6 Biochemical and Immunological Taxonomy of the Mollusca

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I. Introduction

The application of experimental techniques to molluscan taxonomy has both a pragmatic and an empirical foundation. Pragmatic because most of the studies have centred upon species which have some economic significance to man, either as transmitters of disease or as sources of food. Empirical because, in the early stages, techniques tended to be applied at random in attempts to supplement the not wholly satisfactory approaches of traditional morphology. A strong stimulus to some of the biochemical studies has been the parasitologists' desire to take a worm's eye view of the Mollusca. For these reasons this review is limited to a few molluscan groups and the conclusions which have been reached are restricted in their application. The emphasis has been upon species discrimination and the characterization of infra-specific categories rather than on the relationships of the phylum as a whole or even of classes and orders within the phylum. In many cases the techniques employed have been tested almost to destruction and this allows some assessment to be made of the utility of the methods themselves. It must be remembered that any such

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assessments are confined to the context of the application of the techniques and that direct extrapolation to other groups may not be justified. This point becomes clear in this review as it is seen that some methods which work well within one family of gastropods are simply not applicable to other families.

The material is arranged under method headings. Such a presentation leaves a certain amount to be desired but the results from most of the early exploratory investigations do not readily lend themselves to any other treatment. Fortunately some of the recent contributions have been of a more precise nature both with respect to the methods used and to the taxonomic problems involved. Many of the methods have become sufficiently well established for them to be applied as routine adjuncts to some taxonomic studies and where several techniques have been applied to a single problem it is possible to make appropriate evaluations of their respective merits. Although such mixed approaches will involve a certain amount of cross-reference within the text they also serve to draw together current progress in the experimental field.

II. Chromatographic Methods

The earliest applications of chromatographic methods to taxonomic work were directed towards the study of amino acids in the hope that basic differences in the protein composition of different species of organisms might be revealed. In the preparation of material for analysis two general approaches were used, either pieces of tissue or small, whole animals were squashed directly onto paper or extracts were prepared from which soluble proteins were precipitated so that the resultant solution contained only amino acids and small peptides. The second method gave more precisely quantifiable results and clearer separations of the ninhydrin-positive materials but the squash technique, because of the presence of a variety of substances in the tissues, provided the opportunity for investigating other biochemical differences. Of these other substances the compounds which fluoresce in ultra-violet light have attracted most attention and it was this approach which was first used in molluscan studies by Kirk *et al.* in 1954.

A. TISSUE EXTRACTS

In their pioneer contribution Kirk and his co-workers examined a number of species of stylommatophoran land snails from Western Australia (including several species of the native genus *Bothryembryon*) and found that each species yielded a characteristic pattern of fluorescent bands. They examined samples of *Theba pisana* and *Helix aspersa* from widely separate localities and were unable to find any signs of geographical variation but since neither of these species is native to Australia and must, therefore, be relatively recent introductions, one would not expect to find marked population differences. The technique used by Kirk *et al.* (1954) was a horizontal disk method using improvised apparatus. Results obtained in this way were not easily reproducible but development of the Kawerau dish with mechanically cut slotted disks led to great improvements (Wright *et al.*, 1957). A further modification came with the discovery that all of the fluorescent materials found in chromatograms made from squashed fragments of molluscan foot are present in the body-surface mucus. Using mucus only Wright (1959a) carried out a survey of the British species of *Lymnaea* and was able to demonstrate the existence of species-specific patterns and of generalized patterns characteristic for species groups corresponding to some of the older generic divisions of *Lymnaea*. Thus *L. peregra* and *L. auricularia*, members of the former genus *Radix*, have in common a brilliant blue band between Rf 0·4–0·45 while *L. palustris*, previously included in *Stagnicola*, has two bands, one pink and the other lilac between Rf 0·5–0·65. No fluorescent substances were found in the mucus of *L. stagnalis* or *L. truncatula*.

