological or electrophoretic methods, despite the fact that considerable differences were observed between the blood sera of P. vulgata and P. intermedia using these methods.

It was concluded that hybridisation is not occurring to a significant extent on the south coast of Britain and that the 'transitional' forms are in fact variable forms of P. vulgata.

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CHAPTER 1

INTRODUCTION AND LITERATURE SURVEY

INTRODUCTION

Distribution and Specific Characters of the Three Species of Limpet

There are three species of the genus Patella found on British coasts, namely P. vulgata, P. intermedia and P. aspera. P. vulgata is found in prolific numbers right round the south and west coasts of Britain but, being restricted to rocky coasts, it is rarely found on the east coast. P intermedia and P. aspera, however, have a more restricted range and are found only on the south-west coast extending from mid-Wales round to the Isle of Wight. This is probably due to the fact that P. intermedia and P. aspera are less tolerant of cold temperatures than P. vulgata, substantiated by the fact that P. aspera and P. intermedia breed during the summer months whereas P. vulgata continues to be in breeding condition well into the winter.

The three species also differ in their distribution on the shore (Das and Seshappa 1948). P. vulgata is found mainly between the Low Water Spring (L.W.S.) and the High Water Spring (H.W.S.), some individuals occurring in the wash zone. P. intermedia however, is never found above the H.W.S. level. P. aspera lives at a much lower level of the shore, rarely being found above the High Water Neap (H.W.N.) except in rock pools. Davies (1969) found this distribution was related to abilities to withstand desiccation. Despite these differences the overlap between the habitats is quite large.

The specific characters of the three species have been described in detail by Fischer-Piette (1935 and 1948) and Eslick (1940) and Evans (1953) and usefully summarised by Fretter and Graham (British Prosobranch Molluscs, 1962).

The three Patella species differ firstly in foot colour. This is dark grey to black in P. intermedia, bright orange in P. aspera and yellow-green in P. vulgata. However this character shows much intra-specific variation and taken on its own is not a useful criterion for identification.

More important are the small tentacles situated at the edge of the mantle. These are transparent in P. vulgata but opaque and white in P. aspera and P. intermedia. This character appears to show no variation and is very useful for distinguishing two of the species from the third.

When the limpet body is removed from the shell a distinctive mark can be seen at the apex of the shell. This mark is called the head scar and its colour differs for the three species. In P. vulgata the scar is silver, in P. intermedia it is dark brown and mottled and in P. aspera the scar is orange. This is also a useful specific character.

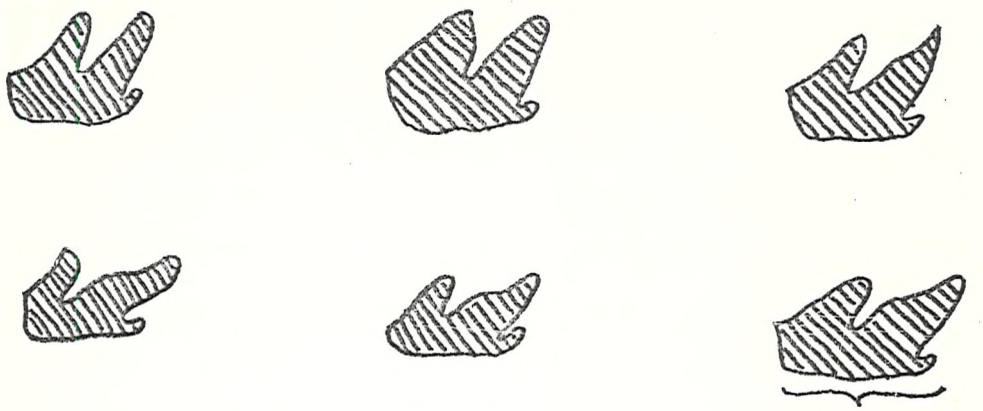
The colour of the remaining shell also varies between species. In P. vulgata the shell lining is a yellow-green colour and is translucent and has only very faint marginal rays if any. In P. aspera the shell lining is white and pearly and again has faint marginal rays. In P. intermedia the shell lining is also pearly but has heavily pronounced, brown marginal rays.

Perhaps the most important specific character is the form of the radula which is a long ribbon of tissue bearing small rasping teeth used for scraping the algal film off the rock surface. The ratio of radula length over the maximum diameter of the shell is different for the three species. The highest value for this ratio is found in P. intermedia, the lowest in P. aspera while P. vulgata exhibits an intermediate value between the two.

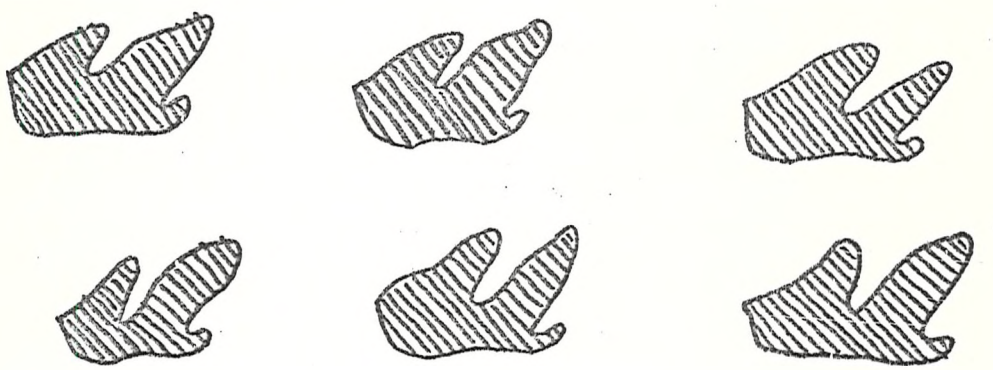
Another specific feature is shown by the pluricuspid teeth found on the radula which, although they vary greatly in size and shape, can be sorted into groups and used to distinguish between the three species. The teeth can be removed from the radula by treatment with caustic soda and examined under the microscope. Examples of typical groups found in the three species are shown in figure 1. It can be seen that in P. vulgata the first cusp is never larger than the second and the cusps are generally small,

Fig. 1 Typical Groups Of Pluricuspid Teeth Found In P. vulgata, P. aspera and P. intermedia.

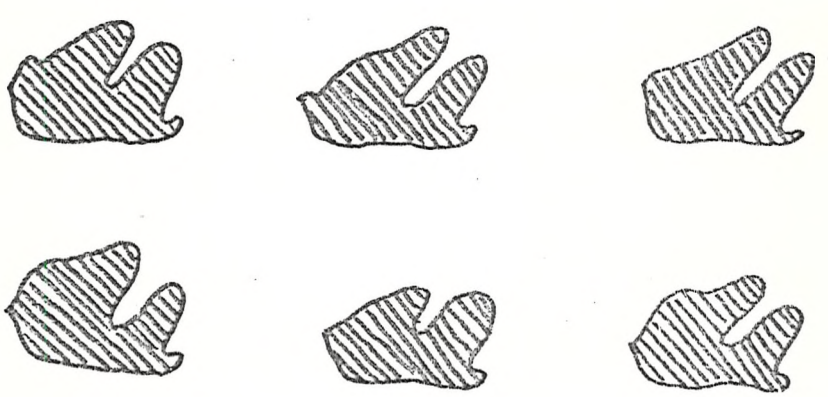
a) P. vulgata



b) P. intermedia



c) P. aspera



thin and elongated. In P. intermedia the cusps are larger and broader but again the first cusp is never larger than the second. In P. aspera the cusps are more blunt and broadened and unlike the teeth of the other two species the first cusp is quite commonly larger than the second.

The external appearance of the shell does differ for the three species, and having collected limpets for a while it becomes easy to pick out the three types. However the differences are not easy to describe. Both P. intermedia and P. aspera tend to be longer than wide and have flattened shells. P. intermedia often has a triangular outline and has numerous, pronounced ribs running from the apex to the edge of the shell. P. vulgata invariably has a smooth, regular shell with a relatively high apex. It would also appear that P. intermedia never reaches the large sizes attained by the other two species.

An interesting difference between the three species concerns an ectoparasite, Urceolaria patellae, which settles on the gills of the limpet. This parasite has two strains one of which is symbiotic with zooxanthellae and one which does not show this symbiosis. The strain without the zooxanthellae shows a preference for P. vulgata while the strain with zooxanthellae invariably settles on P. intermedia or P. aspera. This could be a reflection on the different habitats of the three limpets but this is unlikely since there is a large overlap of habitats.

Although most of these characters show a certain amount of variation, by using a combination of all of them it becomes possible to separate the three species without much difficulty. The external features of the three limpet species are shown in figures 3, 4 and 5.

Work Done by Fischer-Piette and by Evans on The French Coast

In 1946 and 1947 Fischer-Piette carried out a survey of the

limpet population from the Brittany coast to the Basque coast of France, in order to establish which species were present in these areas and to find useful criteria for separating the various species. He listed the three species present as P. vulgata, P. aspera and P. intermedia and gave a detailed description of the external features of each.

However he considered these external characters as inadequate criteria for accurate classification of the limpets and used the ratio of radula length over shell length as an additional criterion. His results for this ratio for the Brittany coast and the Basque coast are given below.

	Brittany coast	Basque coast
P. aspera	1.10	1.10
P. vulgata	1.75	1.75
P. intermedia	2.10	2.20

Fischer-Piette concluded from these results that the differences in the ratio for the three species were sufficient to provide a criterion for the separation of the three and that the ratios remained fairly constant from the Brittany coast to the Basque region.

However Fischer-Piette also used the ratio of the radula length over the cube root of the shell volume. This he thought would be less susceptible to the individual irregularities of the shell margin. The results for this ratio are given below:-

	Brittany coast	Basque coast
P. aspera	2.90	2.87
P. vulgata	3.90	4.38
P. intermedia	4.71	4.66

It can be clearly seen that as one moves south from Britanny to the Basque coast the mean values of the ratios for P. vulgata and P. intermedia are moving closer together while the mean value for P. aspera remains constant. These results led Fischer-Piette to believe that maybe the limpet populations from the Basque coast were exhibiting a lesser degree of differentiation than populations from the Brittany coast.

He continued his study with a survey of the form of the pluricuspid teeth from limpets of the two areas, and he discovered that as one moves south to the Basque region the form of the pluricuspid teeth becomes more variable.

Fischer-Piette went on to describe many 'transitional' forms, found only in the Basque region, not only displaying a mixture of characters of the three species previously mentioned but also some individuals showing similarities to the Mediterranean species P. caerulea. This species is described as having a delicate shell with intricate ornamentation and as having an intense blue translucent shell lining.

On the evidence of this survey Fischer-Piette put forward the idea of one large polymorphic species or possibly inter-breeding group, P. vulgata-intermedia-aspera-caerulea, which is capable of breaking up into separate species on some parts of the coast but not on others. However in 1948 Fischer-Piette claimed that by establishing the breeding cycles of the 'transitional' forms and comparing them with the breeding cycles of the three species, he was able to identify all the Basque 'transitional' forms as belonging to the species P. intermedia. He then suggested that the extreme variability of this species on the Basque coast obscures the fact that both P. aspera and P. vulgata maintain their identity equally as well there as they do in Brittany. In addition to giving rise to P. vulgata-like and P. aspera-like forms, in the same region, P. intermedia also varies towards P. caerulea. Fischer-Piette then substituted his original root-

stock for one of P. intermedia-caerulea. Thus it is P. intermedia only which develops 'transitional' types, both P. vulgata and P. aspera maintaining their identity even on the Basque coast. From the P. intermedia-caerulea root-stock emerges P. intermedia as a distinct species to the north, P. caerulea to the south.

In 1958 Ronald G. Evans studied the limpet populations from five regions on the west coast of France. As each limpet was collected it was given a preliminary identification on the basis of externally visible features such as shell shape, colour of foot and mantle tentacles and intensity of the marginal rays. The classified limpets were then numbered and preserved for further examination. This involved the lining of the shell, the ratio of radula length over shell length and the shape of the pluricuspid teeth. Using these additional criteria the numbered individuals were reclassified and the preliminary and final identifications were compared for each area. For all areas north of the Basque region the two identifications coincided to the extent of 100%. However in the Basque region the figure for correct preliminary identification was less than 90%. The most common fault was identifying individuals of P. intermedia and P. aspera as P. vulgata.

Evans found that the most common 'transitional' shells and pluricuspids were of a P. vulgata-intermedia type. Also the radula length over shell length fraction (R/C) is similar for both species as is their vertical zonation between the tide marks. He therefore rejected Fischer-Piette's theory of the existence of a P. intermedia-caerulea root-stock and suggested an alternative complex involving a P. vulgata-intermedia root-stock. Neither Fischer-Piette or Evans explain what they mean by the concept of a root-stock but presumably they refer to an ancestral species from which the three present day species have evolved.

Work of Fischer-Piette and Evans On The South Coast of Britain

In 1935 Fischer-Piette surveyed the limpet population on

the south coast of Britain. He stated that, on the Channel coast, although the three species form distinct entities on the west coast (Cornwall), there occurs a progressive loss of specific distinctions as one proceeds east until on the Isle of Wight the limpets are in a disordered state and conditions are approaching those described for the Basque coast even to the appearance of P. caerulea characters in some individuals, although this species never actually occurs on British shores.

Fischer-Piette's survey of the Channel limpets was not exhaustive since he used only external characters and his samples were small. Therefore in 1953 Evans decided to confirm these results for the English coast, using larger samples, in order to throw some light on the interrelationships of these three species and also to obtain information on the breeding cycles.

As in his work on the French coast Evans took large random samples from the shore and separated the individuals into species using external characters only. He later checked these preliminary identifications using the whole range of characters including the colour of the head scar, the R/C ratio, the form of the pluricuspid teeth and the strain of ciliate present on the gills. He then compared the accuracy of his original observations for different areas. He found that the percentage identified correctly using external characters only was larger for populations on the west coast (Cardiganshire) than for the Isle of Wight and put this down to the increasing number of 'transitional' forms as one moves eastwards along the coast.

Evans was now convinced of the existence of 'transitional' types. Some individuals show a mixture of characters on the external shell which are difficult to express verbally. 'Intermediacy' is sometimes observed in the shell lining and is more easily described. Thus some shells with the opaque green lining characteristic of P. vulgata would have the mottled head scar of P. intermedia. Alternatively, the dark internal rays of

P. intermedia shells are found at the margins of otherwise aspera or vulgata-like shells.

Evans also found a variation in the ratios of radula length over shell length for different areas. Thus the R/C values, which normally differ largely for the three species, move closer together as one moves east towards the Isle Of Wight. This was mainly due to the values for P. intermedia and P. vulgata becoming lower while the values for P. aspera remained constant. However these values never actually merge and can still be used for specific identification on the Isle Of Wight.

The results concerning the pluricuspid teeth were useful also. Evans sorted the teeth for each species into about five groups for each. He found that on the west coast (Aberystwyth) a large majority of the teeth fell into two groups for each species whereas on the Isle Of Wight the teeth became very much more variable and the number of teeth falling into the less common groups increased.

Discussion Of Theories Put Forward To Explain These Phenomena.

In 1938 Fischer-Piette suggested that these limpets belonged to one large group capable of splitting up into several species, morphologically and ecologically distinct. This splitting is realised in certain districts of the coast line, (there are no 'transitional' types there despite overlapping habitats), but not in other districts. In these last districts there is much variation, leading to unaccustomed types, each special to a locality. These divergent types are potential species, as shown by P. caerulea (partially taking shape on the Isle Of Wight, succeeding, but only locally, on the Biscayan coast, and fully succeeding in the Mediterranean). So geographical isolation is not necessary for the formation of new species of limpets but, of course, the natural tendency of the complex to split into particular types is greatly favoured where geographical isolation occurs as it is not held back by arrivals from other localities.

However, Fischer-Piette did not give any evidence of geographical isolation on the south coast and therefore did not explain why this splitting has occurred in some areas and not in others.

On the same theme as Fischer-Piette, Huxley in 1942 suggested that in the case of the three Patella species on the south coast there is an ecological divergence which in some regions has led to complete speciation, in others only to the partial separation of adaptive types. He suggested that this is a primary condition and constitutes a step towards complete separation, rather than from a secondary condition resulting from hybridisation of three previously differentiated types.

In 1953 Evans, continuing this idea, stated that, on this interpretation of the divergence being of a primary nature, the 'transitional' forms would represent the residuum of the stock from which the more highly correlated groups are being developed. The

fact that P. intermedia, P. aspera and P. caerulea each appear to be more closely related to P. vulgata than they are to each other may be interpreted as an indication that they are divergent types springing from a common P. vulgata-like root-stock. On this interpretation one may consider the P. vulgata population at Bognor reef, with some indication of P. intermedia and P. aspera facies but with neither of these appearing as a distinct species, as representing the initial stage in the separation of the three types. The populations on the Isle Of Wight show a further stage in the evolution of P. aspera and P. intermedia. On the island P. aspera has already almost completely succeeded in isolating itself from the complex, while P. intermedia has not achieved complete isolation as far west as Torquay, but in Cardigan Bay the separation is virtually complete. P. caerulea never, on British shores, achieves the status of a distinct species.

There are obvious difficulties involved in viewing the situation in this way. One of them is the explanation of the mechanism of progressive separation, and the introduction of discontinuities into the complex as one proceeds further west along the Channel coast, for neither geographical isolation nor complete ecological isolation seems to be necessary. Although the P. vulgata populations of Newhaven and Brighton display variation, practically none of it seems to be in the direction of P. aspera, P. intermedia or P. caerulea, yet at Bognor reef such variations are common, in shell, radula and pluricuspid features. It is difficult to know what force is at work selecting variations which (on the present indication) lead to the emergence of P. aspera and P. intermedia further west.

A possible selection force could be temperature variations. There is a considerable increase in sea temperatures from the Brittany to the Basque coast of France. However this explanation would not be valid for the south coast of Britain because, although summer sea temperatures increase from west to east, winter sea

temperatures decrease from west to east due to the increase of the influence of land temperatures further east along the coast.

It has been observed in some plant and animal species that variation in the population often increases towards the edge of the range of the species, to enable the population to adapt to the more variable environment. Although the Isle Of Wight does represent the edge of the range of P. intermedia and P. aspera, P. vulgata continues a long way east along the coast and it is mainly in P. vulgata that this extensive variability is found. It is unlikely, then, that this theory is relevant to this particular situation.

Manwell and Baker have carried out electrophoretic studies on several groups of molluscs in order to aid in their classification. They discovered a large number of protein polymorphisms present in the populations they studied and in 1968 put forward four theories to explain this phenomenon.

Firstly they suggested that the polymorphisms may have been present in an ancestral species and some of the variants retained in subsequent lines after their divergence. A nice example of this retention of polymorphisms in the course of speciation is provided by the persistence of certain blood group polymorphisms and the taster, non-taster polymorphisms for the detection of phenylthiocarbamate in man and some anthropoid apes. This theory is similar to that postulated by Fischer-Piette and by Evans and demands serious consideration.

Secondly, variants from different species which appear to be identical may actually represent separate mutations. Manwell and Baker do mention that it is very unlikely that mutation would occur with sufficient frequency to be responsible for maintaining such a high percentage of variable individuals in the population. However the possibility cannot be completely ruled out.

Thirdly they suggested that similar selection pressures on

two or more species when they occur in the same habitat (sympatric) may result in the persistence of shared variants which otherwise disappear when the species are in different locations (allopatric). The obvious difficulty with this explanation is that the three species of limpets living on the coasts of Wales and Cornwall, which do not exhibit this range of variation, overlap considerably with respect to their habitat and are therefore presumably subject to similar selection pressures as they are on the Isle Of Wight.

The fourth explanation, and the one which Manwell and Baker think is most likely is that of hybridisation, which is discussed below.

Hybridisation

In 1963 Ernst Mayr, in his book 'Animal Species And Evolution', stated that it is well known that many species can be crossed in captivity but do not produce hybrids where ranges overlap in the wild such as in the case of Drosophila spp.. (Patterson and Stone 1952). Mayr believes this is the basis of two wrong conclusions, namely that hybridisation is common in the animal kingdom and that the possibility of hybridisation indicates conspecificity. He then stressed that, in animals, sterility is only one of many isolating mechanisms and that other mechanisms may be more important in maintaining separation. Therefore the possibility of hybridisation provides a limited amount of information concerning the species status.

Hybridisation has been found to be common in birds. In 1952 Cochrum recorded seventy-five hybrid crosses among North American birds. Very few genuine hybrids have been reported among the mammals and in reptiles it is also rare, although Bailey (1942) reported that hybridisation occurred between the rattle snakes Crotalus homalus and Sistrurus catenatus. In contrast to this, hybridisation is fairly common among the amphibians as in the case

of the North American toad Bufo. Hybridisation has also been found to be very common in fishes, probably due to the fact that reproduction involves external fertilisation in this group.

Not much work has been done on hybridisation in marine invertebrates but the fact that it is so common in fishes which employ a method of external fertilisation makes it likely that hybridisation is also widespread among the marine invertebrates.

When considering the possibility of hybridisation between the three limpet species it is important to determine whether factors such as distribution and breeding cycles make this a valid proposition. It has been shown by Evans and later substantiated in this work that the overlap in habitat on the shore is considerable between the three limpet species and providing mature gametes are released simultaneously by the three species there is no reason why they should not come into contact with each other. It will be seen from later work that there is a considerable period of time during which all three species are at a peak of reproductive activity therefore making hybridisation a possibility at this time of year. There seems, therefore, to be no obvious isolating mechanism at work to prevent the mature gametes of the three species coming into contact with each other.

Much work has been done on hybridisation in Echinoderms and particularly sea urchins. In 1963 Chaffee and Mazia obtained an 18% success rate when crossing the sperm of Strongylocentrotus franciscanus with the eggs of S. purpuratus and a 9% success rate when the cross was performed the other way round. Hultin, in 1948 achieved an 80% success rate when crossing these two species but only after treatment with trypsin.

In 1946 Orton carried out cross-fertilisations of the three Patella species on the shore and obtained viable larvae from these crosses. However it is possible that in the sea water he used

there were sperm of all three species in sufficient numbers to fertilise all the eggs and his results are by no means conclusive.

In 1968 Manwell and Baker suggested that hybridisation was responsible for the pattern of enzyme systems observed in certain populations of Cepaea nemoralis and Cepaea hortensis. One of the populations (Tintagel) consisted entirely of C. nemoralis while the other (Erme Valley) was made up of about two-thirds C. hortensis and one-third C. nemoralis. Electrophoretic studies were carried out on isocitrate malate and 6-phosphogluconate dehydrogenases. In C. nemoralis only a single fast N.A.D.P. iso-citrate dehydrogenase was found in the pure population and thus corresponded to one of the C. hortensis; but in the mixed population a number of C. nemoralis showed a heterozygous form comparable to some C. hortensis. Similar patterns of variation were found to occur in both the 6-phosphogluconate and malate dehydrogenases and in both cases the C. nemoralis from the mixed population exhibited at least some affinities with C. hortensis which were not found in the pure C. nemoralis sample.

In 1963 Sick, Frydenberg and Neilsen studied the haemoglobin patterns of the plaice Pleuronectes platessa and the flounder Pleuronectes flesus, using agar electrophoresis. Many individuals had been discovered that exhibited transitional morphological characters between these two species. All these 'transitional' forms showed the same haemoglobin pattern which was different to the patterns for flounder and plaice and also different to the pattern produced by mixing the haemoglobins of the flounder and the plaice. Artificial hybrids of the flounder and the plaice were compared and showed exactly the same haemoglobin pattern as the transitional individuals. It was concluded that cross breeding was the most likely cause of the haemoglobin patterns and that the 'transitional' forms were in fact true hybrids.

In 1953 Evans offered circumstantial evidence against the possibility of hybridisation. He stated that 'intermediate' shell

forms and pluricuspid teeth are common among the limpet populations both at Bembridge and Bognor reef, yet at these stations P. intermedia and P. aspera are either rare (Bembridge) or absent (Bognor). There is an abrupt reduction in the frequency of 'transitional' P. vulgata shells between Culver Cliff (the eastern-most station at which all three species occur in numbers) and Bembridge, but the frequency of such shells at the latter station is still appreciable (28%). Even though P. intermedia and P. aspera may be rare or absent from this station, these 'intermediate' shells might still be hybrids, as viable sexual products could quite easily be carried round the eastern tip of the island by tidal currents, and Culver Cliff is only a mile or so away. The drop from 45% to 28% would then be explained by a partial barrier only against cross breeding. But the figure (25%) for 'transitional' P. vulgata forms at Bognor reef is practically identical to that for Bembridge. Moreover there are considerably more P. vulgata/intermedia pluricuspids at Bognor reef than there are anywhere else, even on the Isle Of Wight. Yet this station lies twenty miles east of the nearest breeding grounds of P. intermedia and P. aspera.

Evans also pointed out that the same argument could be applied to the appearance of P. caerulea facies among the Basque limpets since the latter species is confined to the Mediterranean coast. Similarly the theory of hybridisation makes it very difficult to explain the appearance of P. caerulea facies on the south coast of Britain.

However in 1958 Evans re-examined this argument and discarded it, since on further reflection it can be seen not to be valid. He states that it is true that 'transitional' forms between two species in an area where only one exists cannot be ascribed to hybridisation at the present time but there still remains the possibility that at some time in the past both species occupied that area and freely interbred. Thus P. caerulea-like facies among the present day P. aspera on the Basque coast might be due to the survival of relict genes of

P. caerulea in the population although the species has disappeared from the area. Thus the same argument can be applied to the appearance of intermedia-like facies in the P. vulgata population at Bognor reef.

Evans finished his paper by saying, 'The problems that arise from the existence of these 'transitional' forms can only be resolved by further work in the field of systematics of Patella both by the analysis and comparison of populations from different areas, and by an experimental approach to such problems as the possibility of hybridisation and the fate of the hybrids.'

Direction Of Further Study Suggested By This Previous Work.

Initially it was necessary to confirm the existence of the 'transitional' forms on the Isle Of Wight by carrying out a survey of the limpet populations on the south coast which would include a numerical analysis of the variable morphological characters of populations from different areas.

Before attempting to hybridise the different species under artificial conditions it was necessary to determine whether hybridisation was feasible in the wild with respect to factors such as distribution on the shore and breeding cycles, since if no overlap could be found to occur between the habitats and times of reproductive activity of the three species the possibility of hybridisation could be discounted.

If hybridisation was found to be feasible in the wild it would then be necessary to carry out experiments to determine whether hybridisation could be successfully achieved under artificial conditions.

Since the possibility of hybridisation being successful in the lab. would constitute no definite proof that this was occurring under natural conditions, it was also necessary to establish additional biochemical criteria for the separation of the three species in order to determine the relationship between the 'transitional' forms and the three species.

CHAPTER II.

A SURVEY OF THE LIMPET POPULATIONS FROM VARIOUS STATIONS ON THE SOUTH COAST OF BRITAIN.

SURVEY OF THE LIMPET POPULATIONS FROM VARIOUS STATIONS ON THE SOUTH COAST

Aims

The purpose of this survey was to confirm that the phenomenon of 'intermediacy' still exists among the limpet populations on the south coast and to establish that the percentage of 'transitional' forms does in fact increase as one approaches the Isle Of Wight, as it is twenty years since Evans worked in this area. It was also necessary to discover what proportion of the populations consisted of these 'transitional' forms.

Distribution is important when considering the possibility of hybridisation and it was important to find out how much the habitats of the three species overlap and whether the extent of this overlap differs from one area to another.

Methods

In order to investigate the existence of 'transitional' forms, random samples of 200 individuals of the three species were collected from several areas including Portland Bill, Swanage, Freshwater Bay and Alum Bay. The same procedure of preliminary identification, as used by Evans, was followed. Thus the limpets were sorted into species using external morphological characters only, these being the colour of the foot, the form of the mantle tentacles, the colour of the shell lining and the external features of the shell. Having classified the limpets using these criteria only, the limpets were taken back to the laboratory where they could be dissected and examined in more detail.

The radula was removed from each animal by pulling the rear portion which can be found coiled up underneath the gonad. The radula was then measured for length, labelled and preserved in

alcohol for later investigation of the pluricuspid teeth.

It was found that the easiest way to look at the pluricuspid teeth was to remove the main body of the radula by treatment with hot sodium hydroxide. The separated teeth could then be placed at various angles on a slide and looked at under the microscope, to be sorted into the appropriate group for that species.

Having confirmed the identity of each individual using these additional criteria of radula length, pluricuspid teeth and colour of the head scar, the accuracy of the preliminary identifications was compared for the different areas. The individuals initially identified incorrectly were taken to be 'transitional' forms and the percentage present in the population could therefore be estimated.

The distribution on the shore of the three species was compared for the Isle Of Wight and Swanage. It was necessary to know the extent to which the habitats overlap in order to assess the probability of the eggs and sperm of different species coming into contact during spawning. Twenty $\frac{1}{2}$m. square quadrats were thrown on various parts of the shore and every individual in each quadrat identified. The number of quadrats containing all three species was taken as a measure of heterogeneity of the population.

Results

The results of the survey to discover the accuracy of preliminary identifications for the different areas are given in table 1. The results are shown as the number initially identified incorrectly at each station. The table also shows the way in which they were identified and to which species they were later found to belong.

It can be clearly seen that the number of individuals identified incorrectly is far greater for the Isle Of Wight than for the other areas. The most frequent mistake was to identify *P. vulgata* as *P. intermedia*. Less common was the mistake of identifying *P. intermedia* as *P. vulgata*. In only one case was *P. vulgata* identified as *P. aspera*.