Michejda and his co-workers in Poland (Michejda, 1958; Michejda and Turbanska, 1958; Michejda and Urbanski, 1958) made fairly extensive investigations into the uses of chromatography in molluscan taxonomy. They compared the ninhydrin-positive and fluorescent patterns obtained from aqueous tissue homogeneates of five species of freshwater prosobranchs and five basommatophoran pulmonates. They found that there was a general agreement between the chromatographic patterns and the accepted relationships of the species concerned. The agreement appeared to be more marked among the prosobranchs than among the pulmonates. They made quantitative comparisons between the ninhydrin-positive patterns obtained from skin, muscle, digestive gland and 'visceral sac' of two species of the prosobranch genus *Viviparus*, *Lymnaea stagnalis* and three species of helicids. Their findings showed that skin and muscle gave similar patterns and that there was close agreement between the patterns given by the other two organ systems. A generalized group relationship emerged from this investigation in that curves of the density of the pattern plotted against Rf value were bi-modal for the helicids, unimodal for the viviparids and 'parabolic' for *L. stagnalis*. The third part of the study consisted of a survey of fifty species of gastropods, mostly land snails among which the Helicidae were particularly well represented, using aqueous homogeneates of both whole snails and foot muscle tissues. These analyses gave indications of family relationships but the conclusions drawn were somewhat tentative due to a certain amount of conflict in the results from different groups.

Subsequent applications of chromatography to molluscan taxonomy have been largely concentrated on two aspects of the freshwater basommatophora but before dealing with these it is appropriate to look at three isolated but interesting contributions from the marine field. Collyer (1961) used foot-muscle squash chromatograms to investigate the significance of minor morphological differences between two British populations of the gastropod

Nassarius. Consistent differences in the ninhydrin-positive patterns from the two populations were obtained. Size of the individuals did not appear to affect the pattern but their nutritional state did, certain fractions being lost in starved specimens. These nutritional differences however proved to be reversible. In the light of these results Collyer was inclined to uphold the distinction between *Nassarius reticulatus* and *N. nitida*. Working with the eastern North American oyster *Crassostrea virginica* Hillman (1964) was able to demonstrate differences in the ninhydrin-positive pattern between populations from Long Island Sound and the James River in Virginia. The materials used in this case were direct squashes and alcoholic extracts of adductor muscle tissue. Rucker (1965) compared the amino acid patterns derived from hydrolysis of conchiolin of eight species of marine molluscs. He found striking differences between the species but, with one exception, the patterns given by *Crassostrea virginica* from six localities were similar. The exceptional sample consisted of dead weathered shells in which some degradation of the conchiolin appeared to have occurred. Thus, in these three examples differences were found at the species level in *Nassarius* and at the population level in *Crassostrea* while examination of different material from *Crassostrea* failed to reveal population differences but suggested the existence of specific characteristics.

B. FREE AMINO ACIDS

Taxonomy has not been the prime objective of most investigations of the amino acids of freshwater gastropods. However, studies concerned with the host as an environment for developing trematode larvae have yielded a certain amount of comparative data. Targett (1962b) compared the free amino acids from various tissues in *Biomphalaria glabrata*, *Lymnaea stagnalis* and *Planorbarius corneus* and showed that there were few significant qualitative differences between comparable tissues from the three species. An interesting exception was methionine which, although present in the digestive glands of all three was found in the 'anterior tissues' of only *B. glabrata*. Also of interest was the absence of arginine and histidine in the anterior tissues of laboratory bred *L. stagnalis*, both of these substances being found in wild-caught individuals of the same species. In an earlier paper Targett (1962a) reported on free amino acids in the blood of several species and some of these results are shown in Table I together with those of other workers for comparison.

To what extent the differences which appear in this table are a true reflection of differences between species and populations or simply result from technical variations it is difficult to say. Gilbertson *et al.* (1967) examined four strains of *B. glabrata* and although they found some quantitative differences between the strains they got almost comparable levels of variation from replicate tests on a single population. There are discrepancies between the findings of Targett (1962a) and Gilbertson *et al.* for *B. glabrata* but the most surprising report is that of Dusanic and Lewert (1963). They claimed that

Table I
Free amino acids in the blood of some freshwater basommatophoran pulmonates

	Biomphalaria glabrata			*Lymnaea stagnalis*		*L. palustris*
	Targett (1962a)	Dusanic and Lewert (1963)	Gilbertson *et al.* (1967)	Targett (1962a)	Friedl (1961) (*L.s. jugularis*)	Morrill (1963)
Alanine	+		+	+	+	+
Aminobutyric acid					+	
Arginine			+			+
Asparagine					+	+
Aspartic acid	+		+	+		+
Cystine	+			+	+	+
Glutamic acid	+		+	+	+	+
Glutamine					+	+
Glycine	+	+	+	+	+	+
Histidine			+		+	+
Hydroxyproline						+
Isoleucine	+		+	+	+	+
Leucine	+	+	+	+	+	+
Lysine	+	+	+	+	+	
Methionine			+			
Ornithine					+	+
Phenylalanine	+		+	+	+	+
Proline		+	+		+	+
Serine	+		+	+	+	+
Threonine	+	+	+	+	+	+
Tryptophan					+	+
Tyrosine	+		+	+	+	+
Valine	+		+	+	+	+

threonine and leucine only appeared after exposure of the snails to miracidia of *Schistosoma mansoni* and that leucine subsequently disappeared 14 hours after exposure and threonine after 24 hours. Both of these amino acids have been found in uninfected snails in all of the other investigations but proline which Dusanic and Lewert report in uninfected *B. glabrata* was not found by Targett either in this species or in *L. stagnalis*.

The conflicting evidence of these reports and the results of our own investigations (Wright and Ross, unpublished) tend to confirm the doubts expressed by Burch (1960) concerning the value of amino acid studies in the taxonomy of freshwater gastropods. It is perhaps significant that only one recent paper (Stiglingh and van Eeden, 1970) has appeared on the subject.

C. MUCUS

The other application of chromatographic techniques to taxonomic problems in the basommatophora, the study of fluorescent substances in mucus, has had slightly more success. Following on Wright's (1959a) basic survey of the British species of *Lymnaea* collections were made of some of the well-known variants of *L. peregra* from mountain lakes in Southern Ireland (Ballantine and Bradley, 1963). Chromatograms of the body-surface mucus from this material revealed fluorescent patterns which differed from that shown by the seven populations previously examined from various localities in England. In the light of these results an intensive survey of *L. peregra* was undertaken throughout western Europe with particular attention concentrated in Scotland and Ireland. Over 700 populations were sampled and seven distinctive pattern types were found. Of these, three were very rare, three have wider but restricted distributions and the seventh is widespread throughout the species' range (Wright, 1964, 1966b). Of the three rare forms one was found only in streams draining from hot springs in Iceland, one in a single lake in the extreme north of Scotland and the third in a Scottish lake and a stream in the north of England. Of the three restricted forms one appears to be largely confined to small lakes on granite rock in the west of Ireland and north-west Scotland (*patternless*), the second is also lacustrine but has a wider distribution in Scotland and Ireland and extends into lakes in the north of England and on high ground in Wales (*no-delta*) and the third is common in streams in Scotland, Ireland and Wales and a few localities in the west of England (*split-beta*); this form also occurs in some lakes in Scotland. The seventh form (*normal*) occurs in all kinds of habitats and has been found throughout the British Isles, all over France and the Iberian peninsula, northern Italy, Turkey and the Atlas mountains in Morocco. The Icelandic, *patternless*, *no-delta*, *split-beta* and *normal* forms have all been bred in the laboratory on standard diets and their patterns have remained unchanged through many generations. Hybrids between some of the possible crosses have been achieved although *patternless* has not yet been successfully mated with *normal*. In nature hybrids between *split-beta* and *normal* occur commonly and specimens with intermediate patterns have been found in streams draining from lakes containing different forms. There is no apparent correlation between the fluorescent mucus pattern and shell shape, the true-breeding involute forms from Lough Crincaum in Ireland and Loch Fleodach Coire in Sutherland both have the *normal* pattern type while the large spireless form originally described as *L. burnetti* from Loch Skene in Dumfriesshire has a *no-delta* pattern like that of many spired Scottish lake populations.

Two very useful lessons can be learned from this study. The first is that some biochemical variation between populations of a single species may be greater than the differences between populations of different species. The second is that the degree of intra-population variation within the range of a species is far from uniform. In the case of *L. peregra* and its close relative *L. auricularia* Wright (1964) suggested that high levels of intra-population variation occurred in the regions in which the two species probably evolved but this may be a special case and not necessarily of general application.