TABLE 1

The Number Of Individuals From A Sample of 200 Identified Incorrectly For Four Stations On The South Coast Of Britain.

	No. *P. vulgata* identified as *P. intermedia*	No. *P. intermedia* identified as *P. vulgata*	No. *P. vulgata* identified as *P. aspera*
Portland Bill	2	0	0
Swanage	2	1	0
Alum Bay	6	1	0
Freshwater Bay	30	12	1

The most commonly seen 'transitional' form was an individual with a vulgata-like internal shell and foot but with the mottled head scar of P. intermedia. These particular individuals were observed exclusively on the Isle Of Wight and were found in no other areas. Another common 'transitional' form found on the Isle Of Wight possessed the green-grey foot of P. vulgata but with the chalky mantle tentacles of P. aspera and P. intermedia.

The characteristic which most clearly shows the 'transitional' nature of individuals on the Isle Of Wight is the ratio of radula length to shell length. The means for the values of this ratio for the three species at the various collecting sites are shown in table 2. In this experiment limpets were not collected from Portland Bill but an additional collection was made from Bembridge on the eastern-most side of the island.

It can be seen that as one nears the Isle Of Wight the values of the ratios for P. vulgata and P. intermedia are becoming smaller while the ratios for P. aspera are becoming slightly larger, the values of the ratios for the three species being closest on the Isle Of Wight. There are no values for P. aspera and P. intermedia at Bembridge due to the scarcity of these species in this area.

It was found to be difficult to sort the pluricuspid teeth into groups but an attempt has been made to survey the differences in form from one area to another. The various groups of teeth found in the three species are shown in figure 2 and the results of percentages of groups found in different areas are given in tables 3, 4 and 5.

TABLE 2

The Mean Value For The Ratio Of Radula Length Over Shell Length For The Three Species of Patells From Four Areas

	Patella vulgata	Extreme values	Patella intermedia	Extreme values	Patella aspera	Extreme values
Swanage	1.610	1.299 - 2.050	1.856	1.436 - 2.314	1.012	0.893 - 1.211
Alum Bay	1.750	1.488 - 2.101	1.623	1.212 - 2.035	1.081	0.931 - 1.321
Freshwater Bay	1.536	1.110 - 1.931	1.455	1.206 - 1.998	1.102	0.822 - 1.104
Bembridge	1.486	1.182 - 1.763	-	-	-	-

Fig. 2 The Different Groups Of Pluricuspid Teeth Found In The Three Patella Sps. On The South Coast Of Britain.

a) P. vulgata

Group I. Group II. Group III.

Group IV. Group V.

b) P. intermedia

Group I. Group II. Group III.

Group IV.

c) P. aspera

Group I. Group II. Group III.

TABLE 3

The Different Groups Of Pluricuspid Teeth Exhibited By P. vulgata From Various Stations On The South Coast.

(shown as percentage of 200 individuals)

	I	II	III	IV	V
Portland Bill	62	-	24	4	10
Swanage	60	24	10	6	-
Alum Bay	63	18	16	3	-
Fresh-water Bay	43	27	19	4	7

TABLE 4

The Percentage Of Different Groups Of Pluricuspid Teeth Exhibited By P. intermedia From Various Stations On The South Coast.

	I	II	III	IV
Portland Bill	86	3	7	4
Swanage	90	-	10	-
Alum Bay	77	15	7	1
Fresh-water Bay	56	22	9	13

TABLE 5

The Percentage Of Different Groups Of Pluricuspid Teeth Exhibited by P. aspera From Various Stations On The South Coast.

	I	II	III
Portland Bill	88	11	1
Swanage	94	3	3
Alum Bay	99	1	-
Fresh-water Bay	90	8	2

From the tables it is obvious that in the case of P. vulgata the less common groups of pluricuspid teeth, i.e. groups II to V inclusive, are found more frequently on the Isle Of Wight than further west along the coast. Similarly in P. intermedia the frequency of rare groups, II to IV, increases as one nears the Isle of Wight, the frequency being highest at Freshwater Bay which is the easternmost station. However in P. aspera the pattern of group distribution appears to remain constant for the four different stations, the most common group, I, being always greatly in predominance.

The results of the study of the distribution of the three species are given in table 6 below.

TABLE 6

The Number Of Quadrats Containing One, Two And Three Species Respectively For The Isle Of Wight And Swanage.

	Quadrats with 1 sp.	Quadrats with 2 sps.	Quadrats with 3 sps.
Isle Of Wight	4	13	3
Swanage	8	11	1

Both at Swanage and the Isle of Wight there is some overlap in distribution of the three species but this overlap is more pronounced on the Isle Of Wight. The high percentage of quadrats containing two species is due to the fact that P. vulgata is so prolific and widely distributed round the shore.

Discussion.

It is very difficult to assess exactly what percentage of the population consists of 'intermediate' types since it is impossible to define precisely what constitutes 'intermediacy' and what constitutes normal intra-specific variation.

From table 1 it can be seen that the total percentage of the population of Freshwater Bay identified incorrectly was 21 percent. This is probably a conservative estimate of the percentage of 'transitional' forms present since there are many other individuals which, although they can be primarily sorted into one species without much difficulty, do show signs of 'intermediacy' to some degree.

Another important fact to be noted is that these problems of identification involved only two species namely P. vulgata and P. intermedia, whereas P. aspera was always identified correctly. This was to be expected since 'intermediacy' always seems to involve a mixture of P. vulgata and P. intermedia characters in the same individual, while P. aspera appears to remain separate and shows very little variation.

This idea is confirmed by the pattern of distribution of the pluricuspid teeth groups. While the distribution of groups in P. vulgata and P. intermedia becomes more heterogenous as one moves towards the Isle Of Wight, the distribution of groups in P. aspera remains very constant.

The results for the values of the ratio of radula length over shell length are rather difficult to interpret. As one might expect the values for P. vulgata and P. intermedia are becoming closer together as one moves onto the Isle Of Wight, this involving mainly a decrease in the value of the ratio for P. intermedia. The value of the ratio in P. aspera is also showing a tendency to move closer to that of the other two species but the change is very small.

The results of the distribution study show that there is a considerable overlap in the habitats of the three species this

overlap being greater on the Isle Of Wight. Therefore geographical isolation cannot be working as an isolating mechanism to prevent hybridisation between the three species.

Thus, it can be concluded that 'transitional' forms do exist in greater numbers on the Isle Of Wight than in more western areas and that the percentage of 'transitional' forms in the population is at least 21 percent. The 'transitional' forms are exhibiting a mixture of P. vulgata and P. intermedia characters while P. aspera remains very distinct. Also with respect to distribution there is no reason why hybridisation should not be occurring.

The external features of the 'transitional' forms as compared to those of P. vulgata and P. intermedia are shown in figures 6, 7, and 8.

Explanation of Fig. 3

In P. vulgata (a) the margin of the shell is approximately circular in outline and the shell surface is rough and irregular. The shell is also less flattened than in the other two species. In P. intermedia (b) the shell commonly has a triangular outline with well pronounced ribs running from the apex to the margin. This species never reaches the large sizes attained by the other two. In P. aspera (c) the shell tends to be flattened and longer than it is wide. This species also has well pronounced ribs running from the apex to the margin of the shell.

Explanation of Fig. 4

In P. vulgata (a) the shell has a translucent brown-green lining and the head scar is silver. There are no marginal rays present. In P. intermedia (b) the shell has an opaque white lining and the head scar is mottled brown in colour. The dark internal rays are well defined particularly near the margin of the shell. In P. aspera (c) the shell lining is pearly and the head scar is orange. The marginal rays are rarely present.

Fig. 3 External Shell Features Of a) P. vulgata
b) P. intermedia And c) P. aspera (x 1)

Fig. 4 Internal Shell Features Of a) P. vulgata
b) P. intermedia And c) P. aspera (x 1)

Explanation of Fig. 5

In P. vulgata (a) the foot is grey-green in colour and the mantle tentacles are transparent and consequently difficult to detect. In P. intermedia (b) the foot is black and the mantle tentacles are a chalky white colour, standing out very distinctly against the dark background of the shell lining. In P. aspera (c) the foot is orange and the mantle tentacles are opaque and often tinged with orange.

Explanation of Fig. 6

The normal features of P. vulgata (a) and P. intermedia (c) are shown compared to the appearance of a 'transitional' individual (b) which has the black foot and marginal rays of P. intermedia and the transparent mantle tentacles of P. vulgata.

Fig. 5 Ventral View Of a) P. vulgata b) P. intermedia And c) P. aspera (× 1)

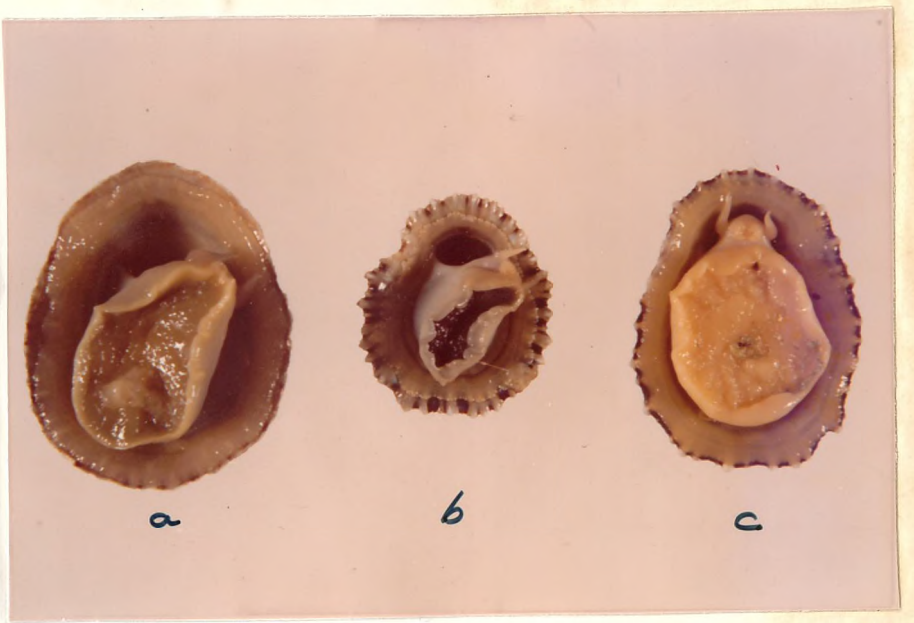
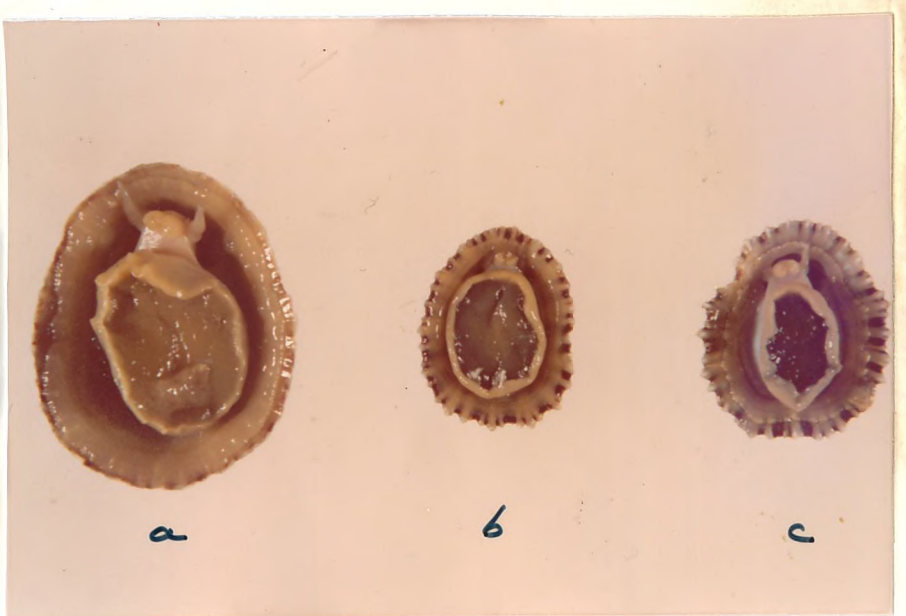


Fig. 6 Ventral View Of a) P. vulgata b) Transitional Form And c) P. intermedia (× 1)



Explanation of Fig. 7

This 'transitional' individual (b) has the distinct marginal rays of P. intermedia (c) but has the green foot and transparent mantle tentacles of P. vulgata (a)

Explanation of Fig. 8

The 'transitional' individual (b) has the marginal rays and white shell lining of P. intermedia (c) and the silver head scar normally found in P. vulgata (a).

Fig. 7 Ventral View Of a) P. vulgata b) Transitional Form And c) P. intermedia (× 1)

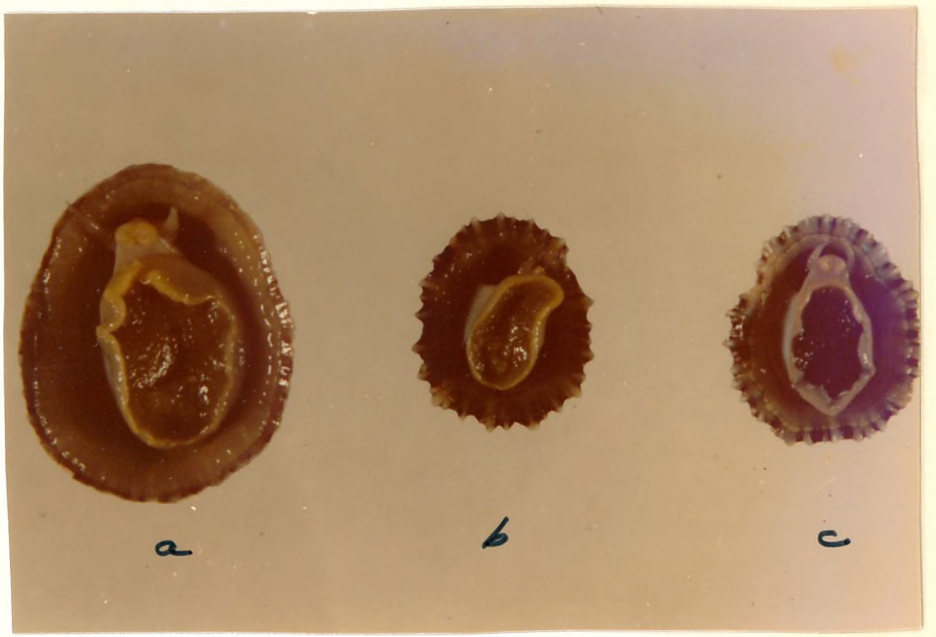
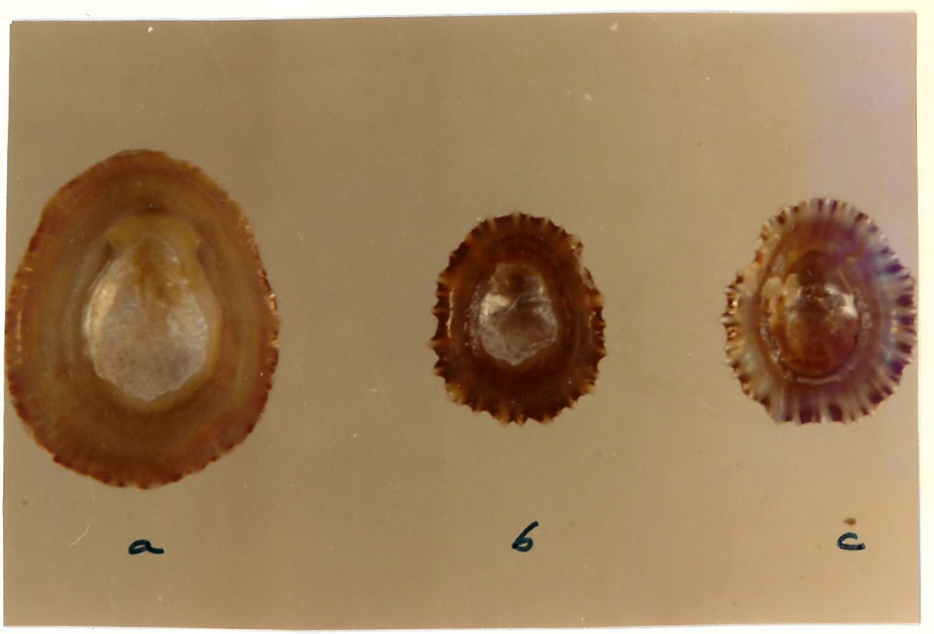


Fig. 8 Internal Shell Features Of a) P. vulgata, b) Transitional Form And c) P. intermedia (× 1)



CHAPTER III

A STUDY OF THE LIMPET POPULATION STRUCTURES OF TWO AREAS USING A SINGLE LINKAGE PROGRAMME

ADDITIONAL SURVEY OF LIMPET POPULATIONS FROM PORTLAND BILL AND THE ISLE OF WIGHT USING A SINGLE LINKAGE COMPUTER PROGRAMME.

Aims

It was decided that it was necessary to carry out a more detailed study of limpet populations from the south coast in order to demonstrate more accurately the increased variability to be found among the Isle Of Wight limpet populations. Therefore a single linkage computer programme was used to compare limpet populations from Portland Bill and from Freshwater Bay on the Isle Of Wight.

Methods

The data was collected from a random sample of eighty individuals from each area. For each individual the following six characters were noted.

The foot colour was recorded as being black, orange or green and the mantle tentacles as transparent, white or orange. After removing the body of the limpet from the shell, the shell lining was recorded as being either green, brown or white and the head scar as silver, mottled or orange. The brown rays running from the margin to the apex of the shell were noted as being present or absent and finally the ratio of the radula length over the shell length was established for each individual. This was recorded as being small (<1.0), medium ($1.0 - 2.0$) or large (>2.0). These data could then be simply transferred to computer cards and run through a single linkage programme.

The data used for the eighty individuals from Portland Bill and from the Isle Of Wight are shown in tables 7 to 10.

TABLE 7

THE SINGLE LINKAGE DATA TAKEN FROM PORTLAND BILL

N	Foot colour			Mantle tentacles			Shell lining			Head scar			Rays		Ratio R/C		
	G	B	O	T	W	O	G	B	W	S	M	O	+	-	1 (M)	2 (L)	3 (S)
1		+		+			+			+				+		+	
2	+			+			+			+				+		+	
3	+			+			+			+				+		+	
4			+			+			+			+		+	+		
5	+			+			+			+				+		+	
6	+			+			+			+				+		+	
7	+			+			+			+				+		+	
8		+			+			+			+		+			+	
9		+			+			+			+		+				+
10			+			+			+			+		+			
11	+			+			+			+				+		+	
12	+			+			+			+				+		+	
13			+			+			+			+		+	+		
14		+			+			+			+		+				+
15	+			+			+			+				+		+	
16			+			+			+			+		+	+		
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27		+			+			+			+		+				+
28		+			+			+			+		+				+
29		+			+			+			+		+				+
30		+			+			+			+		+			+	
31		+			+			+			+		+			+	
32		+			+			+			+		+				+
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34		+			+			+			+		+				+
35			+			+			+			+		+	+		
36		+			+			+			+		+				+
37			+			+			+			+		+		+	
38			+			+			+			+		+		+	
39		+			+			+			+		+				+
40		+			+			+			+		+				+

TABLE 8

THE SINGLE LINKAGE DATA TAKEN FROM PORTLAND BILL

N	Foot colour			Mantle tentacles			Shell lining			Head scar			Rays		Ratio R/C		
	G	B	O	T	W	O	G	B	W	S	M	O	+	-	1 (M)	2 (L)	3 (S)
41	+			+			+			+				+		+	
42	+			+			+			+				+		+	
43		+			+			+			+		+				+
44			+			+			+			+		+	+		
45			+			+			+			+		+	+		
46	+			+			+			+				+		+	
47			+			+			+			+		+	+		
48		+			+			+			+		+				+
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51	+			+			+			+				+		+	
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53	+			+			+			+				+		+	
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69		+			+			+			+		+				+
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71		+		+			+			+				+		+	
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74		+			+			+			+		+				+
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76			+			+			+			+		+	+		
77			+			+			+			+		+	+		
78			+			+			+			+		+	+		
79		+			+			+			+		+				+
80	+			+			+			+				+		+	

TABLE 9

THE SINGLE LINKAGE DATA TAKEN FROM THE ISLE OF WIGHT

N	Foot colour			Mantle tentacles			Shell lining			Head scar			Rays		Ratio R/C		
	G	B	O	T	W	O	G	B	W	S	M	O	+	-	1 (M)	2 (L)	3 (S)
1			+			+			+			+		+	+		
2	+			+				+		+			+			+	
3	+			+				+		+				+		+	
4		+			+		+			+			+			+	
5		+		+			+			+			+			+	
6		+		+			+				+		+			+	
7		+		+				+		+			+			+	
8			+			+			+			+		+	+		
9		+			+			+			+		+			+	
10		+			+		+				+		+			+	
11			+			+			+			+		+		+	
12	+			+			+			+				+		+	
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37			+			+			+			+		+	+		
38		+			+			+			+		+			+	
39			+			+			+			+		+	+		
40	+			+			+			+				+		+	

TABLE 10

THE SINGLE LINKAGE DATA TAKEN FROM THE ISLE OF WIGHT

N	Foot colour			Mantle tentacles			Shell lining			Head scar			Rays		Ratio R/C		
	G	B	O	T	W	O	G	B	W	S	M	O	+	-	1 (M)	2 (L)	3 (S)
41		+			+			+			+		+			+	
42	+			+			+			+			+			+	
43	+			+			+			+				+		+	
44	+			+			+			+				+		+	
45	+			+			+			+				+		+	
46			+			+			+			+		+	+		
47	+			+			+			+				+		+	
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76			+			+			+			+		+	+		
77	+			+			+			+			+			+	
78	+			+				+		+			+			+	
79	+			+			+				+		+			+	
80			+			+			+			+		+	+		

Results

The results are shown in figures 9 to 13 in the form of cluster grouping diagrams.

The computer uses a similarity coefficient to classify the limpets such that two individuals or groups of individuals with a similarity coefficient of 1.0 will be identical and conversely if they have a similarity coefficient of 0.0 they are completely different. The computer starts by grouping individuals with a similarity coefficient of 1.0 and lists all pairs of individuals that are identical to each other. At the next stage (called level 1) the computer uses a lower coefficient such as 0.833 and groups all individuals or groups of individuals which have five out of the six characters in common. In the case of the Portland Bill data this process was continued until all the limpets with a similarity coefficient of 0.166, that is with one out of the six characters in common, were grouped together. Obviously, if the computer continued this process it would eventually group together individuals with a similarity coefficient of 0.0 and the result would be one large group containing all the limpets. Since this would be of no use, the process is not continued to this stage.

In the diagrams for levels 1 and 2 for Portland Bill a majority of the individuals fall into one of the three species groupings. However there are a few individuals that are remaining separate up to level 3, although at level 3 all the individuals have become grouped into one of the three species groups.

By comparison the picture for the Isle Of Wight is very much more complicated. At level 1 there is a much larger number of individuals not falling into one of the main groupings and there is a complex pattern of interrelationships among the groups. At level 2 the individuals have not all become associated into three main groups as in the case for Portland Bill. Instead two large groups and two individuals have remained separate from the remainder of the population.

Explanatory note on linkage cluster diagrams

In each case the individuals within a circle have been grouped together at a previous higher level of similarity coefficient. Thus in Fig. 9 all the individuals grouped within a circle are identical to each other in all six characters. Similarly in Fig. 11 the individuals in each group have at least two out of six characters in common or more.

Where two groups are connected by a line, the groups have the number of characters in common as designated for that level by the similarity coefficient. Thus in Fig. 9 the individuals 72, 56 and 59 have five out of six characters in common with the large group labelled P. vulgata, since the similarity coefficient for that level is 0.833 (= 5/6). Similarly in Fig. 11 the two groups P. vulgata and P. intermedia have one character in common since they are connected at a level with a similarity coefficient of 0.166 (1/6).

The length of the lines connecting the groups has no significance.

Fig. 9 Single Linkage Cluster Groupings For Portland Bill.

Level 1. Similarity coefficient = 0.833

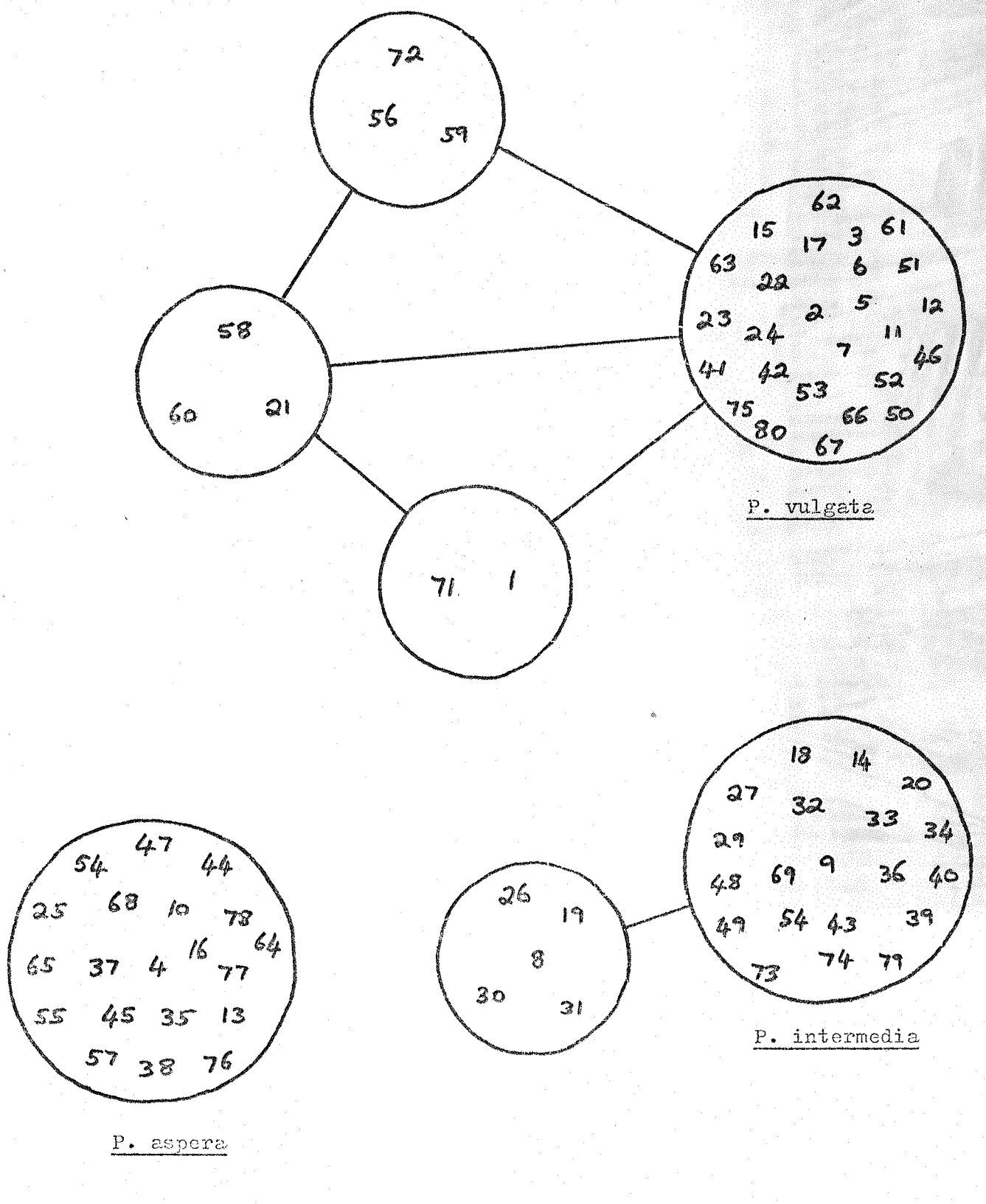


Fig. 10 Single Linkage Cluster Groupings For Portland Bill

Level 2. Similarity coefficient = 0.500

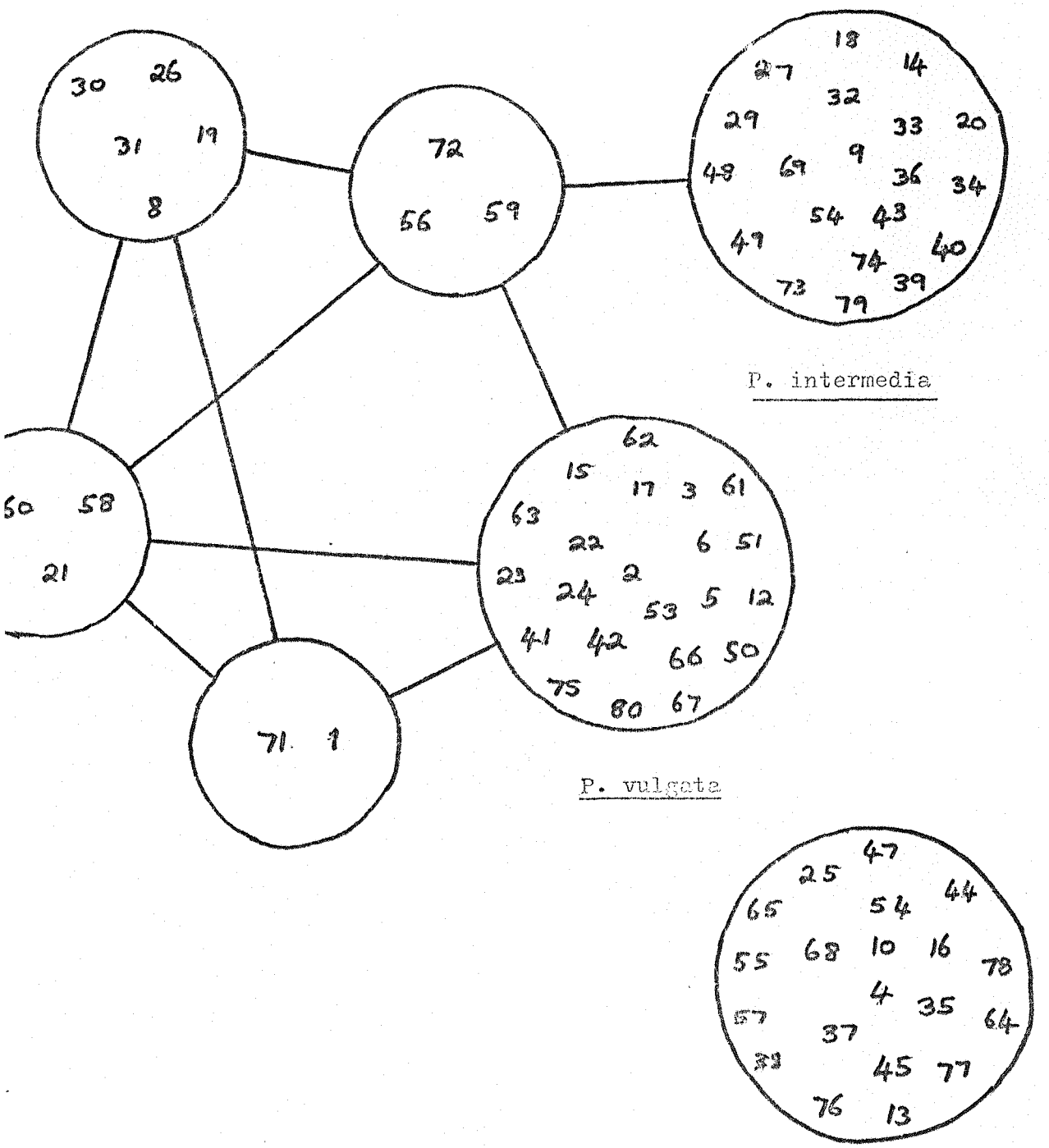


Fig. 11 Single Linkage Cluster Groupings For Portland Bill.