The work on *Lymnaea* was developed to provide background information for comparable studies on the planorbid snail hosts of African *Schistosoma* spp. Preliminary mention of the existence of population differences in mucus chromatograms of *Bulinus truncatus* was made by Wright (1962). Three populations from Iraq and one each from Iran and Israel showed a basically similar three-banded pattern but the innermost of these bands was lacking in a sample from near Cairo and was scarcely visible in a Sudanese population from near Khartoum. In contrast to the fluorescent materials in lymnaeid mucus, which show up clearly in ultra-violet light (365 nm) on freshly developed chromatograms, those of *Bulinus* are usually difficult to detect until the chromatogram is exposed to ammonia fumes. No fluorescent substances have so far been found in the mucus of any species of *Biomphalaria*. Within the genus *Bulinus* the members of the *B. africanus* species group (= *Physopsis*) lack fluorescent substances, species in the *B. forskali* complex have a single fast-moving band (Rf about 0·85 in a butanol-acetic acid-water solvent) which fluoresces a pale greenish-blue colour and members of the *B. truncatus* and *B. tropicus* groups have the basic three-banded pattern between Rf 0·45 and 0·65 previously mentioned for *B. truncatus*. The inner of these bands (1) (Rf 0·45–0·49) is very pale blue or yellowish, the middle (2) is also blue (Rf 0·55–0·58) and the outer (3) is yellow (Rf 0·60–0·65). A population of *B. liratus* from Madagascar and several populations of *B. tropicus* from the South African highveldt have a wide bright blue band (5) which appears only on exposure to ammonia fumes between Rf 0·80 and 0·95 (Wright, 1971). In a recent study of a polyploid complex of *Bulinus* from the Ethiopian highlands Brown and Wright (1972) found that there was considerable variation between different populations in the presence, absence or intensity of the three bands in the basic pattern but that generally speaking the pattern for any particular population was characteristic. In four diploid populations band 5 was found, in one case it was present in four out of six individuals from

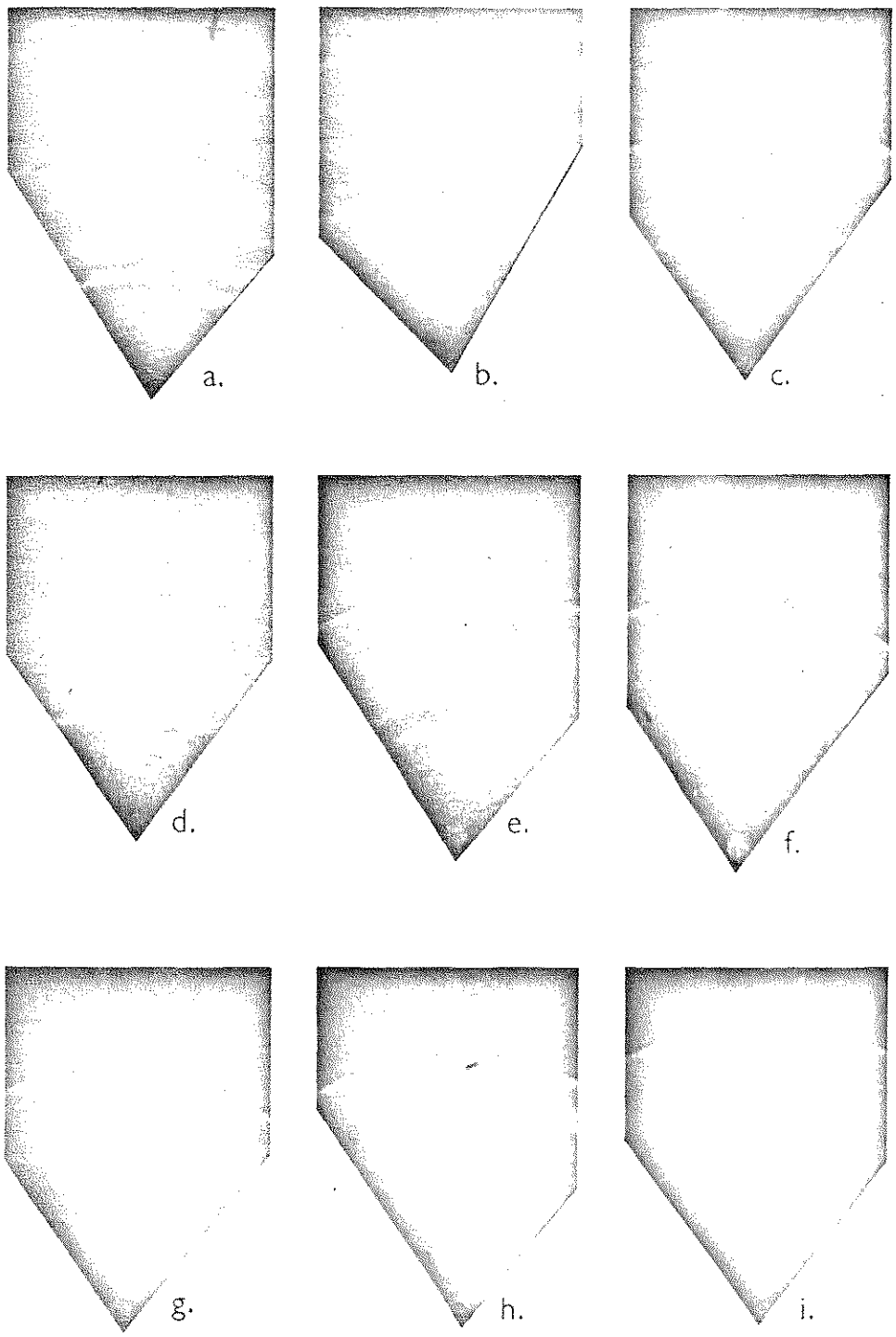


FIG. 1. Paper disk chromatograms of the body-surface mucus of *Bulinus* species, viewed under ultra-violet light after exposure to ammonia fumes (except (i)). (a), (b) and (c) octoploid populations from the highlands of Ethiopia; (d) *B. truncatus* (tetraploid) from Sardinia; (e) *B. truncatus* (tetraploid) from Morocco; (f) *B. natalensis* (diploid) L. Sibayi, South Africa; (g) *B. liratus* (diploid) Madagascar; (h) diploid population from Ethiopia; (i) *B. barayi* (*forskali* group) from Madagascar.

The patterns in (a)–(c) are typical for most populations in the *Bulinus truncatus/B. tropicus* complex. In (a) bands 1–3 are all well developed and roughly equal in intensity, in (b) band 1 dominates and bands 2 and 3 are less well developed while in (c) it is band 2 which dominates. In (d) band 2 is virtually alone, there being only slight traces of bands 1 and 3 while in (e) band 2 is dominant, 3 is stronger than 1 and band 4 is present although weak. In (f) all five bands are present but only band 4 is well developed while in (g) and (h) bands 1, 2, 3 and 5 are all well developed with 5 dominant. In both of these populations band 1 is yellowish in contrast to its blue colour in the other patterns and band 1 may not be homologous throughout. All specimens of *B. liratus* examined have band 5 well developed also all specimens of *B. tropicus* from the highveldt near Potchefstroom in South Africa but it has only been found in three diploid populations from Ethiopia and is not present in all individuals. Specimens in these Ethiopian samples showing band 5 have given rise to offspring lacking the band but selection of those with it has increased its frequency in laboratory culture. The greenish-blue band in (i) is characteristic for all members of the *B. forskali* group and it is visible (as in this specimen) without exposure to ammonia fumes.

a sample and from another locality in 12 out of 49 specimens examined. Individuals with band 5 produced offspring both with and without the band and when present it was always intense, no apparent intermediates being seen. Among tetraploid populations a similar range of variation in the basic three bands occurred but specimens from Lake Margherita show a diffuse band, visible before exposure to ammonia fumes in the region of bands 4 (Rf 0·70–0·80) and 5. Octoploid samples also showed the usual pattern of basic variation and some parents with a three-banded pattern gave rise to offspring in which band 3 was lacking while a single individual from one locality had bands 1–3, a pale blue band 4 and a diffuse blue in the position of band 5 (unlike the bright colour of typical band 5) and gave rise to offspring with similar patterns. A selection of these patterns is illustrated in Fig. 1 and gives some idea of the range of inter-population variation. Until the fluorescent substances have been chemically characterized and the physiological pathways leading to their synthesis are understood we can do little more than point to them as potentially useful taxonomic characters. Below the species level they allow discrimination between populations and races of snails and they also provide a basis for drawing together species at the sub-generic or species-group level.

III. Electrophoretic Methods

Electrophoresis of proteins has been widely used in molluscan taxonomy and has made appreciable contributions of value. Most of the techniques giving improved resolution have been employed (moving boundary and paper electrophoresis gave inadequate resolution) and there has been some diversity in the choice of protein systems selected for examination.