Level 3. Similarity coefficient = 0.166

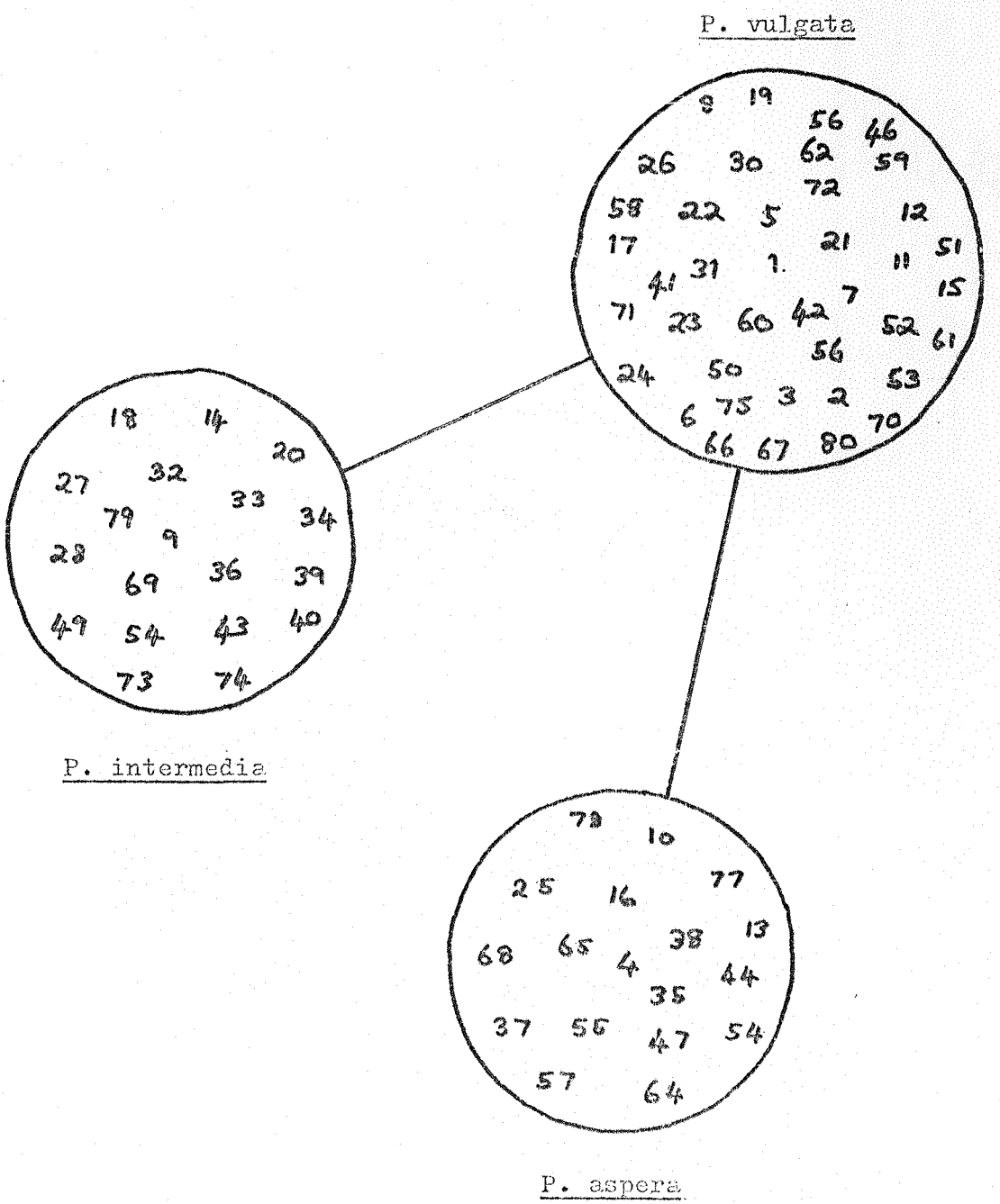


Fig. 12 Single Linkage Cluster Groupings For The Isle Of Wight.

Level 1. Similarity coefficient = 0.833

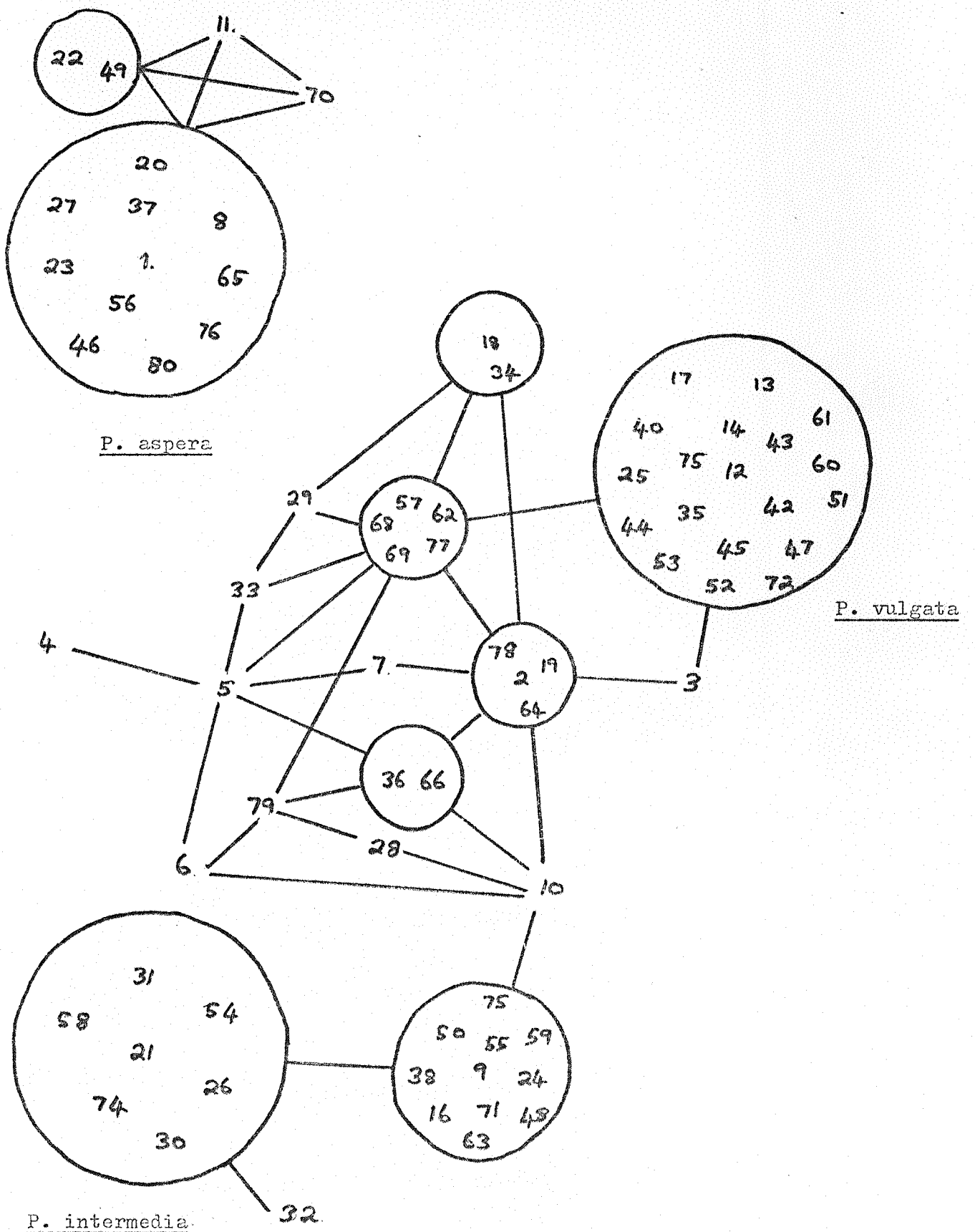
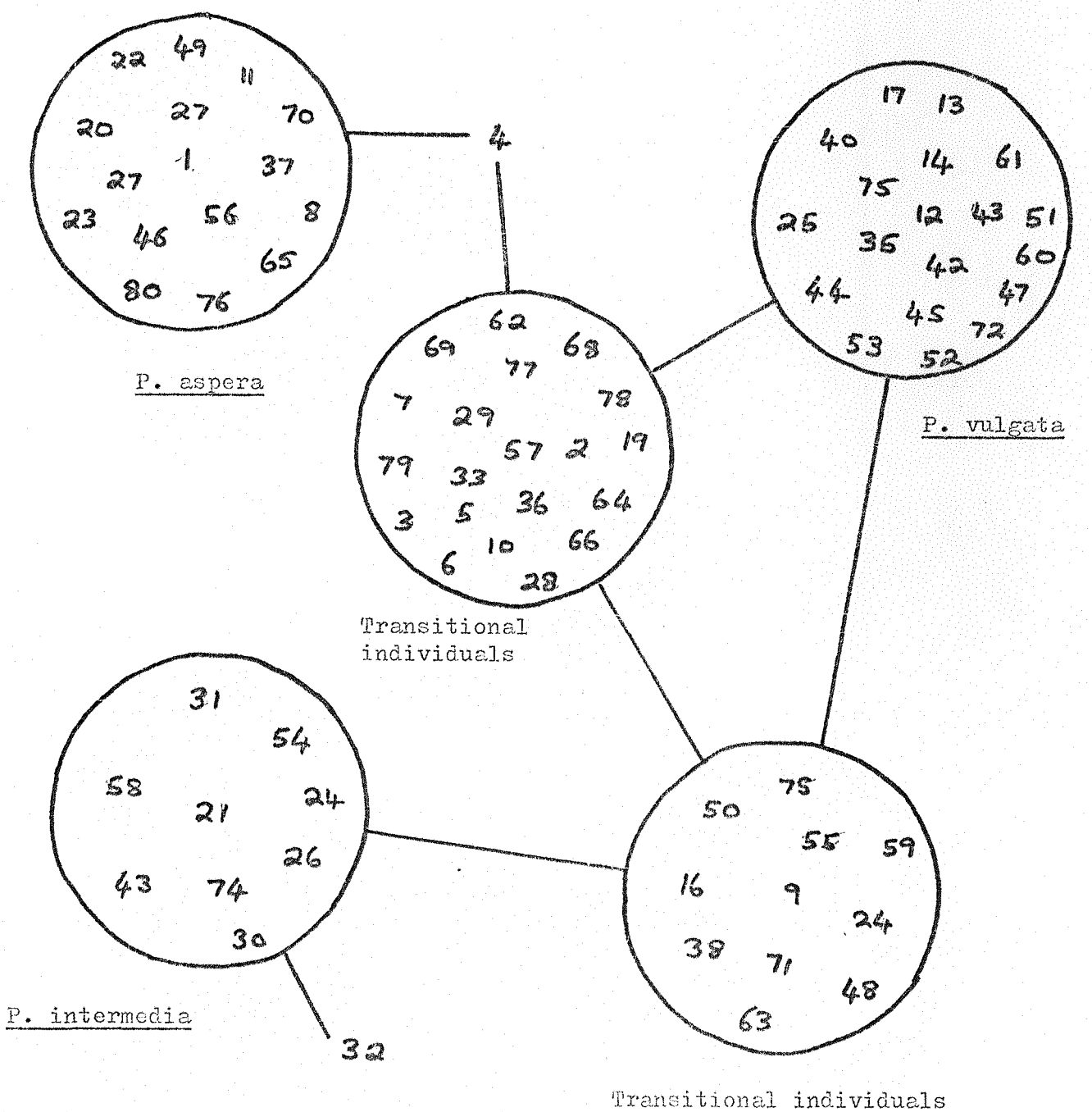


Fig. 13 Single Linkage Cluster Groupings For The Isle Of Wight.

Level 2. Similarity coefficient = 0.333



It is also interesting to note that in the data for both Portland Bill and the Isle Of Wight the P. aspera group has remained completely separate from any other group until the final level.

Conclusions

The Portland Bill data reflects a certain amount of variability, particularly amongst the P. vulgata population with a lesser amount of variability seen in the P. intermedia population. However, it can be clearly seen that the entire population has finally become separable into three clearly defined species groups.

In the case of the Isle Of Wight a much larger amount of variability is exhibited and is mainly due to individuals most closely related to P. vulgata. Finally there remain two fairly large groups separate from the three main groups but which are associated with both the P. vulgata and the P. intermedia populations. These groups represent the high proportion of 'transitional' forms on the Isle Of Wight that are exhibiting similarities to both the P. vulgata and the P. intermedia types.

In both cases the data reflects well the fact that P. aspera is retaining a clearly distinct identity on the Isle Of Wight as well as further west and is showing little involvement with the phenomenon of 'intermediacy' found on the south coast of Britain.

CHAPTER IV

A SURVEY OF THE BREEDING CYCLES OF THE THREE LIMPET SPECIES AND OF THE 'TRANSITIONAL' FORMS

A STUDY OF THE REPRODUCTIVE CYLCES OF THE THREE LIMPET SPECIES AND OF THE 'TRANSITIONAL' FORMS

Aims

The purpose of this study was to investigate the reproductive cycles of the three limpet species in order to establish whether the breeding cycles overlap to any extent, thus making hybridisation feasible.

It was also necessary to establish the reproductive cycle of the transitional forms since this may provide information concerning their relationships to the three species.

In order to do this the reproductive condition of the limpets was surveyed throughout the year and permanent preparations of the gonads were made as records of the different stages in the cycle.

Methods

During each month of the year 200 individuals of the three species and of the transitional forms were collected from Freshwater Bay and the condition of the gonads was investigated by means of dissection. In 1956 Southward, Orton and Dodd invented a scale for defining the state of development of the gonad. This scale consisted of a neutral stage, a developing stage which was divided into five sections and a spawning or regressive stage which was divided into four sections. The methods by which these workers classified the gonads into one of the ten stages was basically subjective and depended on the shape, size and colour of the gonad. Therefore an additional cytological criterion was used. This involved placing a smear of the gonad on a slide in a drop of sea water and examining it under the microscope. This method proved to be a quick way to distinguish between the sections of the development stage since in early development the relative proportion of collagen material to eggs or sperm is greater than in later development, which can be clearly seen in figures 14 to 17.

However, this method is of no use in separating the different spawning sections since, during the spawning stage, the proportion

of collagen to sperm or eggs remains constant, the gonad merely decreasing in size. This fact meant that the method could be used to separate two gonads of comparative size and colour into developing or spawning stages.

In order to study the different stages in more detail and to have positive evidence that the three species were producing fertile eggs and sperm at the same time, permanent preparations of the gonads of the three species were made for each different stage.

A small section of gonad was removed from the animal and fixed in Bouin's fixative overnight. The tissue was then shaken in two changes of 70% alcohol and cleared in Cedarwood Oil. This was found to be preferable to clearing in xylene which tended to make the tissue brittle. When the tissue had cleared it was transferred to liquid paraffin at 60°C for one hour. It was then put in two more changes of liquid paraffin for one hour each. The tissue was then embedded in paraffin in a watch glass and allowed to harden overnight. The tissue could then be section at 7 µm thickness on a Cambridge rocking microtome and the sections stained using the standard Mallory's technique or an iron haemotoxylin stain. The sections were then dehydrated and mounted in Canada Balsam.

Results

The breeding cycle was studied over a period of two years and the results are given in tables 11 to 18 and in the graphs shown in figures 18 and 19.

TABLE 11

PERCENTAGE OF INDIVIDUALS OF P. VULGATA WITH GONADS IN THE VARIOUS STAGES SHOWN THROUGHOUT THE YEAR 1974

N.B. In the case of each month percentages have been taken from a sample of 200 individuals.

	Neut.	I	II	III	IV	V	IV	III	II	I
Jan.	9.5	-	-	-	-	17.0	10.0	23.5	28.0	12.0
Feb.	61.0	-	-	-	-	-	-	2.5	4.0	32.5
Mar.	56.0	-	-	-	-	-	5.5	8.0	10.5	20.0
Apr.	74.0	-	-	-	-	-	-	6.0	3.0	17.0
May	85.0	-	-	-	-	-	-	3.5	2.0	9.5
June	96.0	-	-	-	-	-	-	-	1.0	3.0
July	18.0	53.5	23.5	5.0	-	-	-	-	-	-
Aug.	-	8.5	15.0	42.0	20.5	14.0	-	-	-	-
Sept.	-	3.0	-	11.0	33.0	52.0	-	-	-	-
Oct.	-	-	-	5.5	21.5	73.0	-	-	-	-
Nov.	-	-	-	-	11.0	85.0	4.0	-	-	-
Dec.	-	-	-	-	-	70.0	23.0	7.0	-	-

TABLE 12

PERCENTAGE OF INDIVIDUALS OF P. VULGATA WITH GONADS IN THE VARIOUS STAGES SHOWN THROUGHOUT THE YEAR 1975

	Neut.	I	II	III	IV	V	IV	III	II	I
Jan.		-	-	-	-	63.5	19.5	17.0	-	-
Feb.	4.5	-	-	-	-	28.0	32.0	27.0	2.0	6.5
Mar.	20.0	-	-	-	-	20.0	25.0	10.0	10.0	15.0
Apr.	53.5	-	-	-	-	-	-	2.0	8.5	36.0
May	100.0	-	-	-	-	-	-	-	-	-
June	100.0	-	-	-	-	-	-	-	-	-
July	35.5	60.0	4.5							
Aug.	10.0	52.5	29.5	8.0	-	-	-	-	-	-
Sept.	-	-	18.0	43.5	29.0	9.5	-	-	-	-
Oct.	-	-	-	16.0	45.0	39.0	-	-	-	-
Nov.	-	-	-	-	14.0	80.0	6.0	-	-	-
Dec.	-	-	-	-	-	75.5	19.0	5.5	-	-

TABLE 13

PERCENTAGE OF INDIVIDUALS OF P. INTERMEDIA WITH GONADS IN THE VARIOUS STAGES SHOWN THROUGHOUT THE YEAR 1974

	Neut.	I	II	III	IV	V	IV	III	II	I
Jan.										
Feb.	-	11.5	17.5	18.0	47.5	5.5	-	-	-	-
Mar.	-	4.5	4.0	12.5	34.0	45.0	-	-	-	-
Apr.	-	-	2.0	7.0	37.0	54.0	-	-	-	-
May	-	-	1.5	3.0	32.0	63.5	-	-	-	-
June	-	-	-	-	25.0	75.0	-	-	-	-
July	4.5	-	-	-	23.5	34.0	17.0	17.5	-	3.5
Aug.	-	-	-	-	-	33.0	52.0	15.0	-	-
Sept.	-	-	-	-	-	31.5	45.5	18.5	4.5	-
Oct.	-	-	-	-	-	30.0	42.0	16.0	6.0	-
Nov.	6.5	-	-	-	-	26.5	42.0	19.5	2.0	3.5
Dec.	28.0	-	-	-	-	25.5	36.0	5.5	2.0	3.0

TABLE 14

PERCENTAGE OF INDIVIDUALS OF P. INTERMEDIA WITH GONADS IN THE VARIOUS STAGES SHOWN THROUGHOUT THE YEAR 1975

	Neut.	I	II	III	IV	V	IV	III	II	I
Jan.	35.5	-	-	-	-	18.5	21.0	16.0	4.5	4.5
Feb.	74.0	-	-	-	-	1.5	3.5	7.0	8.0	6.0
Mar.	100.0	-	-	-	-	-	-	-	-	-
Apr.	100.0	-	-	-	-	-	-	-	-	-
May	66.0	28.0	5.5	0.5	-	-	-	-	-	-
June	23.0	20.0	19.5	30.0	2.0	5.5	-	-	-	-
July	4.5	14.0	2.0	20.0	16.5	43.0	-	-	-	-
Aug.	-	-	-	11.0	13.0	72.0	4.0	-	-	-
Sept.	-	-	-	-	-	83.0	15.0	2.0	-	-
Oct.	-	-	-	-	-	41.5	30.0	18.0	10.0	0.5
Nov.	-	-	-	-	-	28.5	29.5	15.0	18.5	8.5
Dec.	10.5	-	-	-	-	25.0	18.0	21.5	10.0	15.0

TABLE 15

PERCENTAGE OF INDIVIDUALS OF P. ASPERA WITH GONADS IN THE VARIOUS STAGES SHOWN THROUGHOUT THE YEAR 1974

	Neut.	I	II	III	IV	V	IV	III	II	I
Jan.	10.0	-	-	-	-	40.5	16.5	18.0	3.5	11.5
Feb.	25.5	-	-	-	-	11.0	19.0	19.0	7.5	18.0
Mar.	62.0	-	-	-	-	-	10.0	5.0	10.0	30.0
Apr.	63.0	-	-	-	-	-	3.5	2.5	14.0	17.0
May	35.5	42.5	17.0	4.0	1.0	-	-	-	-	-
June	15.5	18.5	10.0	8.5	6.5	1.0	-	-	-	-
July	-	5.5	10.0	9.5	40.0	35.0	-	-	-	-
Aug.	-	-	-	-	11.0	74.0	9.0	6.0	-	-
Sept.	-	-	-	-	11.5	52.0	25.0	10.0	1.5	-
Oct.	-	-	-	-	2.0	55.0	27.0	12.0	4.0	-
Nov.	-	-	-	-	-	43.5	31.5	18.0	7.0	-
Dec.	8.5	-	-	-	-	26.0	33.5	20.5	9.0	2.5

TABLE 16

PERCENTAGE OF INDIVIDUALS OF P. ASPERA WITH GONADS IN THE VARIOUS STAGES SHOWN THROUGHOUT THE YEAR 1975

	Neut.	I	II	III	IV	V	IV	III	II	I
Jan.	44.5	-	-	-	-	3.5	14.0	17.0	10.0	11.0
Feb.	92.0	-	-	-	-	-	-	-	-	8.0
Mar.	100.0	-	-	-	-	-	-	-	-	-
Apr.	100.0	-	-	-	-	-	-	-	-	-
May	100.0	-	-	-	-	-	-	-	-	-
June	100.0	-	-	-	-	-	-	-	-	-
July	73.5	13.5	13.0	-	-	-	-	-	-	-
Aug.	41.0	7.5	26.0	4.5	13.5	7.5	-	-	-	-
Sept.	3.0	16.0	20.0	20.5	18.5	5.5	16.5			
Oct.	-	-	-	14.5	11.0	70.0	0.5	4.0	-	-
Nov.	-	-	-	-	-	66.0	24.0	10.0	-	-
Dec.	5.0	-	-	-	-	46.0	14.5	15.0	3.5	16.0

TABLE 17

PERCENTAGE OF TRANSITIONAL INDIVIDUALS WITH GONADS IN THE VARIOUS STAGES SHOWN THROUGHOUT THE YEAR 1974

	Neut.	I	II	III	IV	V	IV	III	II	I
Jan.	22.0	-	-	-	-	15.5	14.0	27.0	5.5	16.0
Feb.	40.5	-	-	-	-	10.0	2.5	19.0	13.5	14.5
Mar.	83.0	-	-	-	-	3.0	0.5	9.0	2.0	2.5
Apr.	100.0	-	-	-	-	-	-	-	-	-
May	72.5	16.5	0.5	11.5	-	-	-	-	-	-
June	49.5	26.0	4.5	8.0	8.0	4.0	-	-	-	-
July	33.5	18.0	20.5	6.5	9.5	12.0	-	-	-	-
Aug.	4.5	29.0	17.0	5.0	14.0	26.0	4.5	-	-	-
Sept.	-	11.0	20.0	5.0	19.5	43.5	1.0	-	-	-
Oct.	-	-	-	24.0	21.0	51.0	-	4.0	-	-
Nov.	-	-	-	4.0	6.0	77.0	13.0	-	-	-
Dec.	-	-	-	-	-	52.0	30.5	7.5	10.0	-

TABLE 18

PERCENTAGE OF TRANSITIONAL INDIVIDUALS WITH GONADS IN THE VARIOUS STAGES SHOWN THROUGHOUT THE YEAR 1975

	Neut.	I	II	III	IV	V	IV	III	II	I
Jan.	9.5	-	-	-	-	25.0	2.5	29.0	13.5	20.5
Feb.	17.0	-	-	-	-	20.0	-	42.5	4.0	16.5
Mar.	33.0	-	-	-	-	-	7.5	20.5	14.5	24.5
Apr.	86.5	-	-	-	-	-	-	-	11.5	2.0
May	100.0	-	-	-	-	-	-	-	-	-
June	100.0	-	-	-	-	-	-	-	-	-
July	92.5	-	7.5	-	-	-	-	-	-	-
Aug.	-	21.5	19.0	31.5	10.0	18.0	-	-	-	-
Sept.	-	1.0	16.0	29.0	27.5	26.5	-	-	-	-
Oct.	-	-	-	5.0	61.0	31.0	-	3.0	-	-
Nov.	-	-	-	-	26.0	64.0	5.0	5.0	-	-
Dec.	-	-	-	-	-	82.5	10.0	-	7.5	-

Explanation of Figs. 14 and 15

In the stage II testis the proportion of connective tissue in the gonad is relatively high. Spermatozoa can be seen projecting into the interior of the seminiferous tubules and there are large areas containing differentiating germ cells.

In the stage V testis the proportion of structural material has considerably decreased. The seminiferous tubules are packed with fully developed spermatozoa and spermatid nuclei can be seen along the walls of the tubules.

c.t. = connective tissue

d.g.c. = differentiating germ cells

s.t. = seminiferous tubules

sp. n. = spermatid nuclei

sp. = spermatozoa

Fig. 14 Section Of P. vulgata Testis At Stage II. (x 100)

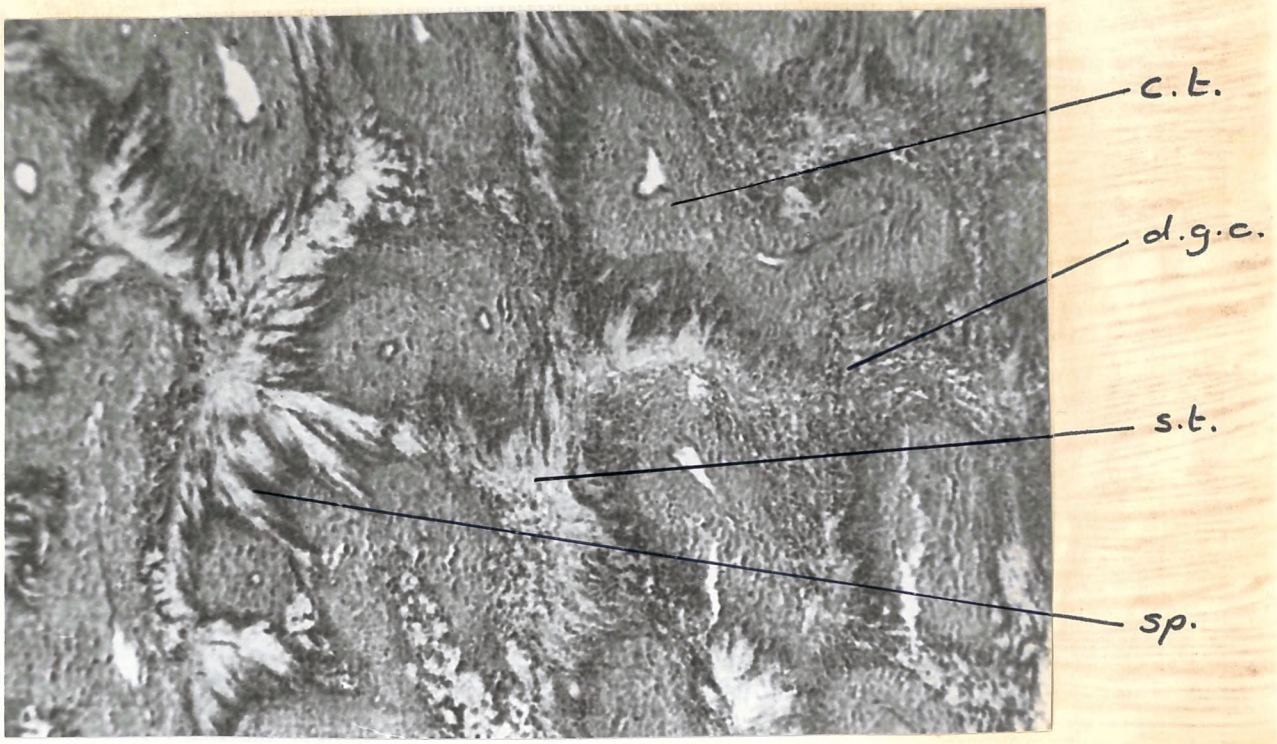


Fig. 15 Section Of P. vulgata Testis At Stage V. (x 100)

Explanation of Figs. 16 and 17

In the stage II ovary the connective tissue trabeculae are well developed and the germinal layers contain germ cells at various stages of oogenesis. There are a few large oocytes present.