A. GENERAL BLOOD PROTEINS

Although blood proteins would seem to be the most obvious source of material they have not been widely used for various reasons. Woods *et al.* (1958) included blood of the oyster *Ostrea virginica* and a squid (*Loligo pealeii*) in a general survey of marine invertebrate sera on starch gel and Wright and Ross (1959) made a preliminary study of the blood of the planorbid *Biomphalaria glabrata* using cellulose acetate as the supporting medium. However, Wright and Ross (1963) found that considerable depletion of the blood protein fractions of *B. glabrata* occurred with increasing age and Targett (1963) noted that the blood proteins other than haemoglobin in a variety of planorbid species were of irregular occurrence. Targett also found that the electrophoretic mobility of the haemoglobin fractions was similar in ten planorbid species (from three separate subfamilies) thus confirming his earlier observations on the similarity of the absorption spectra of various planorbid haemoglobins (Targett, 1962c). More recently Richards (1970) has shown that haemoglobin synthesis in

B. glabrata is subject to genetic variation. In the course of his breeding experiments clones were established in which both juvenile and adult snails were deficient in haemoglobin, others in which the juveniles were deficient but the adults were normal and others in which haemoglobin levels were normal at all ages. Davis and Lindsay (1964, 1967) used polyacrilamide gel disc electrophoresis and found that the 12 fractions which they were able to separate in the blood of *Helix aspersa* decreased quantitatively with increasing size of the snails while there were no qualitative or quantitative changes associated with size in the 20 fractions present in foot muscle extracts of the same species. Furthermore they found that the foot-muscle proteins were capable of indicating differences at the population level in the freshwater prosobranch genera *Oncomelania* and *Pomatiopsis*. In contrast to these observations some workers have reported much less variation in molluscan blood proteins. Michelson (1966b) used disc electrophoresis to study the blood of *B. glabrata* and of the 9–12 fractions which he found he mentioned that there was some variation in only a few of the minor components and that these variations were not correlated with age or sexual maturity. On cellulose acetate at the high pH used by Wright and Ross (1963) and Targett (1963) the haemoglobin fraction is the fastest moving component (about half as fast as baboon serum albumin) but in Michelson's system using a pH of 8·2 the haemoglobin appeared to resolve into as many as four subfractions, all of low mobility with the major one not migrating at all. The fastest moving fraction in Michelson's system stained strongly with bromphenol blue and alcion blue and was also PAS positive suggesting that it may be a protein associated with a muco-polysaccharide. More recently Stadnichenko (1970) has studied blood proteins in *Lymnaea stagnalis*. Refractometer measurements showed an increase in total blood protein associated with increasing size of the snails but electrophoresis in agar gel at pH 8·6 revealed only five fractions, two strongly staining and moving to the anode and three weaker cathodal components. All five of these fractions were present in the three size groups examined (mean shell lengths 17·7 mm, 27·5 mm and 39·2 mm) but some variation in the relative concentration of fractions 2 and 3 (the two on either side of the start line) was noted. Cheng's (1964) claim that the electrophoretic profiles of molluscan blood proteins show specific characteristics was not justified by his results because of the distant relationships between the nine species he examined. Rosenfield and Sindermann (1965) in a preliminary report mentioned that there were differences in the blood proteins of oysters at the generic level but specific distinctions were only apparent in some of the isoenzyme systems. Grossu and Tesio (1971) were able to confirm the distinct status of the morphologically similar slug species *Limax zilchi* and *L. cinereoniger* by finding constant differences in the blood proteins and tissue esterases of individuals of comparable states of maturity.

The suspect nature of blood proteins has led to the adoption of other systems for detailed taxonomic work. A basic dichotomy has arisen in the choice of