The stage V ovary contains very little connective tissue and is packed with fully developed eggs containing germinal vesicles.

c.t.t. = connective tissue trabeculae

d.g.c. = differentiating germ cells

e. = fully developed egg.

g.l. = germinal layer of trabeculae

g.v. = germinal vesicle

o. = oocyte

Fig. 16 Section Of P. vulgata Ovary At Stage II. (x 100)

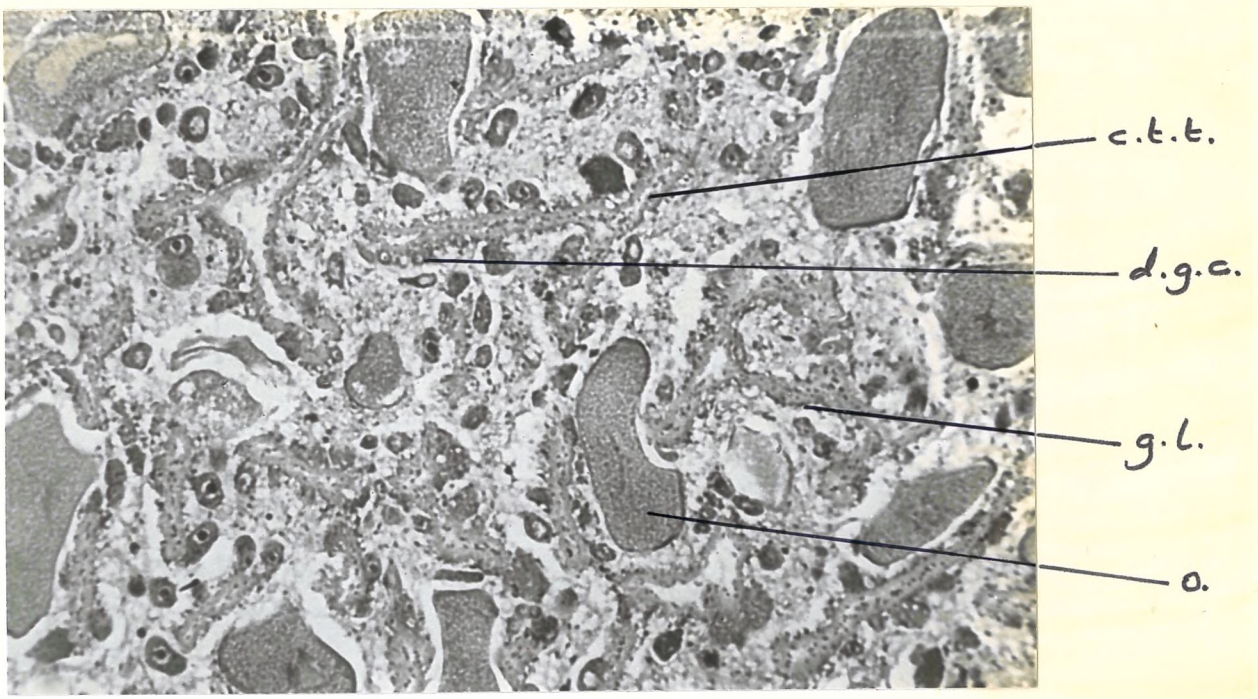


Fig. 17 Section Of P. vulgata Ovary At Stage V. (x 100)

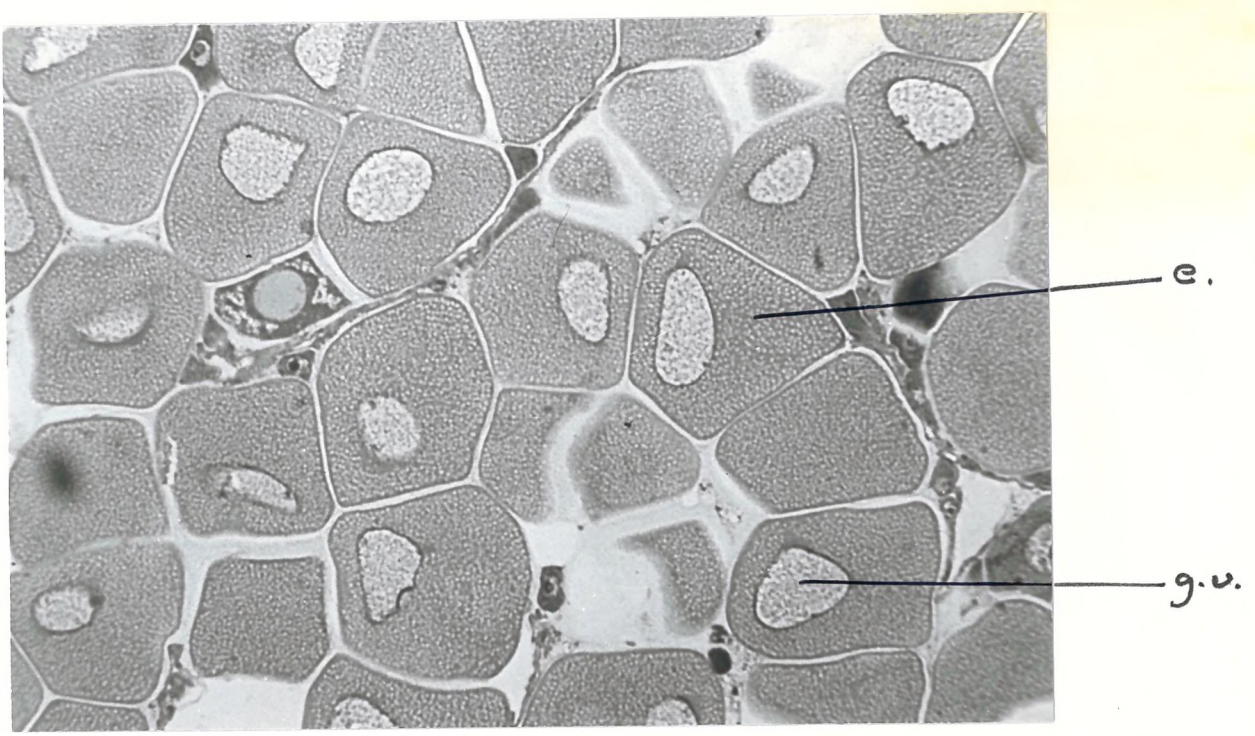


Fig. 18 The Percentage Of Individuals Of P. vulgata P. intermedia, P. aspera and Transitional Forms With Mature Gonads Throughout The Year 1974.

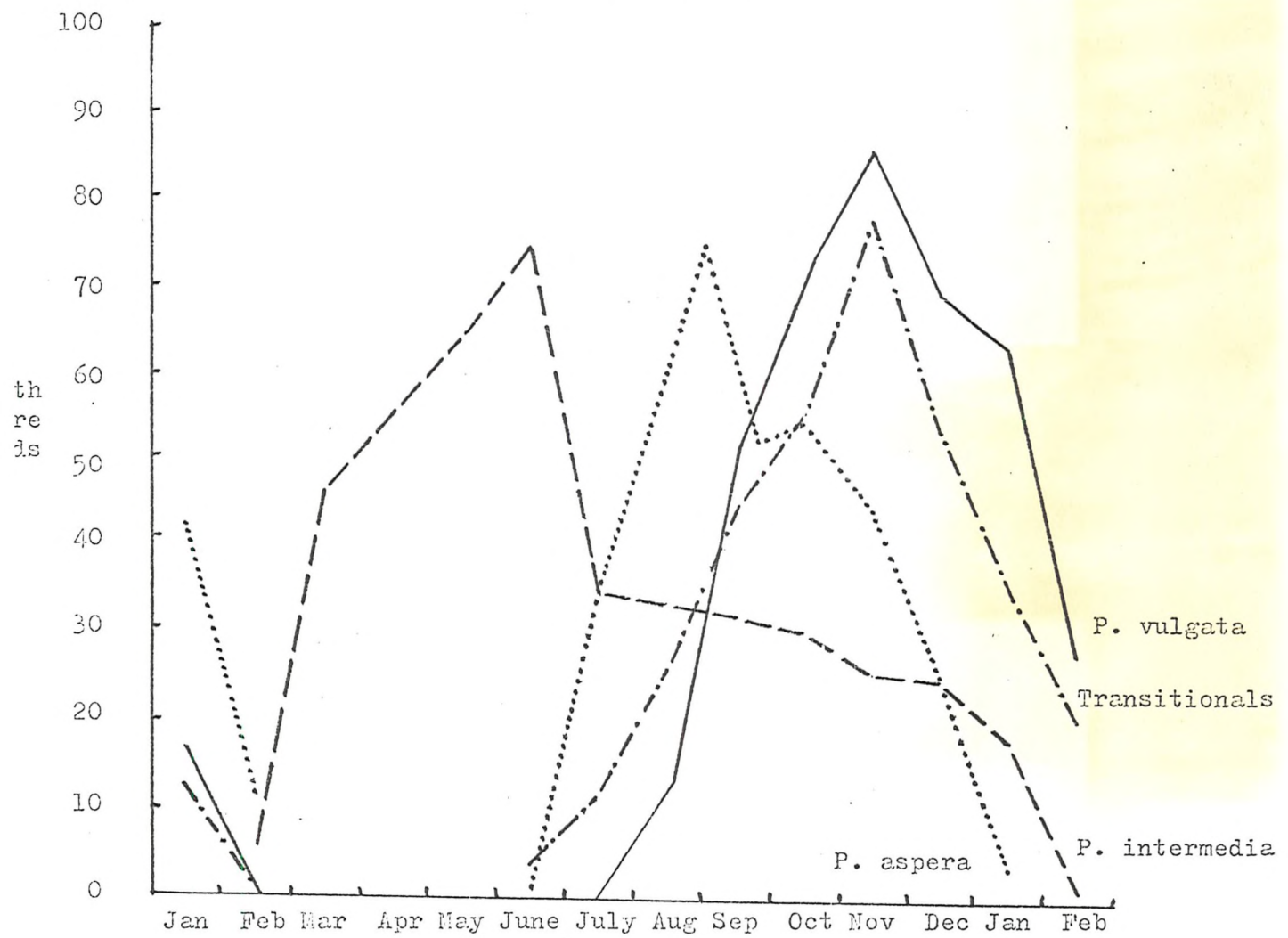
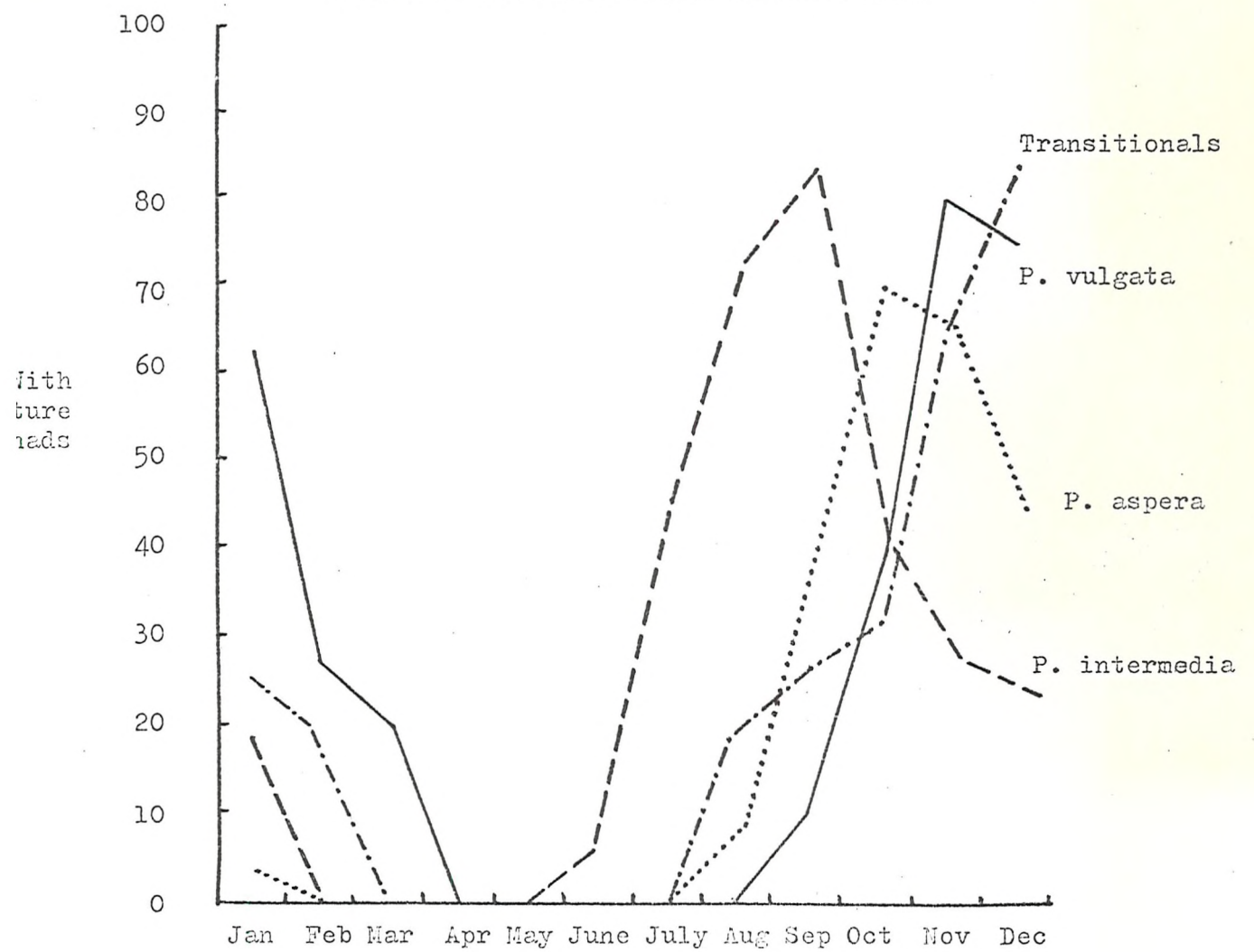


Fig. 19 The Percentage Of Individuals Of P. vulgata, P. intermedia, P. aspera and Transitional Forms With Mature Gonads Throughout The Year 1975.



A comparison of the graphs for 1974 and 1975 clearly shows that the breeding cycles of all three species and of the transitional forms were slightly different for the two consecutive years. In all four groups a peak of reproductive condition was reached earlier in 1974 than in 1975. This type of variation may be normal or may have been influenced by the fact that the winter of 1974 was exceptionally mild. This may have resulted in the limpets remaining in breeding condition till later in the year of 1974, thus causing breeding to commence later in the following year.

In relative terms the pattern of reproductive activity of the four groups was similar during both years. P. intermedia comes into breeding condition first and reaches a peak of activity from June to August. P. aspera reaches a peak of reproductive activity from July to October whereas P. vulgata and the transitional forms are in peak breeding condition from November to January.

Conclusions

It can be seen from these results that although the breeding cycles of the three species differ to some extent, the degree of overlap of reproductive activity is considerable, since from August to November a substantial number of individuals of all three species and of the transitional forms have fully mature gonads. Thus, with respect to the timing of the breeding cycles, there is no reason why hybridisation should not occur between all three species.

It is interesting to note the similarity of the breeding cycles of the transitional forms and of P. vulgata since this might suggest that the transitional forms are more closely related to P. vulgata than to the other two species.

CHAPTER V

HYBRIDISATION EXPERIMENTS BETWEEN THE THREE LIMPET SPECIES AND THE 'TRANSITIONAL' FORMS

HYBRIDISATION EXPERIMENTS

Aims

The purpose of this section of the work was to determine whether hybridisation between the three limpet species could be successfully achieved in the laboratory, in order to either support or eliminate the theory that the transitional forms of limpets found on the Isle Of Wight are in fact hybrids between two different species.

Firstly it was necessary to carry out normal fertilisations of the three species to determine the normal survival rates of the larvae at successive developmental stages. This could then be compared with the survival rates of larvae that are produced by hybrid crosses between the species.

During the same experiments the time taken by the larvae of the normal fertilisations to reach the various developmental stages could be noted and compared with the development rates of the hybrid larvae.

It was later decided to carry out normal fertilisations between the transitional forms since, if they are in fact true hybrids, there is a possibility of their being infertile. The transitional forms were also crossed with the three species because it was thought that this might provide some information concerning how closely they are related to the three different species.

Methods

Normal fertilisations and crosses between the three species were carried out during the summer of 1974 and 1975. The experiments involving the transitional forms were performed later during the summer of 1976. Experiments were continued during several months since the fertility of the different species will obviously vary from one period of the breeding season to another.

As far as possible conditions including temperature were kept as constant as possible for all the breeding experiments in order to make comparisons possible.

Initially 'instant ocean' was used during these experiments but it was later found to be more convenient to use ordinary sea water. This was sterilised in medical flats in an autoclave, in order to kill any Patella sperm that might be present and also to kill any protozoa which may later increase in numbers and use up available oxygen. Since the oxygen is boiled out of the water during autoclaving the water must be shaken before use. The water was kept in an incubator at approximately 12°C until it was used so that it would be at the correct temperature. During the experiment the laboratory must be kept cool in order to prevent the eggs and sperm being killed by fluctuations in temperature. The gonad is removed from the animal and placed gently in a petri-dish containing the sterilised water. The eggs and sperm will then separate naturally into the water and no shaking is necessary. The gonads are then removed from the dish.

Before the eggs and sperm are brought together they must be checked under the microscope to ensure that they are in good condition. Although the eggs tend to be rather angular at first due to being packed into the ovary they should become well rounded after being left in sea water for a few minutes and should also have a clearly defined germinal vesicle. The sperm in the extract, taken from the petri-dish, should be numerous and active.

Having decided that the eggs and sperm are healthy and fully developed, 100 eggs are placed in another petri-dish containing 50 ml. of sterilised sea water and ten drops of sperm extract added to the dish which is then placed in the incubator. The eggs are checked every $\frac{1}{4}$ hour to monitor their progress. Once fertilisation is known to have occurred by the disappearance of the germinal vesicles the excess sperm are decanted off the eggs which are placed in fresh sea water. The eggs are then looked at every half an hour and the time taken to reach the various developmental stages noted, the survival rate at each stage being recorded

at the same time. When the trochophore larva stage is reached they are transferred to finger bowls of sea water in the incubator where they will develop into veliger larvae.

When cross-fertilisation is carried out the same procedure is adopted except that, for example, P. intermedia sperm are added to P. aspera eggs and vice-versa. The controls consist of normal fertilisations of P. intermedia and P. aspera using the same eggs and sperm as used in the crosses. Great care must be taken to ensure that the sperm of the different species is kept strictly separate.

The veliger larvae survived for about two days only since at this stage they begin to actively feed and it was decided that there was no point trying to keep them alive beyond this stage. Photographs of certain developmental stages of P. vulgata are shown in figures 20 to 25.

Results

The results of the breeding experiments are given in the following tables 19 to 54. In the abbreviations for the crosses the female is written first so that the cross V/I involves a P. vulgata female and a P. intermedia male and vice-versa.

The time taken for the germinal vesicles to break down is given in minutes whereas the other time measurements are in hours.

The average survival rates of the larvae of the various crosses are shown in table 55. The average times taken by the various larvae to reach successive developmental stages are shown in table 56. In cases where the eggs or sperm of an individual have proved to be infertile shown by the fact that the normal fertilisations were unsuccessful, the figures for any crosses involving this individual have been omitted.

It can be seen that the percentages of larvae of the normal fertilisations of the three species surviving to the second day veliger stage are all approximately 42%. The most successful

EXPERIMENT 1. JUNE 13TH 1974

TABLE 19

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR VARIOUS CROSSES BETWEEN P. VULGATA, P. ASPERA AND P. INTERMEDIA

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera;

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	0	0	0	95	0	69	70	0	44
First cleavage	0	0	0	80	0	55	62	0	38
Second cleavage	0	0	0	80	0	55	60	0	38
Third cleavage	0	0	0	80	0	50	56	0	35
Trochophore	0	0	0	69	0	46	50	0	30
First day veliger	0	0	0	45	0	15	25	0	12
Second day veliger	0	0	0	39	0	13	21	0	11

TABLE 20

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	-	-	-	10-20	-	10-20	10-20	-	10-20
First cleavage	-	-	-	4-4½	-	4-4½	4-4½	-	4-4½
Trochophore	-	-	-	18-20	-	18-20	18-20	-	18-20
First day veliger	-	-	-	54	-	54	54	-	54

EXPERIMENT 2. JUNE 25TH 1974

TABLE 21

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR VARIOUS CROSSES BETWEEN P. VULGATA, P. ASPERA AND P. INTERMEDIA

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera;

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	0	0	0	90	0	47	73	0	60
First cleavage	0	0	0	81	0	45	70	0	50
Second cleavage	0	0	0	80	0	44	70	0	50
Third cleavage	0	0	0	80	0	44	69	0	49
Trochophore	0	0	0	66	0	39	55	0	40
First day veliger	0	0	0	60	0	39	48	0	36
Second day veliger	0	0	0	60	0	39	48	0	36

TABLE 22

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	-	-	-	10-20	-	10-20	10-20	-	10-20
First cleavage	-	-	-	4-4½	-	4-4½	4-4½	-	4-4½
Trochophore	-	-	-	18-20	-	18-20	18-20	-	18-20
First day veliger	-	-	-	54	-	54	54	-	54

EXPERIMENT 3. AUGUST 2ND 1974

TABLE 23

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR VARIOUS CROSSES BETWEEN P. VULGATA, P. ASPERA AND P. INTERMEDIA

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera;

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	90	78	60	95	55	21	87	45	30
First cleavage	84	61	36	82	40	10	75	29	18
Second cleavage	80	61	28	77	38	10	73	25	16
Third cleavage	79	61	28	72	38	9	70	20	15
Trochophore	64	35	11	58	19	2	58	12	0
First day veliger	64	26	5	51	13	0	58	7	0
Second day veliger	64	26	4	50	13	0	53	7	0

TABLE 24

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	5-10	5-10	5-10	10-20	10-20	10-20	10-20	10-20	10-20
First cleavage	3-3½	3-3½	3-3½	4-4½	4-4½	4-4½	4-4½	4-4½	4-4½
Trochophore	18-20	18-20	18-20	18-20	18-20	18-20	18-20	18-20	-
First day veliger	54	54	54	54	54	-	54	54	-

EXPERIMENT 4. SEPTEMBER 10TH 1974

TABLE 25

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR VARIOUS CROSSES BETWEEN P. VULGATA, P. ASPERA AND P. INTERMEDIA

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	90	82	70	95	67	40	90	66	46
First cleavage	80	68	41	86	54	25	80	51	30
Second cleavage	76	58	40	80	50	19	68	49	30
Third cleavage	76	58	40	79	50	18	68	43	30
Trochophore	61	45	29	63	36	12	65	20	14
First day veliger	50	37	13	55	30	6	43	15	9
Second day veliger	50	37	13	52	21	5	43	15	8

TABLE 26

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	5-10	5-10	5-10	10-20	10-20	10-20	10-20	10-20	10-20
First cleavage	3-3½	3-3½	3-3½	4-4½	4-4½	4-4½	4-4½	4-4½	4-4½
Trochophore	18-20	18-20	18-20	18.20	18-20	18-20	18-20	18-20	18-20
First day veliger	54	54	54	54	54	54	54	54	54

EXPERIMENT 5. SEPTEMBER 27TH 1974

TABLE 27

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR VARIOUS CROSSES BETWEEN P. VULGATA, P. ASPERA AND P. INTERMEDIA

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera;

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	95	60	65	83	70	42	95	55	29
First cleavage	77	48	50	66	60	31	81	42	12
Second cleavage	70	48	50	66	58	31	81	36	12
Third cleavage	70	48	50	59	58	27	80	36	7
Trochophore	51	33	35	43	35	18	64	22	0
First day veliger	50	19	29	34	28	13	60	21	0
Second day veliger	50	10	25	34	28	13	59	21	0

TABLE 28

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	5-10	5-10	5-10	10-20	10-20	10-20	10-20	10-20	10-20
First cleavage	3 $3\frac{1}{2}$	3-$3\frac{1}{2}$	3-$3\frac{1}{2}$	4-$4\frac{1}{2}$	4-$4\frac{1}{2}$	4-$4\frac{1}{2}$	4-$4\frac{1}{2}$	4 $4\frac{1}{2}$	4-$4\frac{1}{2}$
Trochophore	18-20	18-20	18-20	18-20	18-20	18-20	18-20	18-20	-
First day veliger	54	54	54	54	54	54	54	54	-

EXPERIMENT 6. OCTOBER 11TH 1974

TABLE 29

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR VARIOUS CROSSES BETWEEN P. VULGATA, P. ASPERA AND P. INTERMEDIA.

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	85	72	66	90	80	50	95	61	22
First cleavage	84	57	21	77	63	38	82	40	16
Second cleavage	70	57	18	77	62	30	74	40	16
Third cleavage	70	54	18	77	55	30	74	31	8
Trochophore	59	33	7	43	23	4	60	2	5
First day veliger	40	29	2	43	17	0	36	0	1
Second day veliger	38	29	2	40	17	0	30	0	1

TABLE 30

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	5-10	5-10	5-10	10-20	10-20	10-20	10-20	10-20	10-20
First cleavage	3-3½	3-3½	3-3½	4-4½	4-4½	4-4½	4-4½	4-4½	4-4½
Trochophore	18-20	18-20	18-20	18-20	18-20	18-20	18-20	18-20	18-20
First day veliger	54	54	54	54	54	-	54	-	54

EXPERIMENT 7. JULY 20TH 1975

TABLE 31

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR VARIOUS CROSSES BETWEEN P. VULGATA, P. ASPERA AND P. INTERMEDIA.

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera;

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	52	32	0	90	46	0	0	0	0
First cleavage	35	27	0	85	25	0	0	0	0
Second cleavage	35	26	0	80	25	0	0	0	0
Third cleavage	35	21	0	80	13	0	0	0	0
Trochophore	19	12	0	54	11	0	0	0	0
First day veliger	6	7	0	23	3	0	0	0	0
Second day veliger	1	7	0	23	3	0	0	0	0

TABLE 32

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES.

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	5-10	5-10	-	10-20	10-20	-	-	-	-
First cleavage	3-3½	3-3½	-	4-4½	4-4½	-	-	-	-
Trochophore	18-20	18-20	-	18-20	18-20	-	-	-	-
First day veliger	54	54	-	54	54	-	-	-	-

EXPERIMENT 8. AUGUST 10TH 1975

TABLE 33

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR VARIOUS CROSSES BETWEEN P. VULGATA, P. ASPERA AND P. INTERMEDIA

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera;

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	72	60	12	88	65	0	35	15	0
First cleavage	43	30	2	75	28	0	24	1	0
Second cleavage	43	27	2	66	28	0	24	0	0
Third cleavage	43	27	1	62	20	0	24	0	0
Trochophore	31	10	0	44	9	0	17	0	0
First day veliger	30	3	0	26	8	0	5	0	0
Second day veliger	24	3	0	26	8	0	5	0	0

TABLE 34

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES.

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	5-10	5-10	5-10	10-20	10-20	-	10-20	10-20	-
First cleavage	3-3½	3-3½	3-3½	4-4½	4-4½	-	4-4½	4-4½	-
Trochophore	18-20	18-20	-	18-20	18-20	-	18-20	-	-
First day veliger	54	54	-	54	54	-	54	-	-

EXPERIMENT 9. AUGUST 25TH 1975

TABLE 35

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR VARIOUS CROSSES BETWEEN P. VULGATA, P. ASPERA AND P. INTERMEDIA.

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera;

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	95	83	65	90	80	46	71	67	25
First cleavage	80	63	39	85	55	20	59	44	10
Second cleavage	80	60	39	77	43	18	59	31	2
Third cleavage	76	58	39	74	40	18	50	31	2
Trochophore	57	37	21	62	30	1	42	19	0
First day veliger	50	22	3	45	16	0	35	8	0
Second day veliger	49	22	3	45	17	0	35	8	0

TABLE 36

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES.