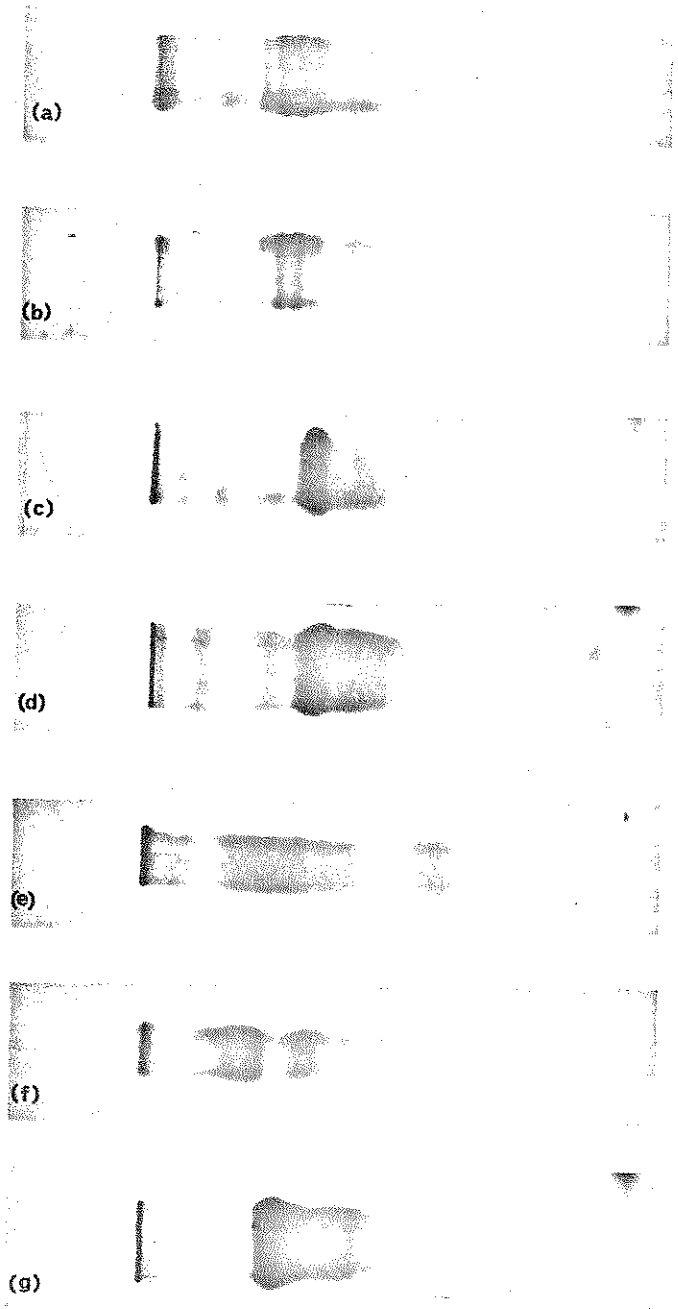
material, dictated largely by the characteristics of the snail groups studied. Wright and Ross (1963) working with planorbid basommatophora turned to egg proteins, while Davis and Lindsay (1964) adopted extracts of foot muscle of freshwater prosobranchs. Both systems have their merits and their disadvantages. To obtain egg proteins in a suitably early state of development necessitates maintenance of living colonies of snails while foot muscles can be collected from wild-caught material without the need for laborious breeding work. Electrophoretic analysis of egg proteins does not damage the individual which produced them and laboratory colonies of known constitution can be established for further work but many freshwater prosobranches are viviparous and in other species eggs are difficult to harvest.

B. EGG PROTEINS

Morril (1964) and Morril *et al.* (1964) made an intensive electrophoretic study of the egg proteins of *Lymnaea palustris*. Using cellulose acetate as the supporting medium they were able to separate nine fractions, four of which were PAS positive and one which proved to be an α-napthyl esterase. In starch gel at the same pH (8·6) 11 protein fractions and two esterases were separated. Extracts of the albumen gland gave protein patterns identical with those of the egg albumen but showed ten esterase fractions two of which were identical with those in the egg contents. The greater resolving power of the starch gel showed slight differences in the patterns between different egg masses from the same individual snail but these were presumed to be due to different total protein concentrations with loss of some of the weaker fractions. There was apparently no selective depletion of particular protein fractions by the developing embryos, a fact also noted by Wright and Ross (1963) for *Biomphalaria glabrata*. Mean values for the total protein content of individual *L. palustris* eggs given by Morril (1964) are 16·6 μg when first laid and 2·6 μg at 7 days.