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	5-10	5-10	5-10	10-20	10-20	10-20	10-20	10-20	10-20
First cleavage	3-3½	3-3½	3-3½	4-4½	4-4½	4-4½	4-4½	4-4½	4-4½
Trochophore	18-20	18-20	18-20	18-20	18-20	18-20	18-20	18-20	-
First day veliger	54	54	54	54	54	-	54	54	-

EXPERIMENT 10. SEPTEMBER 8TH 1975

TABLE 37

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR VARIOUS CROSSES BETWEEN P. VULGATA, P. ASPERA AND P. INTERMEDIA.

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera;

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	92	80	73	95	78	50	89	60	38
First cleavage	76	64	50	80	51	28	75	44	18
Second cleavage	72	64	43	80	37	22	66	25	17
Third cleavage	70	60	43	74	33	20	66	23	17
Trochophore	55	45	29	60	25	9	50	18	7
First day veliger	46	38	21	49	19	6	50	10	2
Second day veliger	46	37	20	49	16	6	48	9	2

TABLE 38

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES.

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	5-10	5-10	5-10	10-20	10-20	10-20	10-20	10-20	10-20
First cleavage	3-3½	3-3½	3-3½	4-4½	4-4½	4-4½	4-4½	4-4½	4-4½
Trochophore	18-20	18-20	18-20	18-20	18-20	18-20	18-20	18-20	18-20
First day veliger	54	54	54	54	54	54	54	54	54

TABLE 39

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR VARIOUS CROSSES BETWEEN P. VULGATA, P. ASPERA AND P. INTERMEDIA

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera;

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	90	0	55	2	0	0	88	63	0
First cleavage	80	0	42	0	0	0	85	36	0
Second cleavage	77	0	40	0	0	0	85	33	0
Third cleavage	77	0	40	0	0	0	84	33	0
Trochophore	60	0	35	0	0	0	70	16	0
First day veliger	55	0	29	0	0	0	61	10	0
Second day veliger	55	0	22	0	0	0	61	4	0

TABLE 40

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES.

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	5-10	-	5-10	10-20	-	-	10-20	10-20	-
First cleavage	3-3½	-	3-3½	-	-	-	4-4½	4-4½	-
Trochophore	18-20	-	18-20	-	-	-	18-20	18-20	-
First day veliger	54	-	54	-	-	-	54	54	-

EXPERIMENT 12. OCTOBER 9TH 1975

TABLE 41

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR VARIOUS CROSSES BETWEEN P. VULGATA, P. ASPERA AND P. INTERMEDIA.

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	86	0	43	33	61	27	93	70	0
First cleavage	69	0	30	26	50	19	75	48	0
Second cleavage	68	0	21	26	42	17	65	41	0
Third cleavage	63	0	8	22	37	17	64	39	0
Trochophore	45	0	1	16	24	4	51	10	0
First day veliger	45	0	1	15	20	2	49	9	0
Second day veliger	44	0	0	15	17	1	49	3	0

TABLE 42

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	5-10	-	5-10	10-20	10-20	10-20	10-20	10-20	-
First cleavage	3-3½	-	3-3½	4-4½	4-4½	4-4½	4-4½	4-4½	-
Trochophore	18-20	-	18-20	18-20	18-20	18-20	18-20	18-20	-
First day veliger	54	-	-	54	54	54	54	54	-

EXPERIMENT 13. JUNE 29TH 1976

TABLE 43

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR CROSSES BETWEEN P. VULGATA, P. ASPERA P. INTERMEDIA AND THE TRANSITIONAL FORMS

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera; T = Transitional;

	T/T	T/V	T/I	T/A	V/T	I/T	A/T
Loss of germinal vesicle	60	67	34	18	75	0	0
First cleavage	52	62	22	10	66	0	0
Second cleavage	49	59	20	8	63	0	0
Third cleavage	49	52	20	8	61	0	0
Trochophore	38	47	15	2	50	0	0
First day veliger	33	40	13	0	39	0	0
Second day veliger	32	40	7	0	34	0	0

TABLE 44

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES

	T/T	T/V	T/I	T/A	V/T	I/T	A/T
Loss of germinal vesicle	5-10	5-10	5-10	5-10	5-10	-	-
First cleavage	3-3½	3-3½	3-3½	3-3½	3-3½	-	-
Trochophore	18-20	18-20	18-20	18-20	18-20	-	-
First day veliger	54	54	54	-	54	-	-

TABLE 45

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR CROSSES BETWEEN P. VULGATA, P. ASPERA, P. INTERMEDIA AND THE TRANSITIONAL FORMS

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera; T = Transitional Forms

	T/T	T/V	T/I	T/A	V/T	I/T	A/T
Loss of germinal vesicle	92	96	73	40	85	66	25
First cleavage	80	79	55	30	77	58	6
Second cleavage	76	70	55	30	77	51	6
Third cleavage	76	70	51	30	72	51	5
Trochophore	54	57	32	22	61	29	1
First day veliger	50	46	18	9	48	10	1
Second day veliger	49	46	18	9	48	9	1

TABLE 46

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES

	T/T	T/V	T/I	T/A	V/T	I/T	A/T
Loss of germinal vesicle	5-10	5-10	5-10	5-10	5-10	10-20	10-20
First cleavage	3-3½	3-3½	3-3½	3-3½	3-3½	4-4½	4-4½
Trochophore	18-20	18-20	18-20	18-20	18-20	18-20	18-20
First day veliger	54	54	54	54	54	54	54

EXPERIMENT 15. JULY 18TH 1976

TABLE 47

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR CROSSES BETWEEN P. VULGATA, P. ASPERA P. INTERMEDIA AND THE TRANSITIONAL FORMS

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera

	T/T	T/V	T/I	T/A	V/T	I/T	A/T
Loss of germinal vesicle	91	95	66	51	88	70	32
First cleavage	79	80	54	29	76	48	23
Second cleavage	79	73	51	28	67	35	22
Third cleavage	68	68	42	17	60	26	21
Trochophore	45	49	10	0	54	20	5
First day veliger	43	40	9	0	48	6	3
Second day veliger	43	39	9	0	47	1	0

TABLE 48

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES

	T/T	T/V	T/I	T/A	T/A	V/T	I/T	A/T
Loss of germinal vesicle	5-10	5-10	5-10	5-10	5-10	10-20	10-20	
First cleavage	3-3½	3-3½	3-3½	3-3½	3-3½	4-4½	4-4½	
Trochophore	18-20	18-20	18-20	-	18-20	18-20	18-20	
First day veliger	54	54	54	-	54	54	54	

EXPERIMENT 16. JULY 27TH 1976

TABLE 49

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR CROSSES BETWEEN P. VULGATA, P. ASPERA, P. INTERMEDIA AND THE TRANSITIONAL FORMS

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera; T = Transitional Form

	T/T	T/V	T/I	T/A	V/T	I/T	A/T
Loss of germinal vesicle	95	93	83	65	96	80	67
First cleavage	81	84	64	38	80	55	46
Second cleavage	79	84	60	37	78	42	31
Third cleavage	75	80	55	37	75	40	29
Trochophore	54	59	36	21	61	30	18
First day veliger	50	53	22	14	51	16	7
Second day veliger	47	46	21	3	51	13	7

TABLE 50

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES

	T/T	T/V	T/I	T/A	V/T	I/T	A/T
Loss of germinal vesicle	5-10	5-10	5-10	5-10	5-10	10-20	10-20
First cleavage	3-3½	3-3½	3-3½	3-3½	3-3½	4-4½	4-4½
Trochophore	18-20	18-20	18-20	18-20	18-20	18-20	18-20
First day veliger	54	54	54	54	54	54	54

EXPERIMENT 17. AUGUST 4TH 1976

TABLE 51

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR CROSSES BETWEEN P. VULGATA, P. ASPERA, P. INTERMEDIA AND THE TRANSITIONAL FORMS

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera;
T = Transitional Forms;

	T/T	T/V	T/I	T/A	V/T	I/T	A/T
Loss of germinal vesicle	90	95	0	55	89	78	60
First cleavage	79	82	0	41	77	50	43
Second cleavage	76	76	0	37	73	37	25
Third cleavage	70	74	0	37	72	33	22
Trochophore	55	59	0	29	60	20	16
First day veliger	48	50	0	20	46	20	10
Second day veliger	47	50	0	4	40	16	0

TABLE 52

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES

	T/T	T/V	T/I	T/A	V/T	I/T	A/T
Loss of germinal vesicle	5-10	5-10	-	5-10	5-10	10-20	10-20
First cleavage	3-3½	3-3½	-	3-3½	3-3½	4-4½	4-4½
Trochophore	18-20	18-20	-	18-20	18-20	18-20	18-20
First day veliger	54	54	-	54	54	54	54

EXPERIMENT 18. AUGUST 12TH 1976

TABLE 53

PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR CROSSES BETWEEN P. VULGATA, P. ASPERA, P. INTERMEDIA AND THE TRANSITIONAL FORMS

N.B. V = P. vulgata; I = P. intermedia; A = P. aspera
T = Transitional Forms.

	T/T	T/V	T/I	T/A	V/T	I/T	A/T
Loss of germinal vesicle	77	75	60	18	80	65	27
First cleavage	69	72	45	3	70	50	13
Second cleavage	66	61	33	1	65	46	11
Third cleavage	65	61	32	1	59	40	10
Trochophore	54	53	23	0	48	28	5
First day veliger	44	40	14	0	41	17	2
Second day veliger	44	40	12	0	41	15	2

TABLE 54

THE TIME TAKEN FOR THE CROSSES IN THE ABOVE EXPERIMENT TO REACH THE VARIOUS DEVELOPMENTAL STAGES

	T/T	T/V	T/I	T/A	V/T	I/T	A/T
Loss of germinal vesicle	5-10	5-10	5-10	5-10	5-10	10-20	10-20
First cleavage	3-3½	3-3½	3-3½	3-3½	3-3½	4-4½	4-4½
Trochophore	18-20	18-20	18-20	-	18-20	18-20	18-20
First day veliger	54	54	54	-	54	54	54

TABLE 55

THE AVERAGE PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR CROSSES BETWEEN P. VULGATA, P. ASPERA AND P. INTERMEDIA

	V/V	V/I	V/A	I/I	I/V	I/A	A/A	A/V	A/I
Loss of germinal vesicle	83.3	68.4	56.5	91.1	66.9	43.5	85.1	59.8	36.7
First cleavage	70.8	52.2	38.3	79.7	46.7	31.5	64.4	41.7	24.0
Second cleavage	67.1	51.3	34.8	76.3	41.6	27.3	70.1	35.0	22.6
Third cleavage	65.9	49.6	33.2	74.0	38.2	26.0	68.1	32.7	20.3
Trochophore	50.2	31.2	21.5	55.3	23.5	15.0	56.5	14.7	12.0
First day veliger	43.6	22.6	12.8	41.9	17.1	9.0	46.5	10.0	7.5
Second day veliger	42.1	21.3	11.1	41.5	15.5	8.7	44.9	8.4	7.4

TABLE 56

THE AVERAGE PERCENTAGE SURVIVAL AT SUCCESSIVE DEVELOPMENTAL STAGES FOR CROSSES BETWEEN P. VULGATA, P. ASPERA P. INTERMEDIA AND THE TRANSITIONAL FORMS

	T/T	T/V	T/I	T/A	V/T	I/T	A/T
Loss of germinal vesicle	84.7	86.9	63.2	40.1	85.5	71.8	42.4
First day cleavage	73.3	76.5	48.0	25.1	74.3	52.2	26.2
Second day cleavage	70.8	70.5	43.8	23.5	70.3	42.2	19.0
Third day cleavage	67.1	67.5	40.0	21.6	66.5	38.0	17.4
Trochophore	50.0	54.0	27.2	12.3	55.6	25.4	9.0
First day veliger	44.6	45.5	21.2	7.2	45.3	13.8	6.6
Second day veliger	44.5	41.8	19.4	2.6	43.5	12.8	5.3

cross is achieved between the female of P. vulgata and the male of P. intermedia with a survival rate of 21.3%. The success rate is lower when the cross is carried out the other way round. A lower survival rate is seen for crosses between P. vulgata and P. aspera this being about 10%. The least successful cross is that of P. aspera and P. intermedia with a survival rate of 8%.

The survival rates of the normal fertilisations of the 'transitional' forms, and the crosses between the 'transitional' forms and P. vulgata are the same at about 42%. The survival rates of the crosses between the 'transitional' forms and P. intermedia are slightly but not significantly lower than those of the crosses between P. vulgata and P. intermedia, and the same is true of crosses between the 'transitional' forms and P. aspera.

In all cases the critical stages at which the survival rates drop most rapidly are the first cleavage of the egg and the formation of the trochophore larva.

The development of the larvae follows a very consistent pattern in which the course of development is determined by the species represented by the maternal parent. Thus, in crosses involving a P. vulgata female, development follows the normal pattern for P. vulgata which develops faster than P. aspera or P. intermedia. However, in a cross involving a P. aspera female or a P. intermedia female, even if crossed with the faster developing P. vulgata male, the larvae develop in the slower time normal to P. aspera and P. intermedia.

Despite the time differences in the initial stages of development all the larvae become synchronised with each other by the time the trochophore larva appears at between 18 and 20 hours.

When one compares these results with those for crosses involving the 'transitional' forms, it can be seen that the 'transitional' forms have a very similar pattern of development to P. vulgata. Thus, the larvae of the normal fertilisations of the

'transitional' forms take the same time to develop as the larvae of the P. vulgata fertilisations. Also the larvae of any crosses involving the female 'transitional' forms show the same rate of development as those of crosses involving the females of P. vulgata.

Conclusions

It can be concluded from these results that hybridisation can, in fact, be successfully achieved under artificial conditions.

It is interesting to note that P. vulgata can be crossed with the other two species with a greater degree of success than when P. aspera and P. intermedia are crossed with each other. This might support the theory that P. aspera and P. intermedia have evolved from a common vulgata-like root stock and are thus more closely related to P. vulgata than they are to each other.

Another important point is that when crossing the 'transitional' forms with P. aspera, P. intermedia and P. vulgata the same success rate is achieved as when using P. vulgata, suggesting a close relationship between the 'transitional' forms and P. vulgata.

Finally the development time of the larvae of P. vulgata and the 'transitional' forms are the same and are faster than those for the larvae of P. aspera and P. intermedia. This again provides evidence that the 'transitional' forms are closely related to P. vulgata and are not behaving as hybrids between P. vulgata and P. intermedia might be expected to.

Fig. 20 Unfertilised Egg Of P. vulgata (x1000)

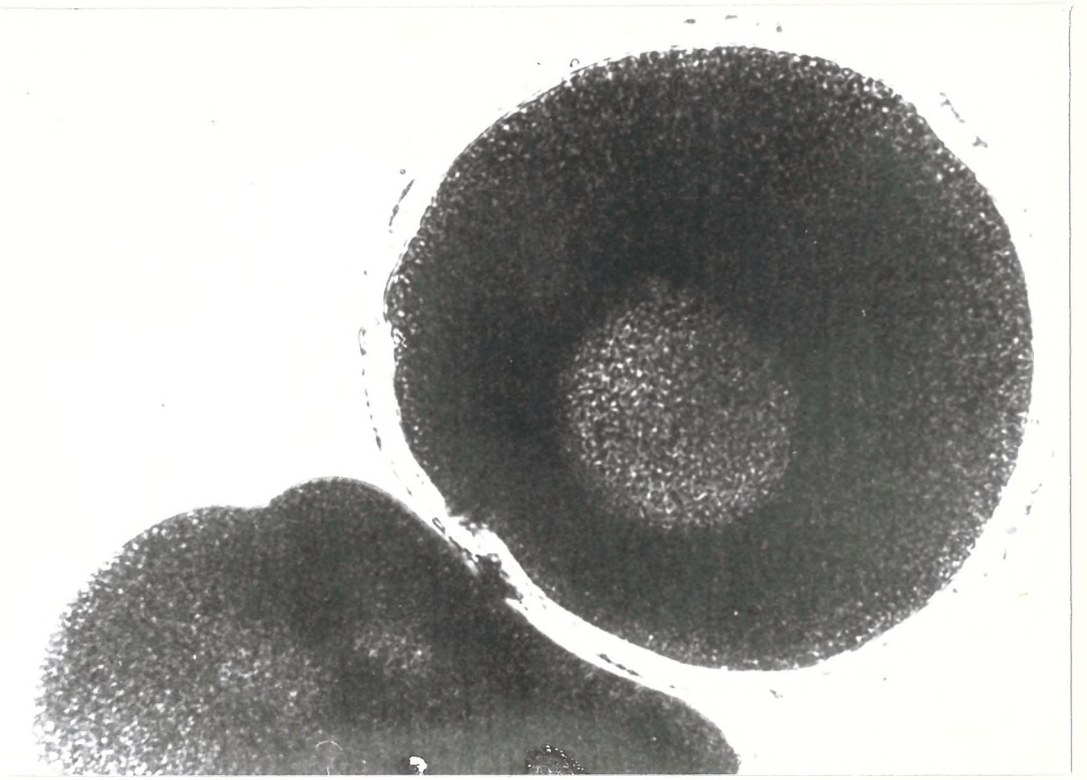


Fig. 21 4-cell Stage Of P. vulgata (x500)

Fig. 22 16-cell Stage Of P. vulgata (x1000)

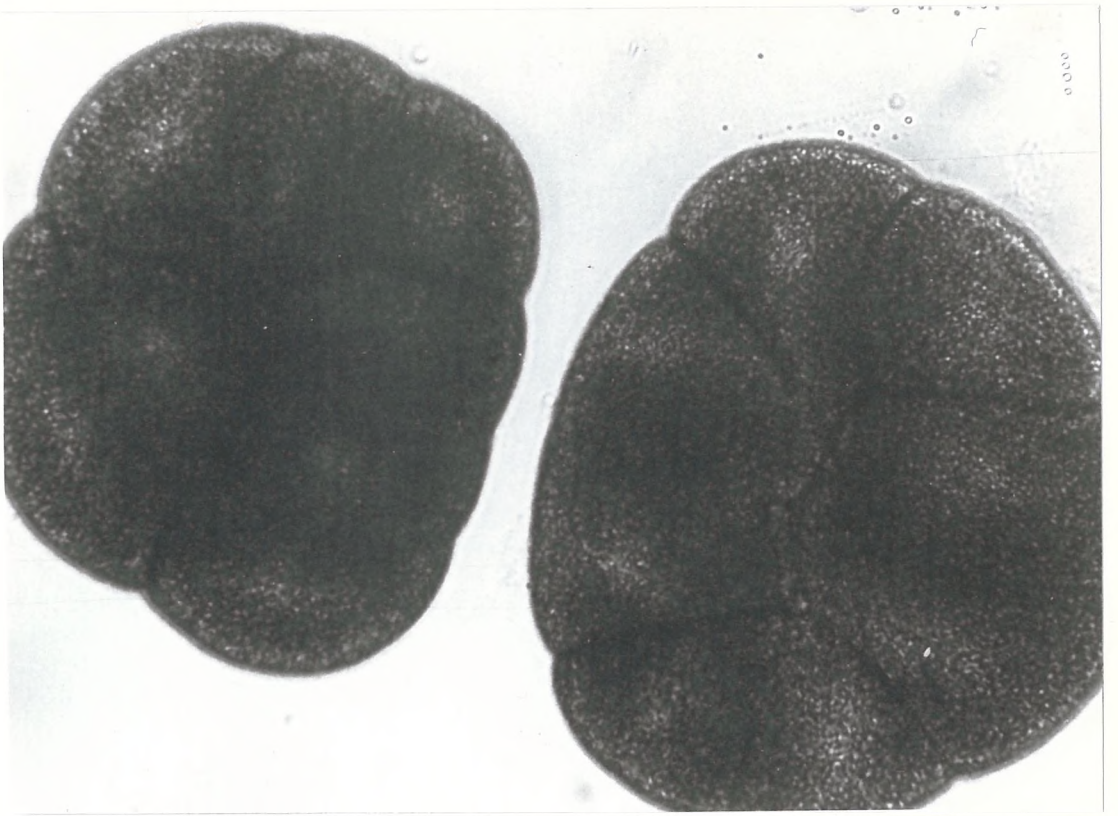


Fig. 23 32-cell Stage Of P. vulgata (x1000)

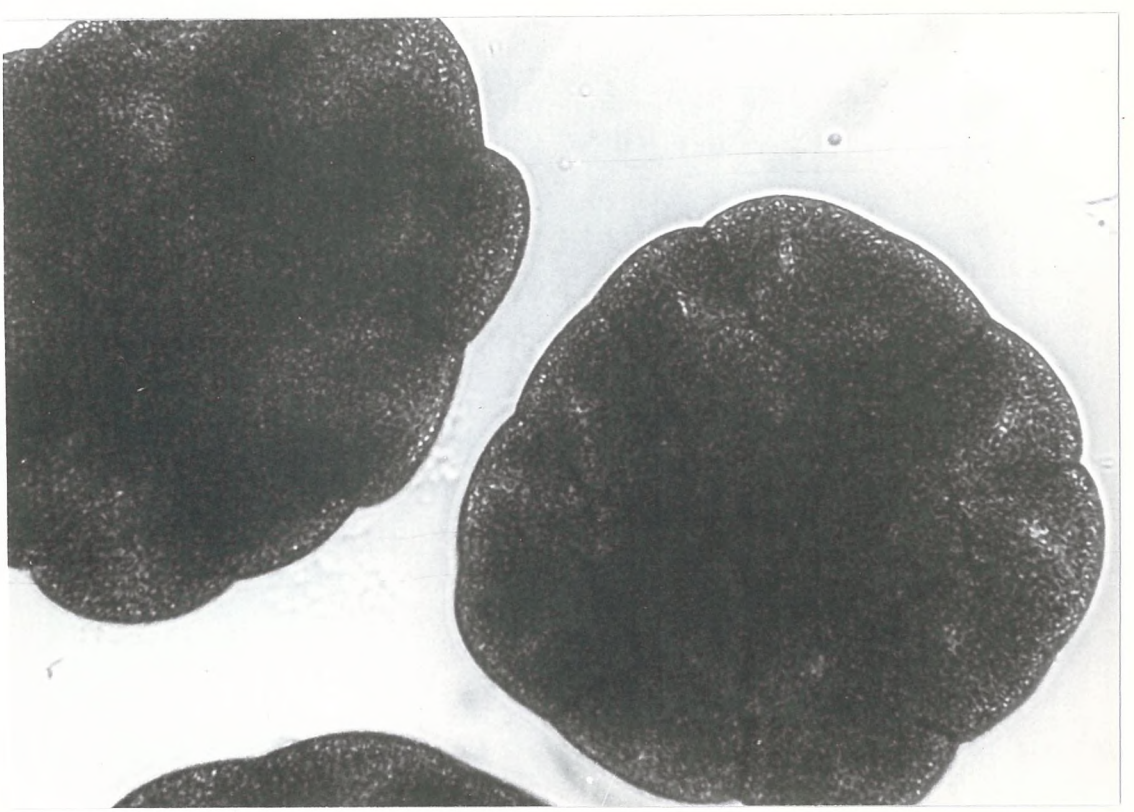


Fig. 24 10 Hour Larva Of P. vulgata (x1000)

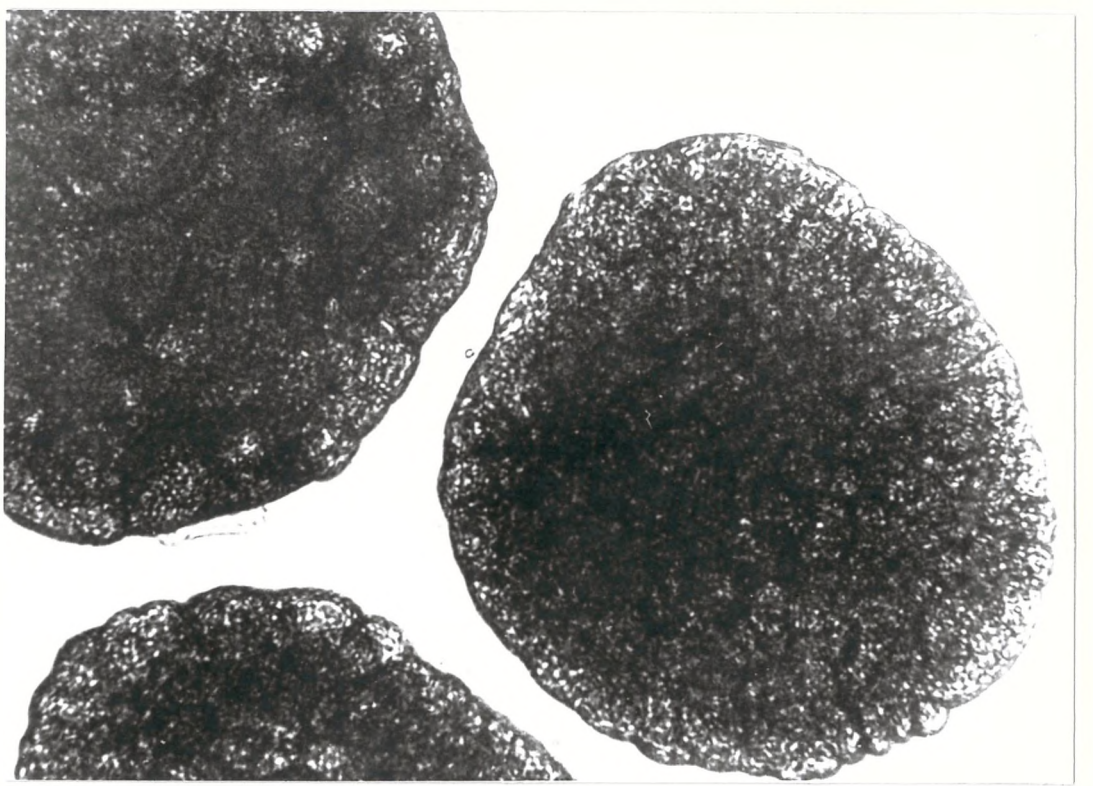
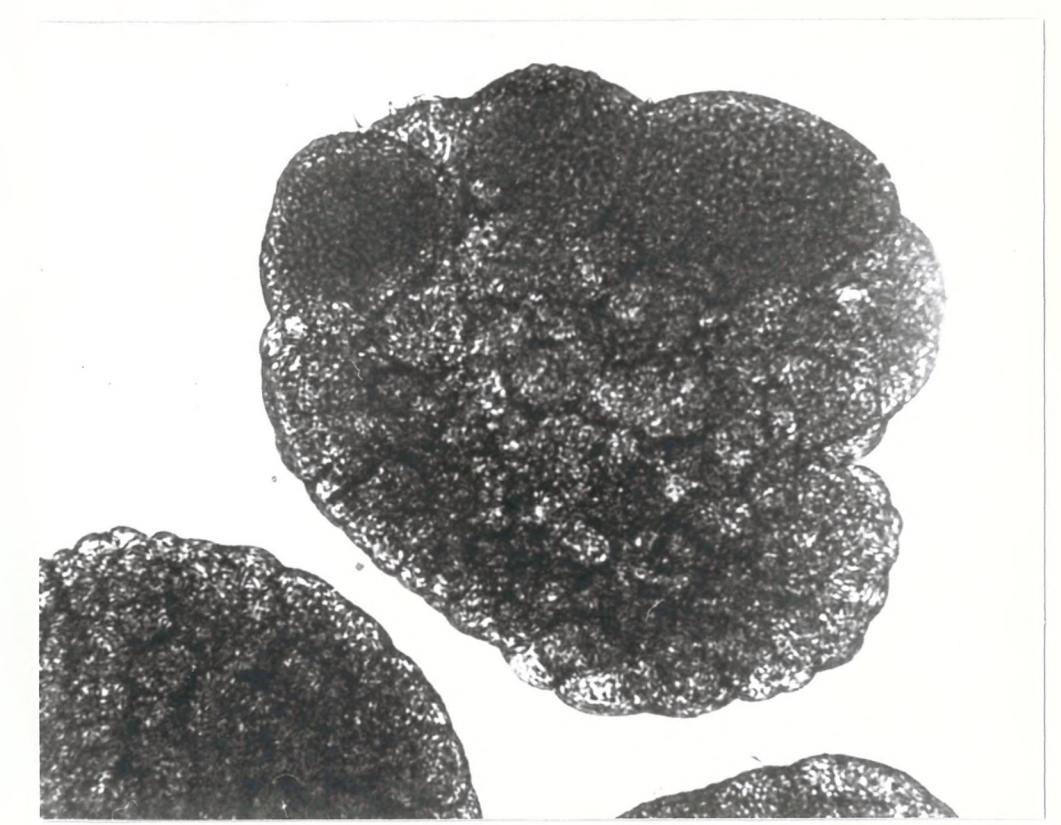


Fig. 25 14 Hour Larva Of P. vulgata (x1000)



CHAPTER VI.

THE USE OF IMMUNOLOGICAL METHODS TO STUDY THE INTERRELATIONSHIPS OF THE THREE LIMPET SPECIES

IMMUNOTAXONOMIC WORK

Aims

The purpose of this section of the work was to produce specific anti-sera to the three limpet species using rabbits. These anti-sera could then be used as a criterion for distinguishing between the three species.

It was hoped that, if effective, these anti-sera could also be used to determine the specific status of the 'transitional' forms.

Methods

Rabbits were used to produce antibodies to the blood serum of the three species of limpet. Two rabbits were used for each of *P. vulgata*, *P. aspera* and *P. intermedia* in case one should die or fail to produce an immune reaction.

Blood was extracted from the pallial vein of the limpets and centrifuged to remove the haemocytes and other impurities. It was necessary to carry out spectrophotometric protein determinations on each of the limpet sera to make it possible to inject an equal and known quantity of protein into each rabbit.

The rabbits were bled prior to injection as a control to ensure that they possessed no natural immune reaction to the limpet sera. For this purpose five mls. of blood was taken from the ear vein of each rabbit into bijoux bottles. Two hours later the clots were separated from the edges of the bottles which were then left overnight at a temperature of $4^{\circ}C$. The next day the serum was pipetted off the clot and centrifuged to remove excess haemoglobin. The serum could then be stored in small aliquots in the deep-freeze until required.

The course of injections continued over a period of two months during which time five injections were given. In some cases the serum was given in pure form, either into the ear vein or sub-cutaneously, and in other cases the serum was homogenised with

Freund's adjuvant and then given sub-cutaneously. Freund's adjuvant contains tuberculin and is designed to attract lymphocytes to the area where the serum is administered.

The details of the injections and their dates are given in table 57.

In order to determine the strength of the immune reaction the rabbits were bled twice during the course of the injections as shown in table 57. For this purpose the three prepared limpet sera were diluted using doubling dilutions down to a concentration of one in sixty-four thousand. A small amount of each dilution was drawn into a micropipette followed by an equal volume of serum containing the antibodies from the appropriate rabbit. The liquids were then drawn further up the tube to cause an air lock and the pipette sealed in a flame. The tubes were then left for two hours after which time the presence of precipitates could be detected at the interfaces of the two liquids. The lowest dilution at which the precipitate could be seen was ascertained and taken as a measure of the strength of the immune reaction. The results of these tests are also given in table 57.

The rabbits were finally bled on the 13th November. About 100 mls. of blood was taken from the ear vein after which the rabbit was killed by the injection of 4 mls. of nembutal and as much blood as possible drawn from the heart using a syringe and then a Pasteur pipette. The blood was left in 250 ml. beakers for two hours and then the clot was separated from the edge of the glass and left overnight. The next day the serum was decanted off, centrifuged and stored in small aliquots in the deep freeze for later use.

The Use Of Ouchterlony Plates.

Immunodiffusion experiments were carried out using Ouchterlony plates to discover whether the rabbits had provided

TABLE 57

DETAILS OF INJECTIONS ADMINISTERED AND RESULTS OF TESTS TO ESTABLISH THE STRENGTH OF IMMUNE REACTION DURING THE TWO MONTH PERIOD.

N.B. S.C. = subcutaneously I.V. = intra-venously

	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Rabbit 5	Rabbit 6
17.9.75	1.8 ml. *P. aspera* serum I.V.	1.7 ml. *P. aspera* serum I.V.	2 ml. *P. vulgata* serum I.V.	2 ml. *P. vulgata* serum I.V.	1 ml. *P. intermedia* serum I.V.	1 ml. *P. intermedia* serum I.V.
24.9.75	2 ml. *P. aspera* + adjuvant S.C.	2 ml. *P. aspera* + adjuvant S.C.	2 ml. *P. vulgata* + adjuvant S.C.	2 ml. *P. vulgata* + adjuvant S.C.	1 ml. *P. intermedia* + adjuvant S.C.	1 ml. *P. intermedia* + adjuvant S.C.
10.10.75	Strength of immune reaction 1/1000	Strength of immune reaction 1/5000	Strength of immune reaction 1/5000	Strength of immune reaction 1/1000	Strength of immune reaction 1/5000	Strength of immune reaction 1/5000
14.10.75	2 ml. *P. aspera* serum S.C.	2 ml. *P. aspera* serum S.C.	2 ml. *P. vulgata* serum S.C.	2 ml. *P. vulgata* serum S.C.	1 ml. *P. intermedia* serum S.C.	1 ml. *P. intermedia* serum S.C.
23.10.75	2 ml. *P. aspera* + adjuvant S.C.	2 ml. *P. aspera* + adjuvant S.C.	2 ml. *P. vulgata* + adjuvant S.C.	2 ml. *P. vulgata* + adjuvant S.C.	1 ml. *P. intermedia* + adjuvant S.C.	1 ml. *P. intermedia* + adjuvant S.C.
5.11.75	Strength of immune reaction 1/40,000	Strength of immune reaction 1/20,000	Strength of immune reaction 1/40,000	Strength of immune reaction 1/40,000	Strength of immune reaction 1/40,000	Strength of immune reaction 1/40,000
8.11.75	2 ml. *P. aspera* serum S.C	2 ml. *P. aspera* serum S.C.	2 ml. *P. vulgata* serum S.C.	2 ml. *P. vulgata* serum S.C.	1 ml. *P. intermedia* serum S.C.	1 ml. *P. intermedia* serum S.C.

specific antibodies to the serum they were injected with, since it was possible that the antibodies would not be sufficiently specific to distinguish between the sera of the three species.

The Ouchterlony plates were prepared using purified ion-agar containing 0.1% sodium azide to prevent bacterial growth. A pattern of wells was later cut out of the agar to accommodate the blood sera of the limpets and the rabbit antisera. The control rabbit sera and the antisera obtained from the final bleeding were each tested against the blood sera of the three species and that of the 'transitional' forms.

After the various sera had been placed in the wells the plates were left for a period of two days until the precipitin lines appeared at which point the plates were photographed. The plates were then rendered permanent by washing in phosphate buffered saline to remove excess soluble protein, drying on a hot plate and staining in azocarmine for one hour.

The results of these immunodiffusion experiments are shown in figures 26 to 31. Each photograph is provided with a plan of how the plates were set out on which interpretations of the pre-cipitin lines present are also given.

It is clear that all three pre-injection control rabbit sera produced no reaction against the blood sera of the three species establishing that the rabbits possessed no natural immunity to the limpet blood sera.

However all three sera from the injected rabbits showed a reaction against the blood sera of the three species and of the 'transitional' forms. It can also be seen that in each case the rabbit anti-sera showed a stronger reaction against the serum of the species of limpet the rabbit was initially injected with. For instance in Plate 9. the anti-intermedia serum from rabbit 6. showed a stronger reaction against the serum of P. intermedia

Fig. 26 Plan Of Immunodiffusion Plates 1 and 2.

V = P. vulgata serum; I = P. intermedia serum; A = P. aspera serum;

T = serum of 'transitional' form.

Interpretations of the precipitin lines present are shown.

Plate 1.

Centre well contains pre-injection control serum from rabbit I.

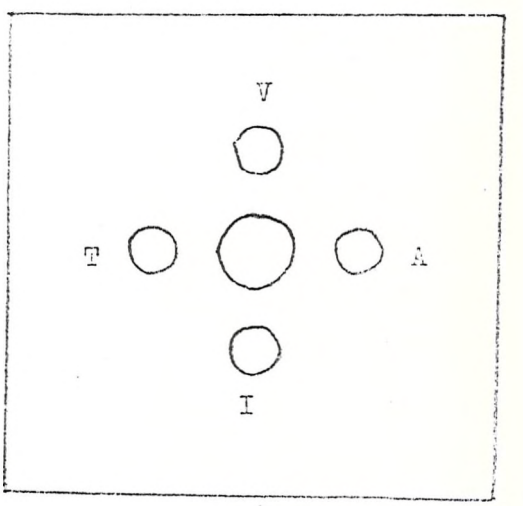


Plate 2.

Centre well contains anti-P. aspera serum from rabbit I.

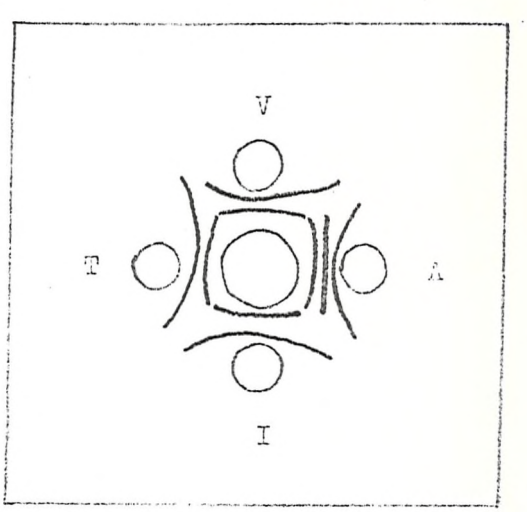


Fig. 27 Ouchterlony plates 1 and 2.

Plate 1

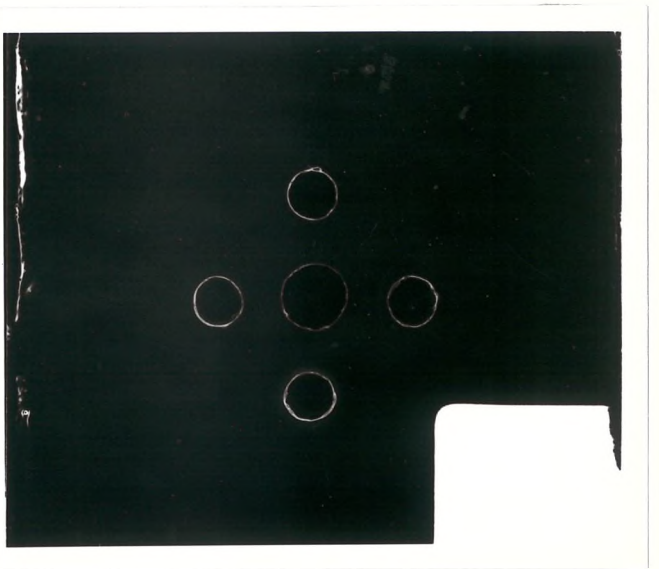


Plate 2

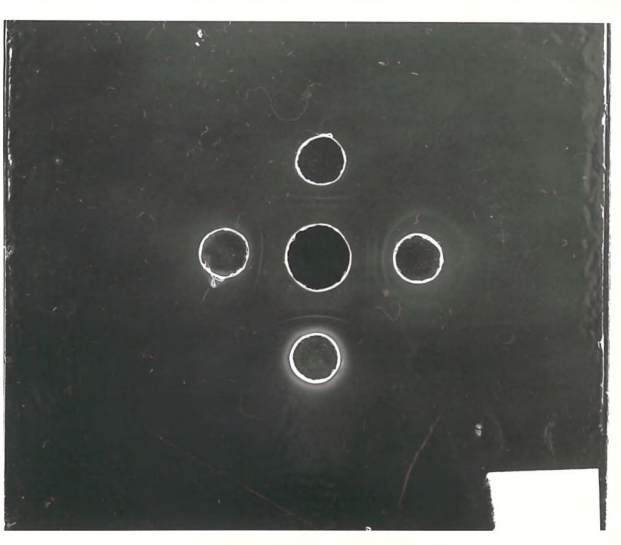


Fig. 28 Plan Of Immunodiffusion Plates 3 and 4.

V = P. vulgata serum; I = P. intermedia serum; A = P. aspera serum

T = serum of 'transitional'form

Interpretations of the precipitin lines present are shown.

Plate 3.

Centre well contains pre-injection control serum from rabbit 3.

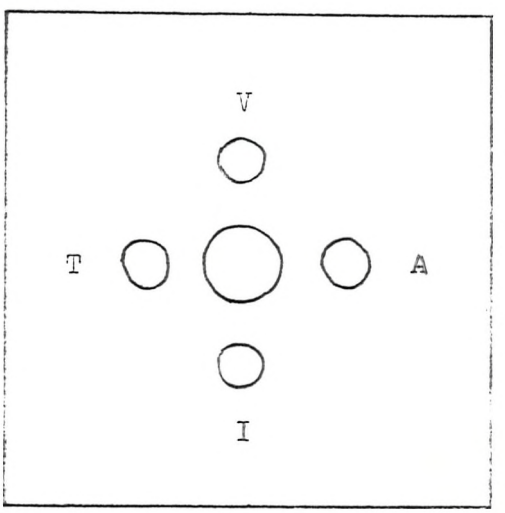


Plate 4.

Centre well contains anti-P. vulgata serum from rabbit 3.

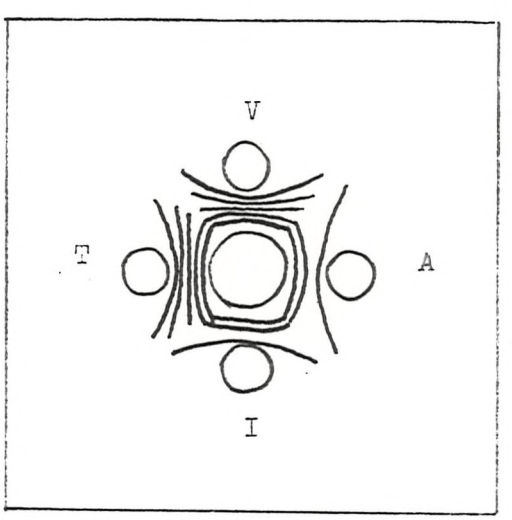


Fig. 29 Ouchterlony plates 3 and 4.

Plate 3

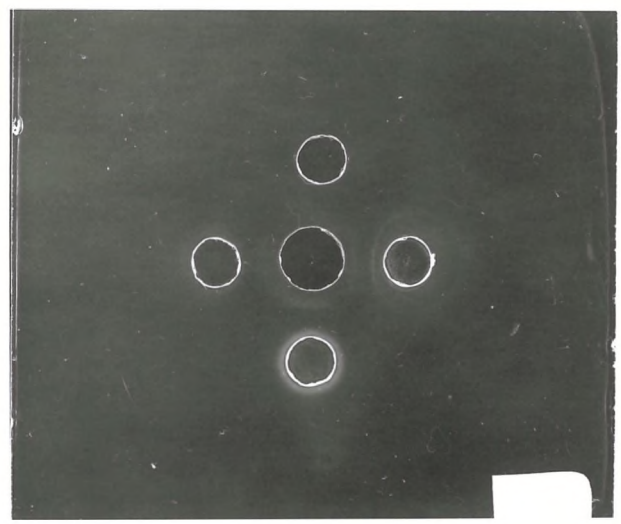


Plate 4

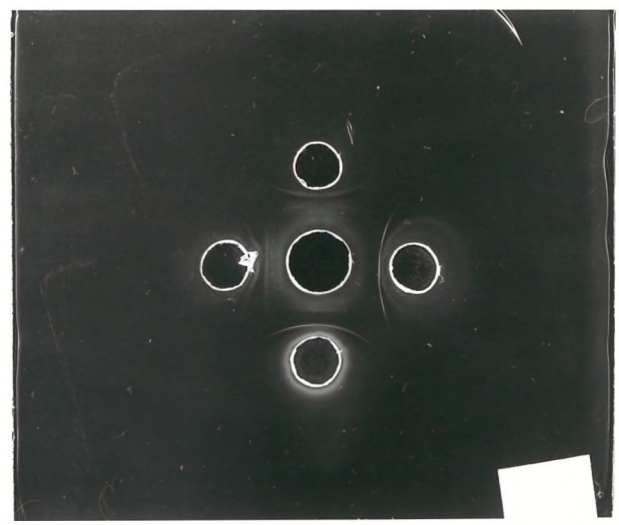


Fig. 30 Plan Of Immunodiffusion Plates 5 and 6.

V = P. vulgata serum; I = P. intermedia serum; A = P. aspora serum;

T = serum of 'transitional' form.

Interpretations of the precipitin lines present are shown.

Plate 5.

Centre well contains pre-injection control serum from rabbit 5.

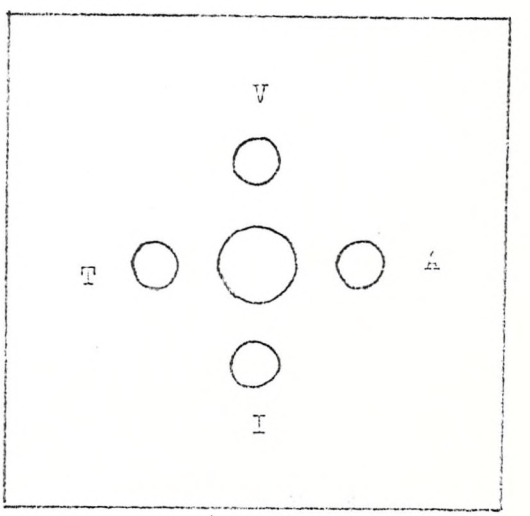


Plate 6.

Centre well contains anti-P. intermedia serum from rabbit 5.

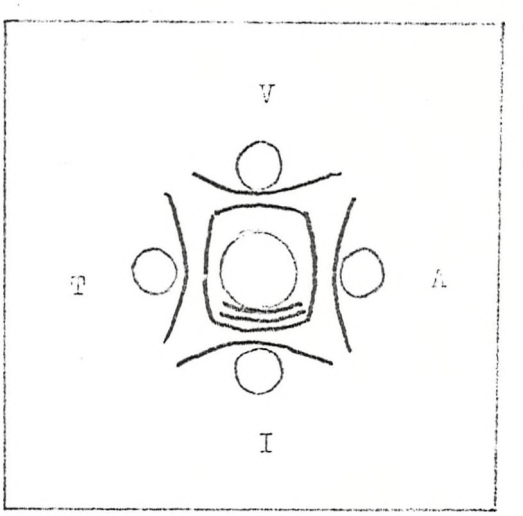


Fig. 31 Ouchterlony plates 5 and 6.

Plate 5

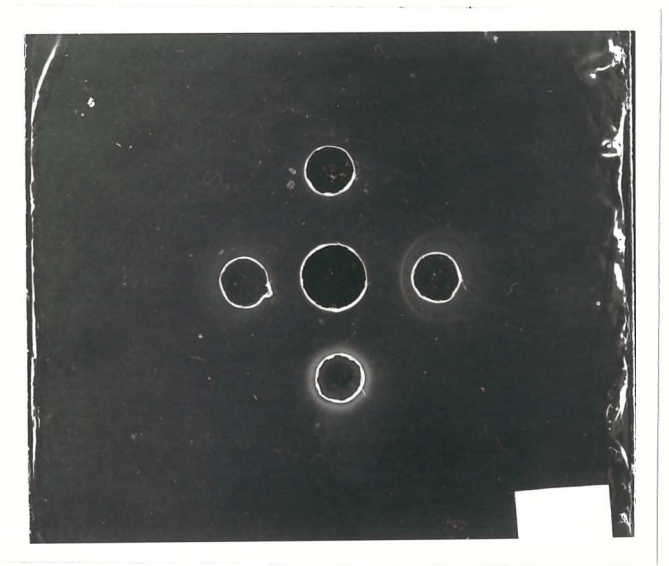
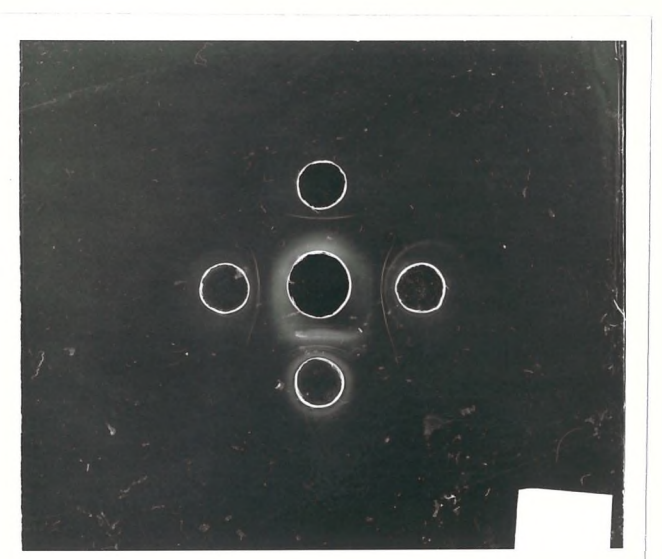


Plate 6



than to the other two species or the 'transitional' form.

In Plate 6 it would seem that the blood sera of the 'transitional' form and of P. vulgata have produced the same reaction against the anti-vulgata serum of rabbit 4 which would suggest that this particular 'transitional' individual possessed more antigens in common with P. vulgata than with the other two species.

Since from these results it was obvious that the anti-sera were capable of distinguishing between the three species it was possible to produce specific anti-sera to each of the three limpet species.

The Production Of Specific Anti-sera To The Blood Of P. Vulgata And P. Intermedia.

An anti-serum specific to the blood serum of P. vulgata was produced by adding two mls. of P. aspera serum and P. intermedia serum to two mls. of the anti-vulgata serum extracted from rabbit 4. This mixture was shaken and left for 24 hours during which time it was hoped that the antigens common to all three limpet species would react with and precipitate out the common antibodies leaving a serum containing antibodies that would be active only against antigens possessed by P. vulgata and not by the other two species. After 24 hours the precipitate was centrifuged off and the remaining serum concentrated threefold by freeze drying since it was found to be inactive at the normal concentration.

A specific antiserum to the blood of P. intermedia was produced in the same way, that is by adding the blood sera of P. vulgata and P. aspera to the anti-intermedia serum from rabbit 6.

The process was not repeated for P. aspera since, as previously stated, this species does not seem to be involved in the phenomenon of 'intermediacy'.

Ouchterlony plates were again used to test whether these anti-vulgata and anti-intermedia sera had indeed been rendered specific, by this absorption process, to P. vulgata and

P. intermedia respectively.

The results of these experiments are shown in figures 32 to 37. The photographs are each provided with a plan to show how the plates were set out. Interpretations of the precipitin lines present are also included in the plans.

The plates show that the attempt to produce an antiserum specific to one species only has been successful. In plate 1 the specific anti-vulgata serum in the centre well has produced a reaction only against the blood serum of P. vulgata and no reaction at all has occurred against that of P. intermedia. Similarly in Plate 2 the specific anti-intermedia serum in the centre well has shown a reaction only against the blood serum of P. intermedia. In plates 3 and 4 the outer wells contain the blood sera of individual 'transitional' forms which have produced a strong reaction against the specific anti-vulgata serum in the centre wells. It would seem, therefore, that these twelve 'transitional' individuals possess at least some antigens normally specific to P. vulgata alone.

A Survey Of The Antigenic Properties Of A Number Of 'Transitional' Forms Using Specific Anti-vulgata And Specific Anti-intermedia sera.

Having produced specific antisera to P. vulgata and P. intermedia it was now possible to discover whether they show a reaction against only the specific anti-vulgata serum or only the specific anti-intermedia serum, or whether they show a reaction against both which might be expected of a hybrid individual.

For this purpose immunodiffusion experiments were carried out in agar set onto microscope slides. Two long wells were cut along the outer edges of the slides into which the specific anti-vulgata and specific anti-intermedia sera were placed. Along the centre of the slide ten small wells were cut each of which contained the blood serum of an individual 'transitional' form. The

Fig. 32 Plan Of Immunodiffusion Plates 7 and 8 Used To Test The Specific Anti-vulgata And Anti-intermedia Sera.

V = P. vulgata serum; I = P. intermedia serum;

Interpretations of the precipitin lines present are shown.

Plate 7

Centre well contains specific anti-vulgata serum from rabbit 3.

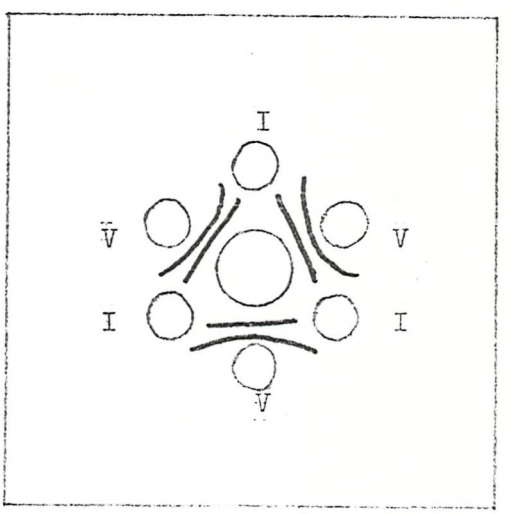


Plate 8

Centre well contains specific anti-intermedia serum from rabbit 5.

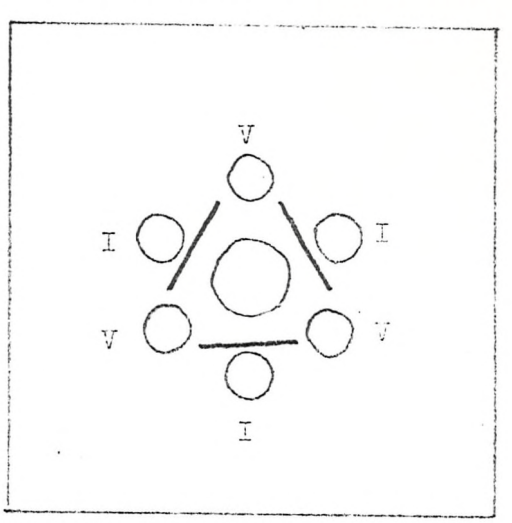


Fig. 33 Ouchterlony plate 7.

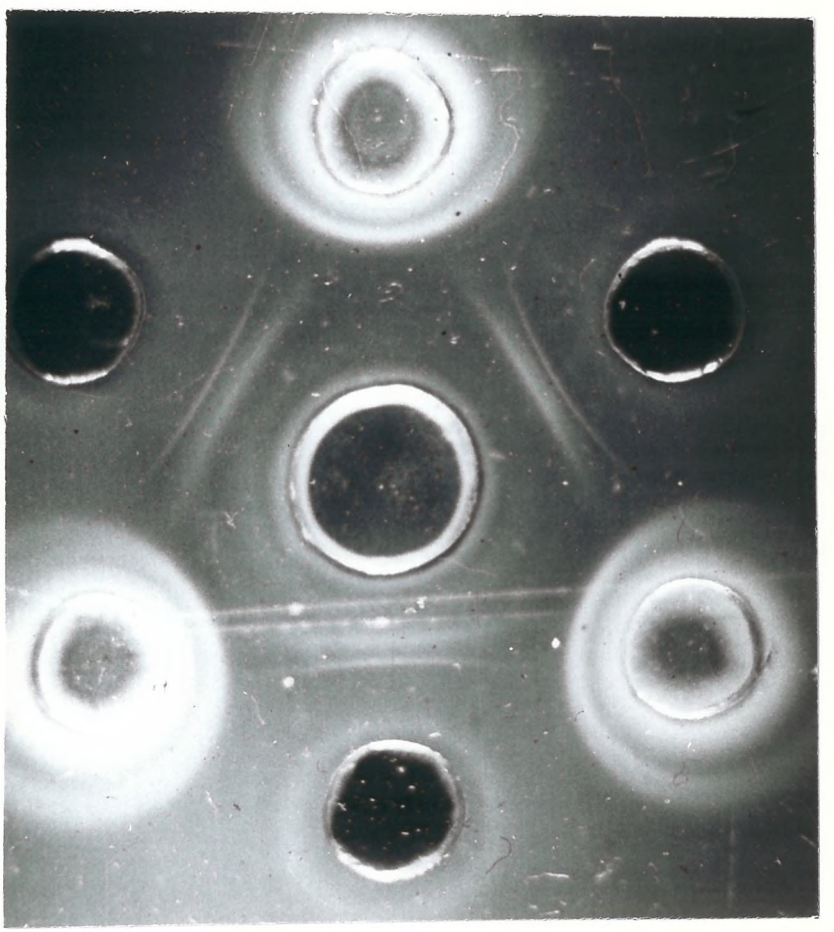


Fig. 34 Ouchterlony plate 8.

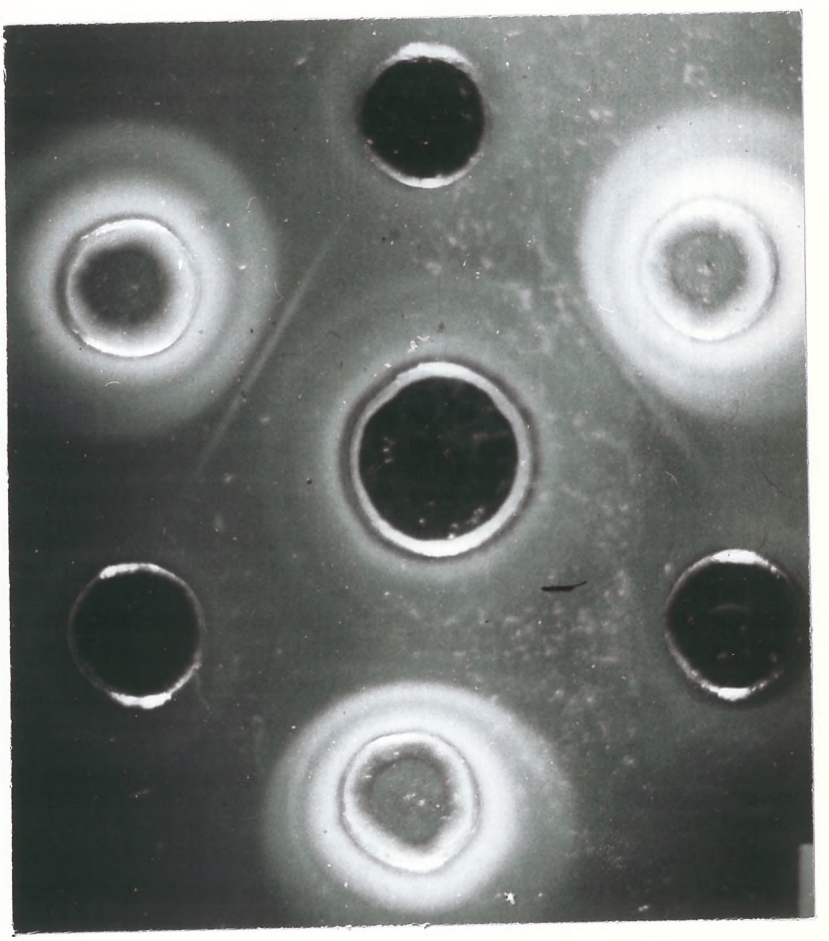


Fig. 35 Plan Of Immunodiffusion Plates 9 and 10 Used To Test Twelve Individual Transitional Forms.

T = Serum Of Transitional Form.

Plate 9.

Centre well contains specific anti-vulgata serum.

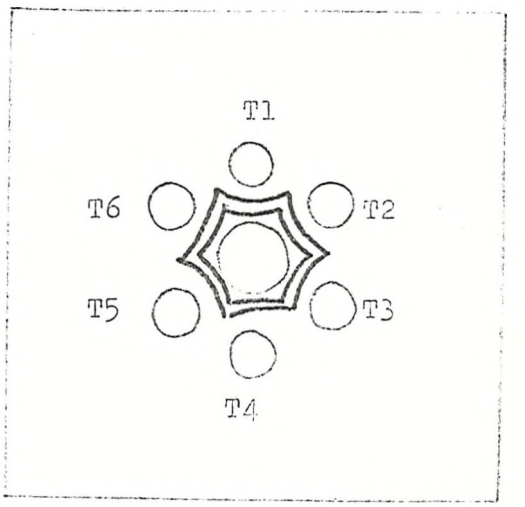


Plate 10.

Centre well contains specific anti-vulgata serum.

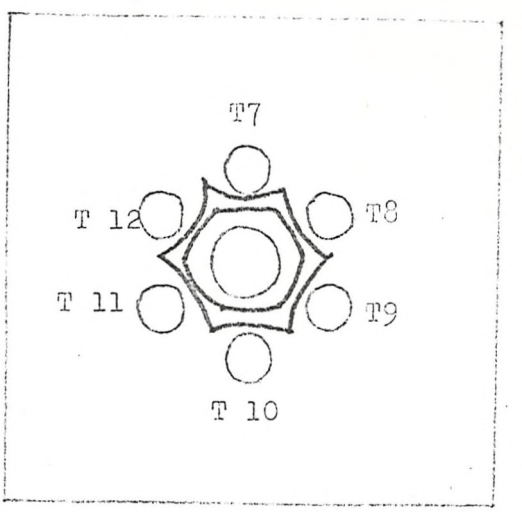


Fig. 36 Ouchterlony plate 9.

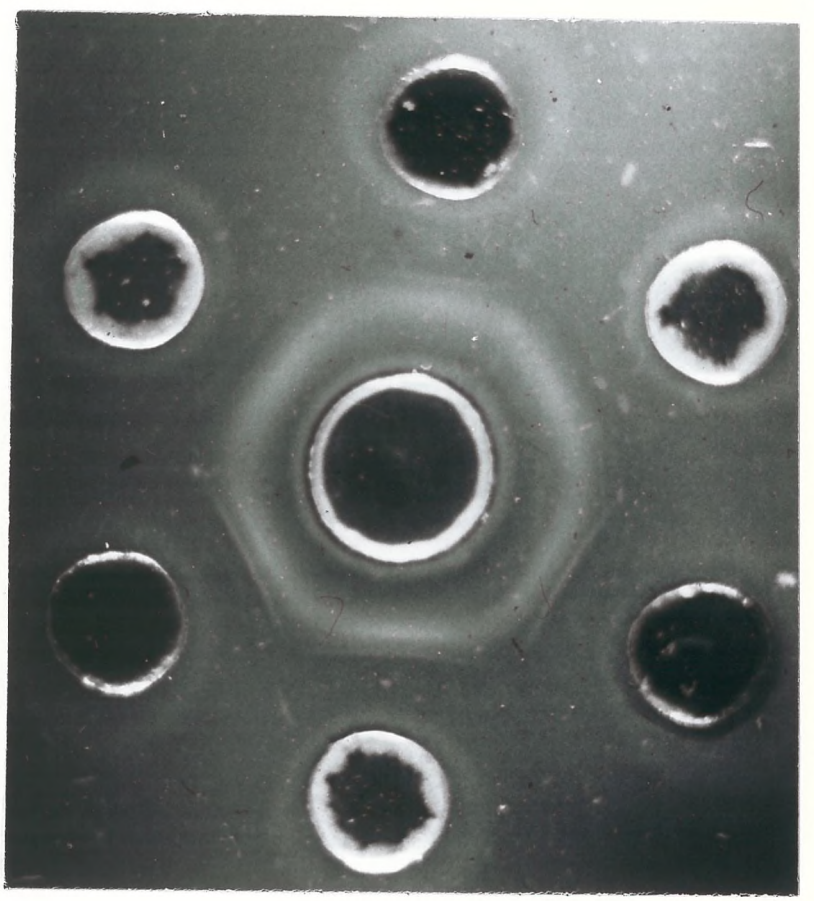
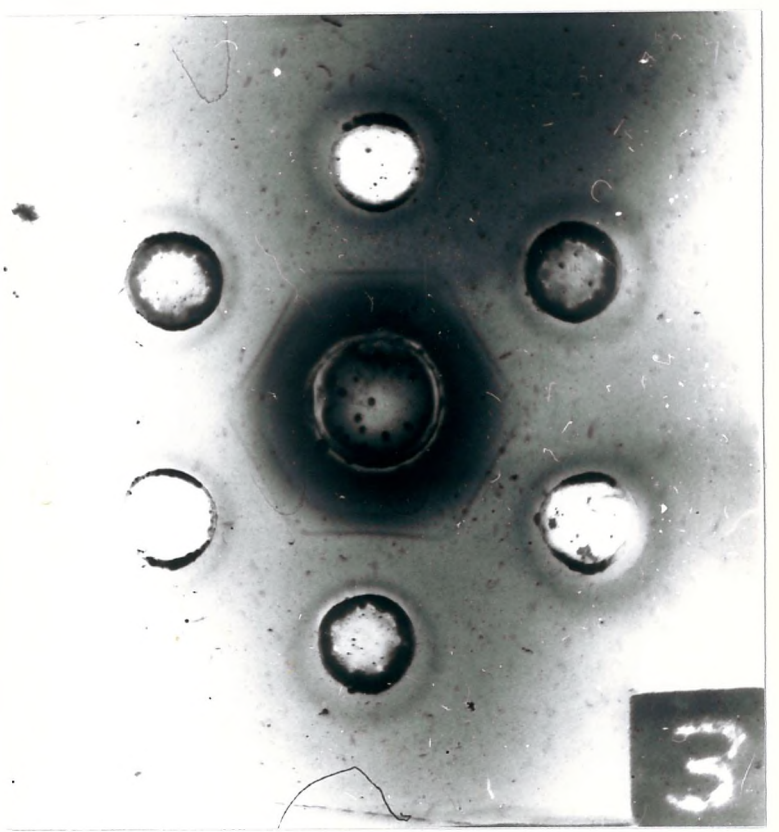


Fig. 37 Ouchterlony plate 10.



slides were then treated using the same procedure as before.

The morphological characters of the eighty-five individuals tested are shown in tables 58, 59 and 60.

Results

The results of the survey of the eighty-five 'transitional' individuals are shown in figures 38 to 41 which again are provided with plans showing the way in which the various sera were set out onto the slides, and with interpretations of the precipitin lines present.

It can be seen that each of the 'transitional' individuals has shown a strong reaction against the specific anti-vulgata serum in the upper well but shows no reaction at all to the specific anti-intermedia serum in the lower well.

Conclusions

It is difficult to say exactly how much evidence the antigenic properties of the three species as compared to those of the 'transitional' forms provides.

It would seem that the wide variety of 'transitional' forms tested, which included individuals showing similarities to P. intermedia in five out of the six characters used, all possessed a similar antigenic pattern to that of P. vulgata and possessed none of the antigens specific to P. intermedia.

It was thought at first that if the gene or genes controlling antigen production in P. vulgata were dominant then a hybrid individual would exhibit similarities to only P. vulgata for this character. However since, as has been previously shown, the 'transitional' forms are as fertile as any of the three species one would expect to find a small proportion of 'transitional' individuals with the double recessive gene combination appearing in the population during successive generations. This would result in a small proportion of 'transitional' individuals exhibiting an antigenic pattern similar to that of P. intermedia.

Since this has not been found to occur, the immunological

TABLE 58

THE MORPHOLOGICAL CHARACTERS OF THE INDIVIDUAL 'TRANSITIONAL' FORMS 1 - 30 USED IN THE SURVEY

	Foot colour			Shell lining			Head Scar			Mantle tentacles			Margin-al rays		R/C value
	G	B	O	G	B	W	S	M	O	T	W	O	+	-	
1		+		+			+			+			+		1.9
2	+				+			+		+				+	1.5
3			+	+			+			+				+	0.9
4		+			+		+			+				+	1.8
5	+				+		+			+				+	1.5
6	+			+			+				+		+		1.7
7		+		+				+		+			+		1.7
8		+		+			+			+				+	1.4
9	+				+			+		+			+		1.7
10	+			+			+			+			+		1.5
11	+			+			+			+			+		1.9
12		+		+			+			+			+		2.0
13	+				+		+				+		+		1.9
14		+		+				+		+				+	1.8
15	+				+		+				+		+		1.8
16	+			+				+		+				+	1.4
17	+				+			+			+			+	1.7
18		+		+			+				+		+		1.9
19		+		+			+			+				+	1.8
20	+			+			+			+			+		1.6
21	+			+			+			+				+	1.6
22		+		+				+			+		+		2.0
23	+			+			+				+		+		2.0
24	+			+			+			+				+	1.9
25		+			+			+		+			+		1.9
26	+			+				+		+				+	1.2
27	+			+			+			+			+		1.6
28		+		+				+		+			+		1.9
29		+		+			+			+				+	1.7
30		+			+			+		+			+		2.0

TABLE 59

THE MORPHOLOGICAL CHARACTERS OF THE INDIVIDUAL 'TRANSITIONAL' FORMS 31 - 60 USED IN THE SURVEY

	Foot colour			Shell lining			Head scar			Mantle tentacles			Marginal rays		R/C value
	G	B	O	G	B	W	S	M	O	T	W	O	+	-	
31	+				+			+		+			+		2.0
32		+		+			+			+				-	1.7
33		+		+				+		+			+		1.9
34	+			+			+			+			+		1.6
35	+			+				+		+				+	1.2
36		+			+			+		+			+		1.9
37	+			+			+			+				+	1.9
38	+			+			+				+		+		2.0
39		+		+				+			+		+		2.0
40	+			+			+			+				+	1.6
41	+			+			+			+			+		1.6
42		+		+			+			+				+	1.8
43		+		+			+				+		+		1.9
44	+				+			+			+			+	1.7
45	+			+				+			+			+	1.4
46		+		+			+			+			+		1.9
47	+				+			+		+				+	1.5
48	+				+			+			+		+		1.8
49		+		+			+			+			+		2.0
50	+			+			+			+			+		1.9
51	+				+			+		+			+		1.7
52		+		+			+			+				+	1.4
53		+		+				+		+			+		1.7
54	+			+			+				+		+		1.7
55	+				+		+			+				+	1.5
56	+				+		+			+				+	1.8
57			+	+			+			+				+	1.1
58		+		+				+			+		+		1.9
59	+			+			+			+			+		1.4
60		+			+			+		+			+		2.0

TABLE 60

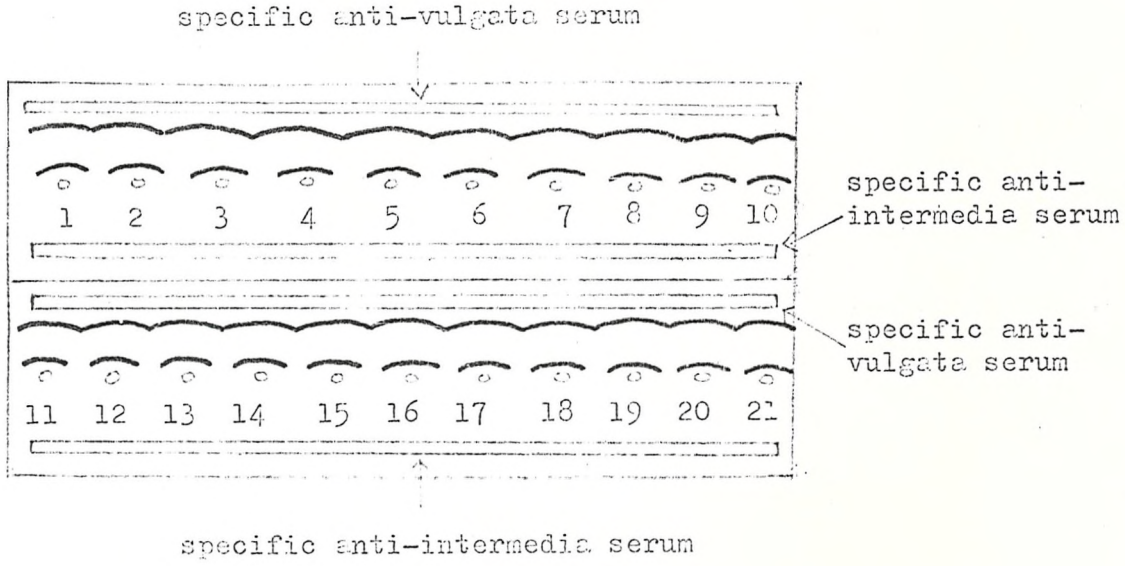
THE MORPHOLOGICAL CHARACTERS OF THE INDIVIDUAL 'TRANSITIONAL' FORMS 61 - 85 USED IN THE SURVEY

	Foot colour			Shell lining			Head scar			Mantle tentacles			Marginal rays		R/C value
	G	B	O	G	B	O	S	M	O	T	W	O	+	-	
61		+			+			+		+			+		1.9
62	+			+				+		+			+		1.8
63		+		+			+			+				+	1.6
64		+		+			+				+		+		2.0
65	+			+				+		+				+	1.7
66	+				+		+				+			+	1.8
67		+			+			+		+			+		2.0
68		+		+			+			+				+	1.7
69		+		+				+		+			+		1.9
70	+			+			+			+			+		1.6
71	+			+				+		+				+	1.2
72		+			+			+		+			+		1.9
73	+			+			+			+				+	1.9
74	+			+			+				+		+		2.0
75		+		+				+		+			+		2.0
76		+		+			+			+				+	1.8
77	+			+				+			+		+		1.8
78	+				+			+		+				+	1.5
79	+				+		+				+		+		1.9
80		+		+			+			+			+		2.0
81			+	+			+			+				+	1.3
82	+				+		+			+				+	1.5
83		+			+		+			+				+	1.8
84	+				+			+		+				+	1.5
85		+		+			+			+			+		1.9

Fig. 38. Plan Of Immunodiffusion Plates 11 to 14 Used To Test The Eighty-Five Individual Transitional Forms.

Interpretations of the precipitin lines present are shown.

Plates 11 and 12



Plates 13 and 14

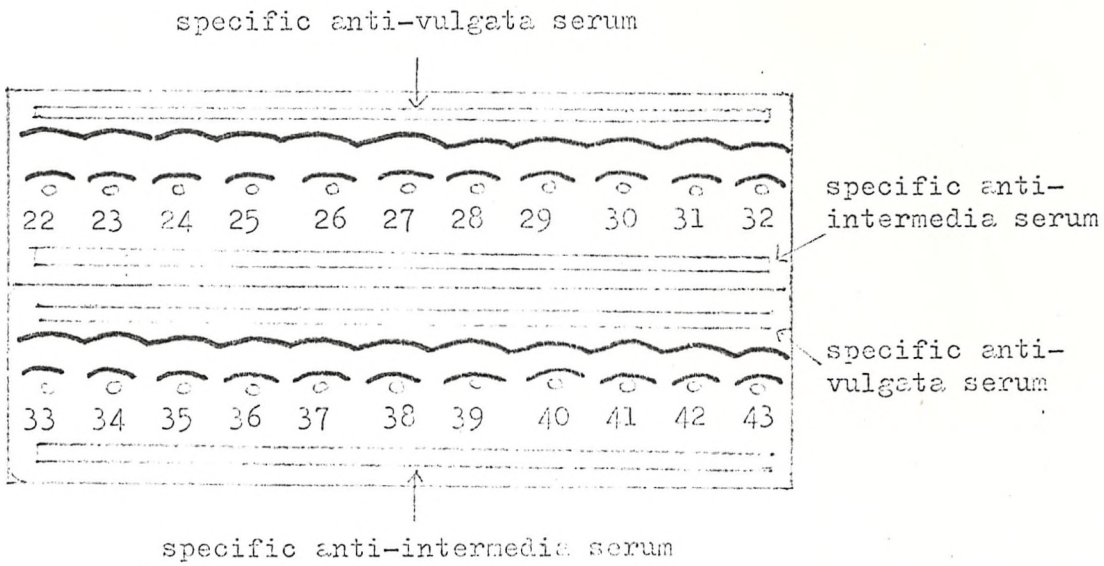
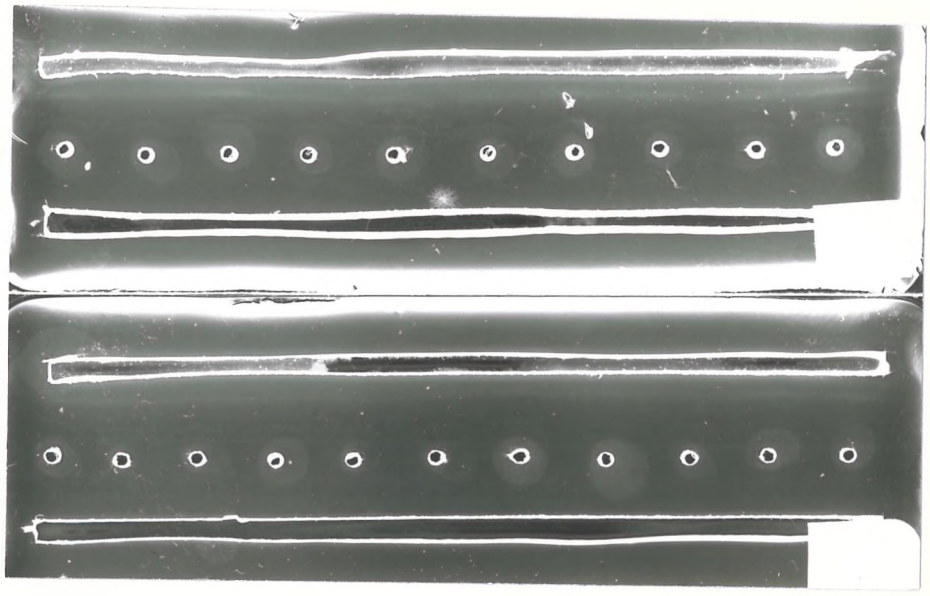


Fig. 39

Plates 11 and 12



Plates 13 and 14

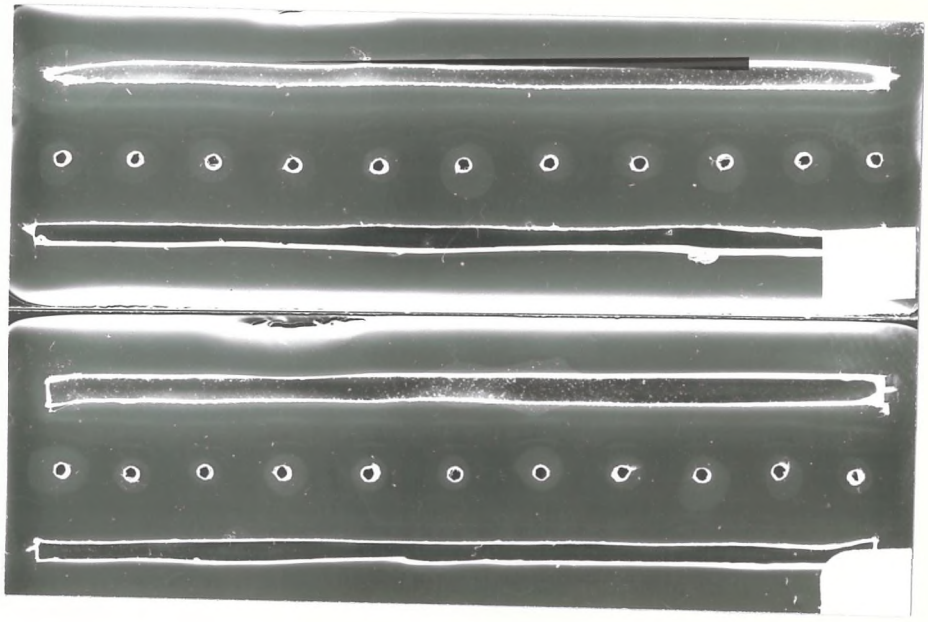
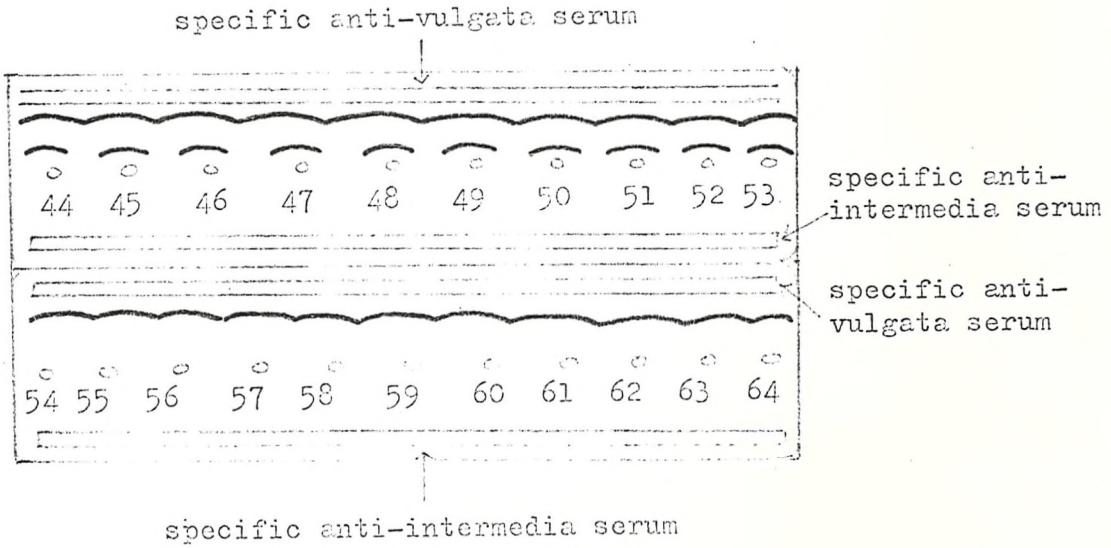


Fig. 40 Plan Of The Plates 15 to 18 Used To Test The Eighty-Five Individual Transitional Forms.

Interpretations of the precipitin lines present are shown.

Plates 15 and 16



Plates 17 and 18

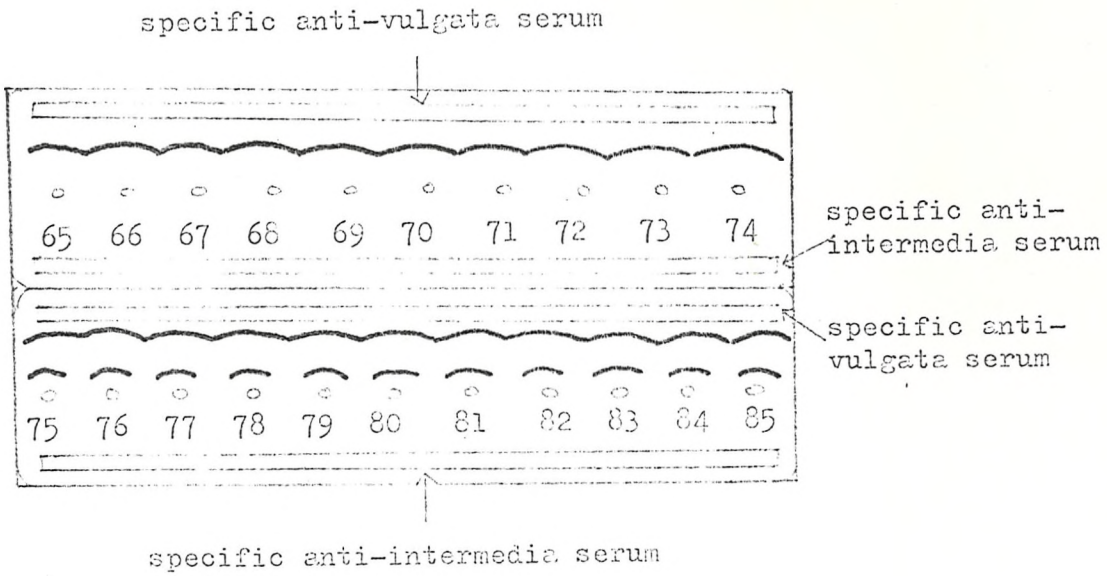
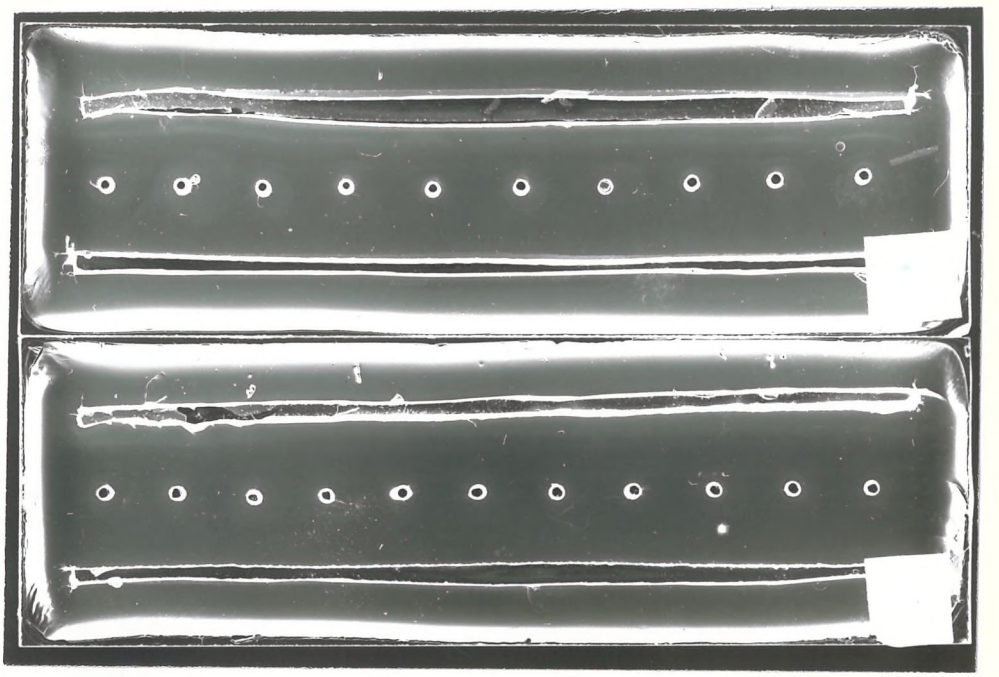
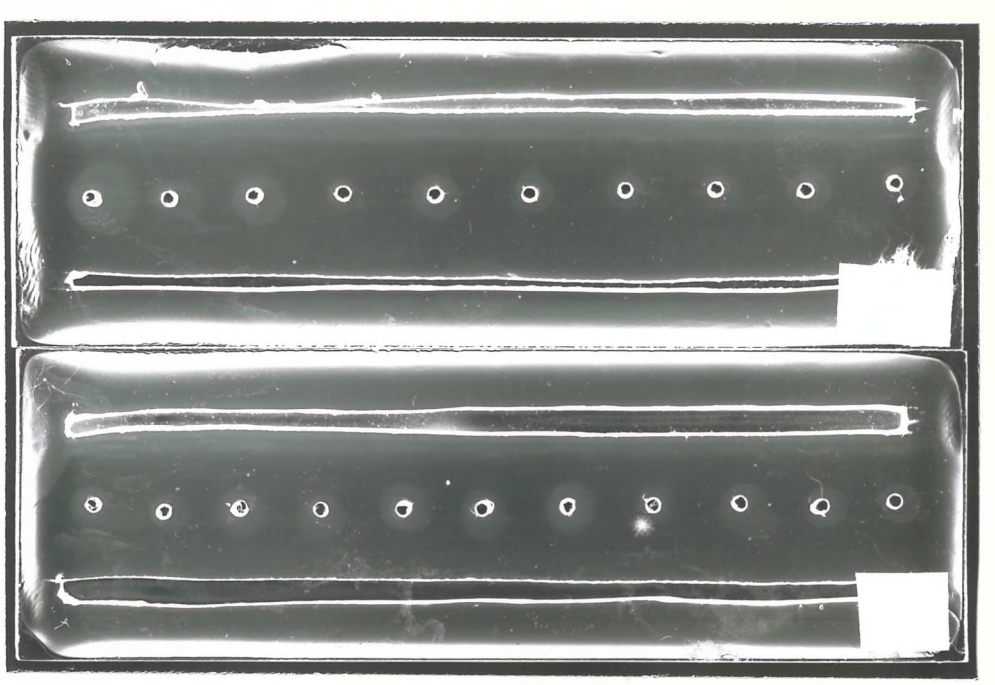


Fig. 41

Plates 15 and 16



Plates 17 and 18



work strongly implies that these 'transitional' individuals are in fact merely variants of the species P. vulgata.

As individuals with five out of the six P. intermedia characters have been found to possess the antigenic pattern specific to P. vulgata, one concludes that P. vulgata is an extremely variable species as compared with P. intermedia which exhibits very little variation.

CHAPTER VII.

ELECTROPHORESIS OF THE BLOOD SERA OF THE THREE LIMPET SPECIES AND OF THE 'TRANSITIONAL' FORMS.

ANALYSIS OF THE BLOOD PROTEINS OF P. VULGATA, P. INTERMEDIA, AND THE TRANSITIONAL FORMS BY ELECTROPHORESIS.

Aims

The purpose of this part of the work was to discover whether P. vulgata and P. intermedia could be distinguished from each other by the banding pattern of blood proteins separated during electrophoresis.

If a difference was observed it would then be possible to establish whether the blood proteins of the 'transitional' forms produce a similar pattern to that of P. vulgata or P. intermedia or whether they produce a pattern intermediate between the two which would support the theory that the 'transitional' forms are hybrids of P. vulgata and P. intermedia.

Hopefully, therefore, this electrophoretic work would provide confirmation of the results of the immunological experiments.

Methods

Disc electophoresis was carried out using polyacrylamide gels as laid out by Davis in his paper 'Disc Electrophoresis' (1964). The sample and spacer gels were omitted and the sample was mixed with a few drops of 80% sucrose solution containing 0.001% bromophenol blue and layered straight onto the small pore separation gel. The current was adjusted to 2ma/tube until the sample had entered the separation gel and the current was then increased to 4ma/tube. Electrophoresis was continued until the free bromophenol blue had migrated 3 cm. into the gel which normally took approximately 40 minutes.

The gels were then stained for 15 minutes in a solution of 0.5% of naphthalene black in 7% acetic acid. 7% acetic acid was used for destaining which took approximately 4 days to be completed.

It was necessary to prepare the blood samples carefully

in order to achieve good separation of the proteins. For each sample blood was collected from the pallial vein of an individual limpet and centrifuged to remove the haemocytes. The concentration of protein in the serum was established using a standard spectrophotometric method and was found to be approximately 3.0 microgrammes/ml. Since the protein concentration was so low it was necessary to concentrate the serum ten times by freeze drying to produce a comparable level of protein concentration to that of the protein samples used by Davis. A volume of 0.1 ml. of the sample was layered onto the gel so that the total amount of protein running through each gel was 3.0 microgrammes.

The results, in the form of photographs provided with interpretations of the bands present, are shown in figures 42 to 47.

Results

One can see from the photographs that there was always a large amount of background staining present in the gels which was probably due to the presence of denatured proteins and, unfortunately, there was insufficient time to improve the technique any further.

However, despite this problem, the gels do demonstrate a marked difference between the patterns produced by the proteins of P. vulgata and P. intermedia and they also show that the pattern produced by the blood serum of the 'transitional' forms is, as far as one can tell from the photographs, the same as that for P. vulgata.

Electrophoresis was carried out about twenty times altogether, using different concentrations of sample and different buffer systems, with varying degrees of success. However, in each case the patterns of banding produced by the blood serum of P. vulgata and the 'transitional' forms were identical and always different from those resulting from electrophoresis of the blood serum of P. intermedia.

Explanatory note on experiments I, II amd III illustrated in Figs. 42 to 47.

The three experiments represent three electrophoretic runs carried out on different days. As far as possible, conditions were kept constant for each run.

The sample of blood serum on each gel was pooled from three individuals, since it was not possible to collect sufficient blood from one individual. Thus, in Fig. 42, the first P. vulgata gel contained a sample pooled from three individuals of P. vulgata. The second P. vulgata gel contained a sample pooled from a different group of three individuals. The same method was used for all the gels in the three runs.

There is a marked difference in the banding patterns produced during the separate runs. The most likely reason for this is that the pH. values of the buffers used for the three runs were slightly different. A slight variation in pH. value would cause the proteins to move at a different rate through the gel and produce slightly different banding patterns. In some cases protein bands have fused with each other, so that although two bands are present, only one can be observed. This effect is particularly marked in experiment III.

Fig. 42. Representation Of The Banding Patterns Produced By Electrophoresis Of The Blood Sera Of P. vulgata, P. intermedia And The Transitional Forms In Experiment I.

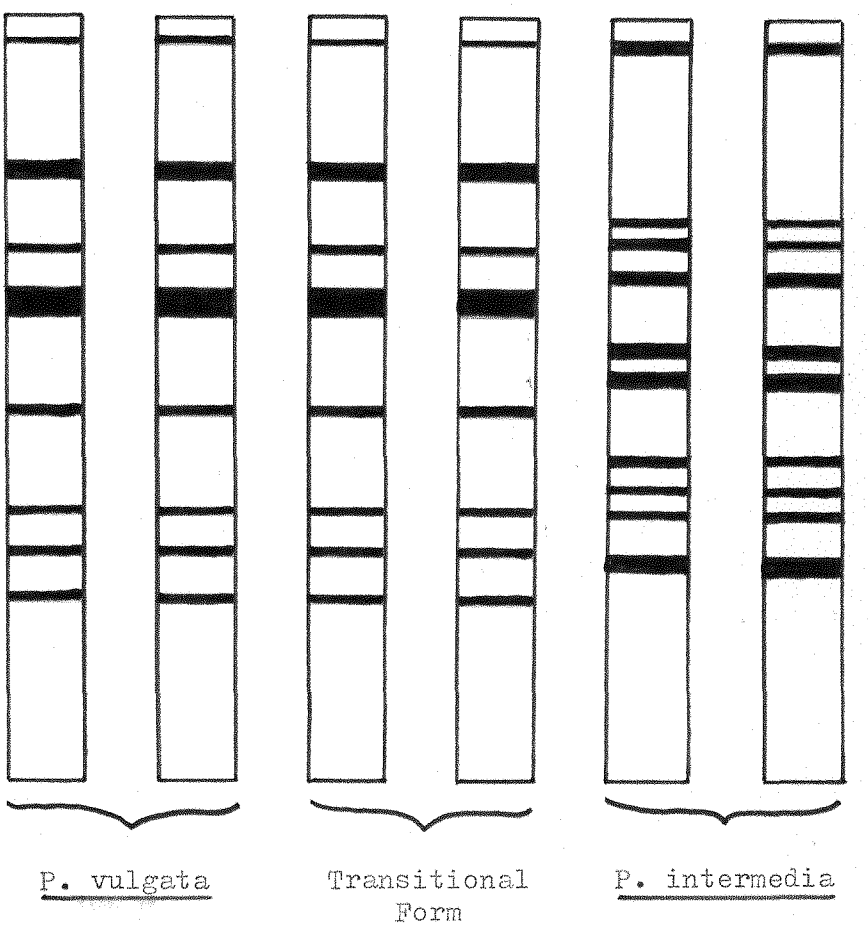


Fig. 43. Representation Of The Banding Patterns Produced By Electrophoresis Of The Blood Sera Of P. vulgata, P. intermedia And The Transitional Forms In Experiment II.

Fig. 44. Representation Of The Banding Patterns Produced By Electrphoresis Of The Blood Sera Of P. vulgata, P. intermedia And The Transitional Forms In Experiment III.

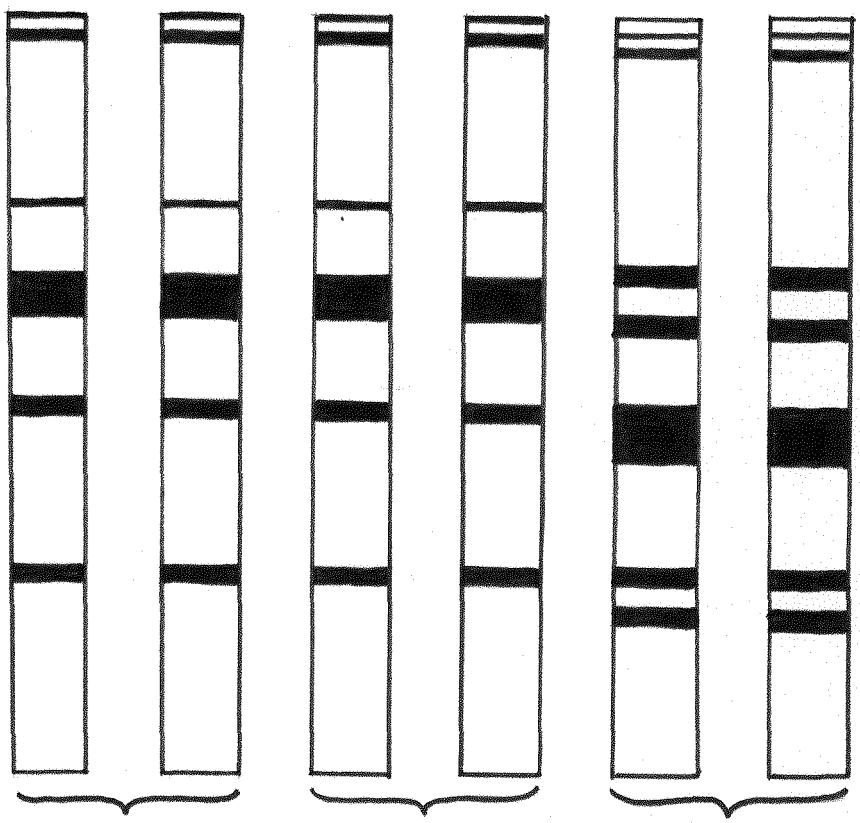
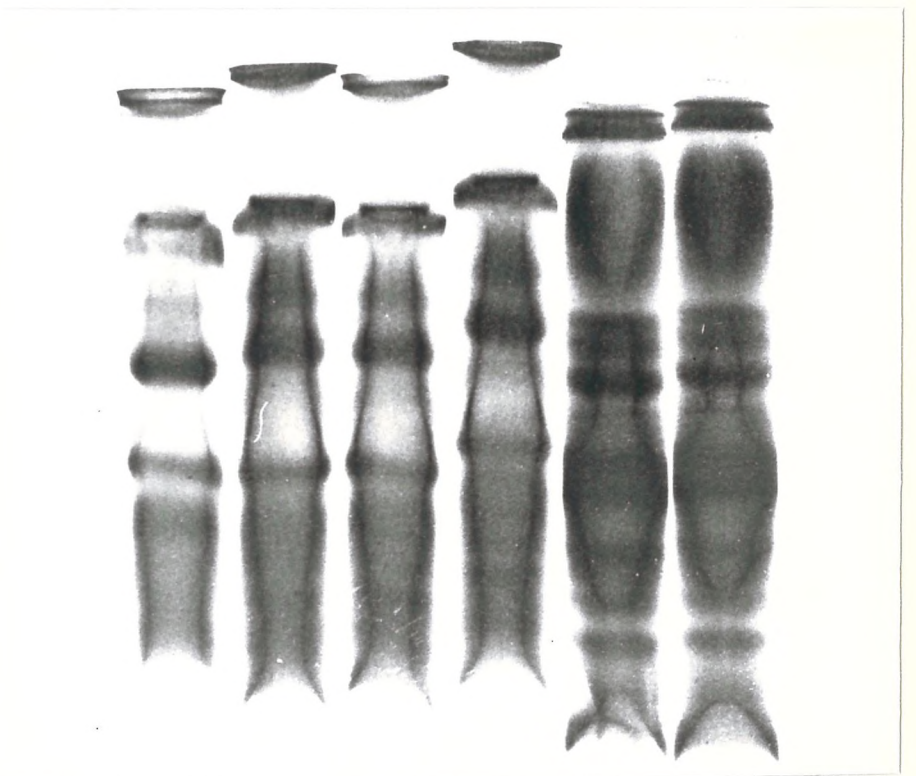


Fig. 45 Photograph Of The Banding Patterns Produced By Electrophoresis Of The Blood Sera Of P. vulgata, P. intermedia And The Transitional Forms In Experiment I.



P. vulgata Transitional P. intermedia
Forms

Fig. 46 Photograph Of The Banding Patterns Produced By Electrophoresis Of The Blood Sera Of P. vulgata, P. intermedia And The Transitional Forms In Experiment II.

P. vulgata Transitional P. intermedia

Forms

Fig. 47 Photograph Of The Banding Patterns Produced By Electrophoresis Of The Blood Sera Of P. vulgata, P. intermedia And The Transitional Forms In Experiment III.

P. vulgata Transitional P. intermedia

Forms

Conclusions

Unfortunately there was insufficient time to complete this work with respect to cutting out the background staining and investigating which particular types of protein were represented by the bands in the gel. Despite this problem, however, it is thought that the results are sufficient to provide evidence that the 'transitional' forms are probably not hybrids of *P. vulgata* and *P. intermedia*, and that with respect to the blood proteins, at least, the 'transitional' forms are similar, if not identical to, *P. vulgata*.

CHAPTER VIII.

DISCUSSION

DISCUSSION

The results of the survey of the limpet populations on the south coast of Britain clearly show that individuals displaying a mixture of characters of two or more species do occur in certain areas. These 'transitional' individuals obviously form an increased proportion of the population as one moves east along the coast, culminating at the Isle Of Wight where the greatest percentage of 'transitional' forms is found. The percentages of 'transitional' forms to be found at Portland Bill, Swanage, Alum Bay and Freshwater Bay are estimated at 1%, 1.5%, 3.5% and 21.5% respectively.

The evidence for the existence of these 'transitional' forms is supported by the increased variability found in characters such as the ratio of radula length to shell length and the pluricuspid teeth as one moves from west to east along the coast.

The results of the single linkage computer programme possibly give a clearer picture of the population structures at Portland Bill and at Freshwater Bay. At Portland Bill the population is demarcated into three groups representing the three species and only a few individuals remain separate from these clearly defined groupings. However on the Isle Of Wight five groupings are defined with two individuals defying classification into even these groups.

These results confirm those found by Evans and Fischer-Piette when they were working in this area. However in certain respects conditions appear to differ from those described by these workers. Evans described 'transitional' forms present on the south coast of Britain as having a mixture of characters of P. vulgata and the Mediterranean species P. caerulea. However, in this work, no evidence has been found to suggest that P. caerulea facies appear in the limpet population in any of the areas studied.

Results show also that P. aspera displays very little intra-specific variation and can be clearly defined as a distinct species

even on the Isle Of Wight. This is demonstrated by the fact that factors such as the ratio of radula length to shell length and the distribution of pluricuspid teeth groupings remain almost constant for P. aspera from one area to another, while these factors may vary to a large extent for P. vulgata and P. intermedia. Also in only an extremely small number of cases were P. aspera characters found to occur in otherwise P. vulgata-like or P. intermedia-like forms. Similarly, no examples were found of P. aspera-like individuals displaying characters normally found in the other two species. The reason for this fact can possibly be explained by the relative distributions of the three species. Whereas the habitats of P. vulgata and P. intermedia overlap to a very large extent, P. aspera is restricted to a much lower position on the shore, possibly due to a low resistance to desiccation, and therefore is more rarely found living in areas occupied by the other two species. Since this is the case P. aspera is more likely to become separated from the other two species, whether one accepts the theory of hybridisation or of incomplete speciation as the cause of 'intermediacy'.

It can, therefore, be assumed that P. caerulea, and to a lesser extent, P. aspera can be eliminated from the study of 'intermediacy' on the south coast of Britain.

Several types of 'transitional' forms involving characters of P. vulgata and P. intermedia have been found during the course of this work, particularly on the Isle Of Wight. These include P. vulgata-like individuals with either the black foot, the mottled head scar, the brown shell lining, the opaque mantle tentacles or the marginal rays of P. intermedia. Similarly, P. intermedia-like individuals were found with the green shell lining or the silver head scar of P. vulgata. However these forms were far less common.

Evidence from the survey of the limpet populations suggests that a majority of the 'transitional' forms are of the P. vulgata type, possessing one or more characters normally found in

P. intermedia. The reverse situation was found to occur only very infrequently.

The study of the distribution of the limpets on the shore reveals that there is an overlap in the habitats of the three species but particularly between those of P. vulgata and P. intermedia. For some reason the extent of this overlap appears to be greater on the Isle Of Wight than in areas further west and a possible explanation for this situation is given later.

The breeding cycles of the three limpet species on the Isle Of Wight do vary to some extent. P. aspera and P. intermedia seem to be primarily summer breeders whereas P. vulgata will continue breeding right through the year. However, during both the years when the breeding cycles were studied, P. aspera and P. intermedia continued to show reproductive activity during the winter but at a reduced rate. The reason for this extension of the breeding cycle may have been that the winters of 1974 and 1975 were exceptionally mild. P. intermedia reached a peak of reproductive activity in May and June but a few individuals remained in breeding condition until the following January. P. aspera was found to have a shorter breeding season and reached a peak of reproductive activity in August. The breeding cycles of P. vulgata and of the 'transitional' forms appeared to be almost identical and both reached their peak of reproductive activity during November and December. Despite these differences there is a considerable period of time when all three species show some degree of breeding activity thus making the possibility of hybridisation feasible.

Results of the hybridisation experiments show that the three species can be crossed artificially with a reasonable degree of success. By studying the survival rates of the larvae of the different crosses it can be seen that P. vulgata can be crossed with P. intermedia and P. aspera with a greater degree of success than

when P. aspera and P. intermedia are crossed with each other, suggesting that P. aspera and P. intermedia are more closely related to P. vulgata than they are to each other. Also the larvae of the cross between P. vulgata and P. intermedia have a greater survival rate than of any other cross which might suggest that P. vulgata is more closely related to P. intermedia than to P. aspera. However, as mentioned in the introduction to this work, it is difficult to know how much valid information is provided by the results of crossing different species under artificial conditions.

More importantly the results show that with respect to the survival rates of the larvae, the 'transitional' forms produced the same results as P. vulgata in both the normal fertilisations and in the crosses with the other two species. With the same reservations as explained before this fact seems to provide evidence for a very close relationship between P. vulgata and the 'transitional' forms.

Another fact discovered during the hybridisation experiments was that the larvae of P. aspera and P. intermedia take a longer time to reach the initial stages of development, so that in P. aspera and P. intermedia the first cleavage of the eggs was observed after four hours whereas the larvae of P. vulgata reached this stage after only three hours. The reason for this could be explained by the fact that P. aspera and P. intermedia are normally summer breeders and their larvae would normally be developing in sea temperatures of about 16^{o}C. Since all the experiments were carried out at a temperature of 12^{o}C it may have been that the development of the larvae of these species may have been retarded. On the other hand P. vulgata is an autumn and winter breeder and the larvae would normally be subjected to temperatures of 12^{o}C or below and therefore were probably developing normally in the laboratory. Despite this fact it was thought to be reasonable to compare the development times of the 'transitional' forms with those of the three species

in order to help establish the relationships between them. Since the 'transitional' forms showed the same pattern of development as P. vulgata this was taken as good evidence of a close link existing between them.

It was because this difference existed between the 'transitional' forms and P. vulgata and the other two species with respect to the time taken for the larvae to develop, that another fact about the development of the larvae was noticed. This was that, in every case, the development of the larvae is determined by the species represented by the maternal parent. Thus, the larvae of a cross between a P. vulgata female and a P. intermedia male would develop in the shorter time normally common to P. vulgata, whereas if the cross was performed the other way round the larvae would take the longer time to develop as usually seen in P. intermedia and P. aspera. This phenomenon has been observed in other animals, for example, certain species of sea urchins and may be worthy of further investigation. However it is not particularly relevant to this work.

The results of the immunological work are fairly clear cut. Specific antisera to P. vulgata and P. intermedia were successfully produced and could be used to distinguish between the two species. Each of the eighty-five 'transitional' forms produced a strong reaction against the specific anti-vulgata serum and no reaction at all against the specific anti-intermedia. This would seem to provide good evidence that the 'transitional' forms are not hybrids between P. vulgata and P. intermedia since if this were the case one would expect to find some reaction against the specific anti-intermedia serum in at least a small proportion of the 'transitional' forms. It was thought at first that the failure of hybrids to show a reaction against the antisera of the two species could be explained if the gene or genes controlling the antigenic properties were dominant in P. vulgata. However, since the 'transitional' forms have proved to be fertile and are presumably continuing to contribute to the gene pool of the population, one would expect to find a small proportion of individuals, with the double recessive gene combination, which

would show a reaction against the specific anti-*intermedia* serum. Not only do the results imply that it is not very likely that hybridisation is occurring, but they also show that again the 'transitional' forms are exhibiting many affinities with *P. vulgata*.

It was somewhat surprising to find that *P. vulgata* and *P. intermedia* could be easily distinguished from each other by ordinary electrophoretic methods. It is thought that the considerable differences in the blood protein composition are due to the different blood pigments in the two species. When blood is extracted from the limpets it can be clearly seen that the blood of *P. intermedia* is much more heavily pigmented than that of *P. vulgata*, and is a dark orange colour. In contrast the blood of *P. vulgata*, and of the 'transitional' forms, is almost colourless. It is not known exactly which proteins differ between the two species but there is little doubt that the blood proteins of the 'transitional' forms produce the same banding pattern in the gel as the blood proteins of *P. vulgata*. If one was examining the blood proteins of a hybrid between two species one would expect to find a pattern intermediate between that of the two species as found by Sick, Frydenberg and Nilsen (1963) in their work on *Pleuronectes* spp. as described in the introduction to this work. Since this is clearly not what is happening in the case of the *Patella* 'transitional' forms, this is further strong evidence to suggest that they are not in fact hybrids.

Thus it is concluded, from the evidence of the breeding cycles and the hybridisation experiments and from the biochemical tests used, that the 'transitional' forms are merely phenotypic variations of the species *P. vulgata* and that a vast proportion of the variation seen in the limpet populations particularly on the Isle Of Wight is attributable to this species.

It is proposed, therefore, that while it is possible that some hybridisation is occurring, this cannot be the major cause of the presence of these 'transitional' forms. The evidence for this proposal is that if the 'transitional' forms were true hybrids or

the results of introgressive hybridisation in the population, there would be a more even distribution of P. vulgata and P. intermedia characters exhibited by these individuals. Thus hybrids of P. vulgata and P. intermedia would be expected to show some affinities with P. intermedia with respect to either the breeding cycles, the development of the larvae, or the biochemical properties of the blood. However the only similarities to P. intermedia exhibited by the 'transitional' forms are in a few morphological characters.

Having eliminated the theory of hybridisation as a causative factor for the existence of 'transitional' forms, it is now necessary to offer an alternative explanation for the enormous amount of variation in the P. vulgata population.

The most likely theory seems to be that P. intermedia and P. aspera have evolved from a common vulgata-like ancestral species and that, whereas this process of speciation is virtually complete in areas such as the coasts of Wales and Cornwall, this process is still taking place further east along the coast. Moreover it would seem that P. aspera has succeeded in becoming a distinct species even on the Isle Of Wight and it is only P. vulgata and P. intermedia that have not succeeded in becoming fully separated from each other. The idea of a P. vulgata-like common ancestor is supported by the results of the hybridisation experiments which demonstrate that P. intermedia and P. aspera are more closely related to P. vulgata than they are to each other.

It is now necessary to try and explain the reason for this discontinuity between the limpet populations of the different areas of the south coast.

It is suggested that the situation might be explained by comparing the types of shore found in the two areas. A very noticeable feature about the rocky shores of the Isle Of Wight is that below the steeply shelving base of the cliffs the shore is very flat,

gently shelving and uniform, and virtually all parts of the shore provide equally exposed conditions. However the types of rocky shore found on the west coast are very different. In these areas the shore is steeply shelving with large outcrops of rock which provide features such as rock pools and sheltered land facing positions, resulting in a much more variable environment. A simplified representation of the two types of shore are shown in figure 48. Thus in the case of the Isle Of Wight the two individuals a) and b) are subjected to very similar environmental conditions. They are both in an exposed position and would be submerged for approximately the same time during high tide. In contrast the individuals c) and d) are subject to very different conditions although they are living the same distance apart as a) and b). Individual c) occupies a much more sheltered position than d) and probably has to deal with greater fluctuations in temperature and salinity than d). Also if c) was living in the rock pool it would remain submerged for a longer period than d) which is living lower down on the shore.

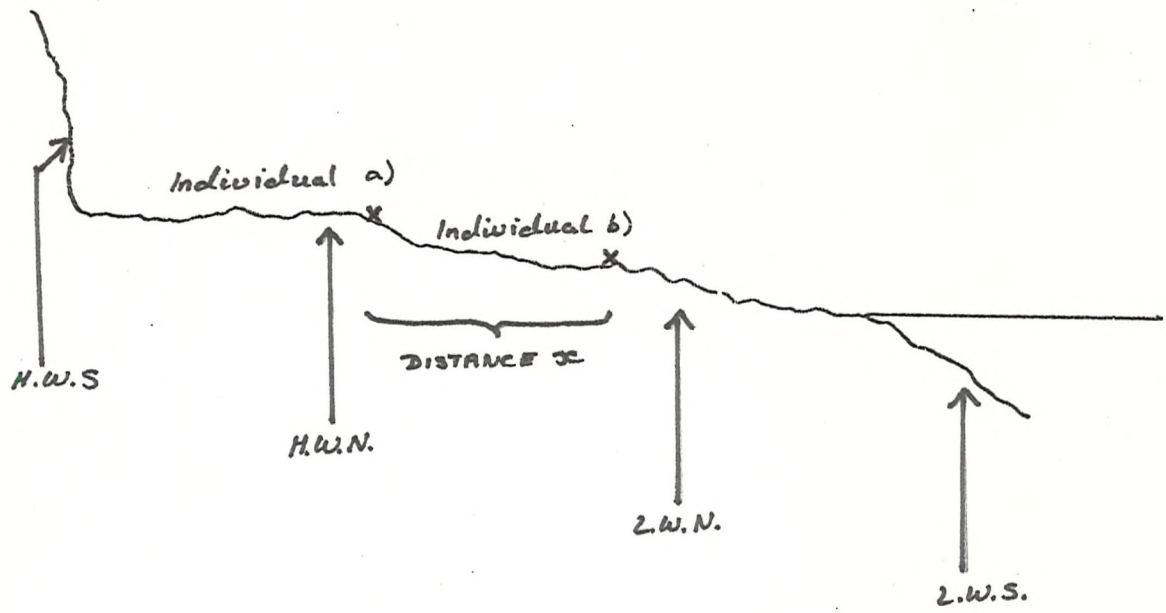
For this reason it is possible that on the west coast a considerable degree of ecological isolation has resulted between the three species thus allowing speciation to occur more rapidly than in eastern areas of the coast where the thræspecies are not subject to the same degree of ecological isolation due to the more uniform conditions pertaining in these areas.

This would also explain why the habitats of the three species were found to overlap to a greater extent on the Isle Of Wight than at Swanage.

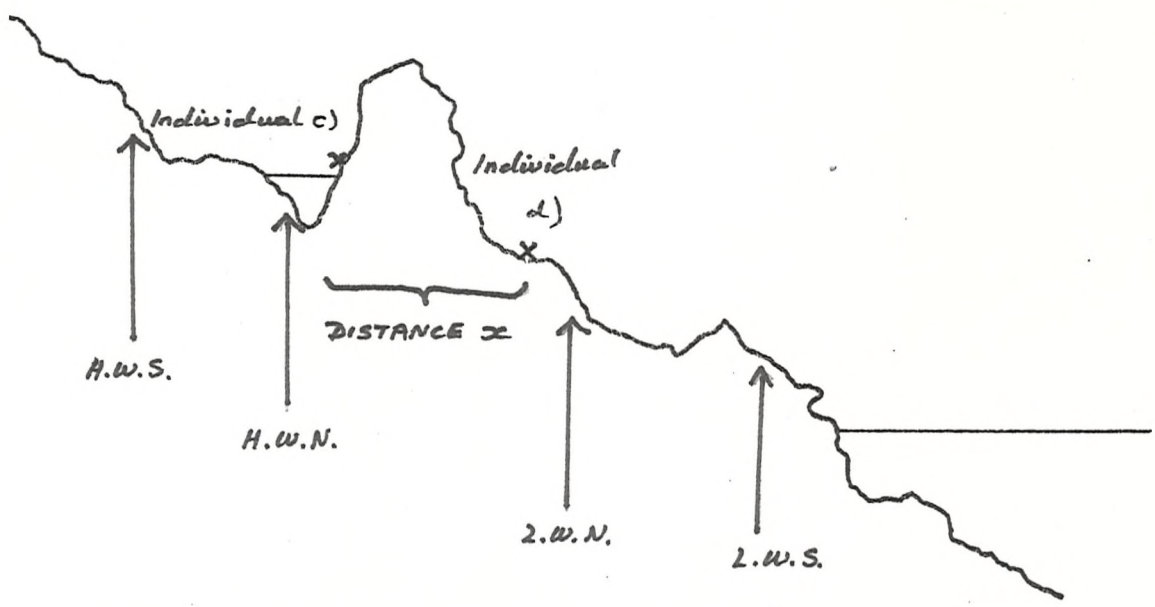
It is possible that such an explanation could be relevant to the situation on the west coast of France. Unfortunately information on the relative topographies of the north and south coasts of France could not be found, so it is not possible to extend this theory to

Fig. 48 The Comparative Structures Of Typical Rocky Coasts Found On The Isle Of Wight And In Cornwall.

I. Isle Of Wight.



II. Cornwall



these areas. However it would be interesting to find out whether the structures of the shores on the west coast of France vary in the same way.

To conclude, the present work indicates that the 'transitional' forms on the Isle Of Wight are the result of incomplete separation of the two species in this area and that there is scope for further investigation to discover, fully, the reasons for this observed lag in speciation as compared to areas further west along the south coast of Britain.

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