FIG. 2. Egg proteins of *Bulinus* species separated by electrophoresis on cellulose acetate. (a) *B. truncatus*, Sebha Oasis, Libya. (b) *B. truncatus*, Hedera, Israel. (c) *B. tropicus*, Potchefstroom, South Africa. d *B. liratus*, Basybasy, Madagascar. (e) *B. reticulatus*, Kisumu, Kenya. (f) *B. reticulatus*, Masingire, Mozambique. (g) *B. wrighti*, Marbum, South Arabia.

d and b show the typical sub-divided main fraction characteristic for members of the *B. truncatus* tetraploid group. Despite the geographical distance between the origins of these two samples the egg protein patterns are virtually identical. (c) and (d) show the clearly defined major fraction of the *B. tropicus* (diploid) group which contrasts with that of *B. truncatus*. These two populations are closely similar in most respects but there are differences in their egg proteins, particularly the slow-running fractions between the origin and the main fractions. e shows the egg protein pattern for topotype *B. reticulatus* and a similar pattern has been obtained from specimens of Tanzanian origin while (f) illustrates the pattern found in material from Mozambique, Rhodesia and South Africa. These results indicate that the populations of *B. reticulatus* from East Africa are distinct from those of southern central Africa. (g) shows the pattern given by *B. wrighti*, a species which was at one time confused with *B. reticulatus* but now shown to be distinct from it.



Wright and Ross (1965) described and figured the egg protein patterns obtained by electrophoresis on cellulose acetate at pH 11·78 of twelve species of the African planorbid genus *Bulinus* and seven species of *Biomphalaria*. Many of the species were represented by several populations and egg protein patterns at the population, species and species-group level were characterized. The most useful contribution in this work was the finding of a characteristic species-group pattern for the *Bulinus truncatus* complex. Members of this group are intermediate hosts for *Schistosoma haematobium* in northern and western Africa and the Middle East but morphologically they are not easy to separate from the *B. tropicus* complex which do not act as hosts for *S. haematobium*. The egg protein pattern is one of the most reliable features for distinguishing these groups. Another important aspect of this study was the demonstration of differences in egg proteins between populations of a single species. This reflection of physiological differences at the intermediate host population level provided further support for hypotheses on the evolution of trematode strains in parallel with their molluscan hosts (Wright, 1960; 1966a). Wright and Ross (1966) continued the survey of bulinid egg proteins with a detailed study of all of the members of the *B. africanus* species group and most of the *B. forskali* complex. These results reinforced the previous work and, in addition, revealed the close relationship between *B. nasutus* and *B. abyssinicus* and their position slightly apart from other members of the *africanus* group as well as demonstrating a distinction between topotype *B. reticulatus* from Kenya and the form from southern Central Africa. Egg protein patterns have also been used to confirm the unique nature of *B. nyassanus* and the relationship of *B. succinoides* (both of these species are endemic to Lake Malawi) to the *B. tropicus* group rather than the *B. truncatus* complex to which the morphological evidence suggested an affinity (Wright *et al.*, 1967). More recently (Wright, 1971) egg proteins have provided evidence of the diversity of populations of *B. cernicus* on Mauritius, a fact confirmed by divergent characters of the radular teeth, also they have shown the close similarity between populations of *B. bavayi* on Aldabra and Madagascar. Brown and Wright (1972) in a study of the polyploid complex of bulinids in the Ethiopian highlands have shown the existence of two distinct egg protein patterns in octoploid populations as well as the typical *B. truncatus* pattern among tetraploids and two further patterns among diploid populations. Possible intermediates between the two diploid pattern types were found in some mixed populations but no intermediates between the two octoploid patterns have been seen.

C. MUSCLE PROTEINS

It has already been mentioned that the majority of electrophoretic studies of foot muscle proteins have concentrated on the freshwater prosobranchs but two brief notes relating the application of the technique to basommatophoran pulmonates are interesting. Pace and Lindsay (1965) examined thirteen

populations of *Bulinus* belonging to three species complexes as well as three other species in different planorbid genera. They found strikingly little overall variation but the level of variation between two populations of a single species of *Bulinus* was as great as that between the three planorbid subfamilies represented in their material. They concluded that there appeared to be little prospect of the technique being of any value in the Planorbidae. Burch and Lindsay (1969) in examining the relationships of two Indian species of *Lymnaea*, *L. luteola* and *L. acuminata* compared the foot muscle proteins of both species with those of European *L. auricularia* and the holarctic *L. stagnalis*. Existing systems of classification had associated *L. luteola* with *L. stagnalis* on the basis of morphological similarities in the structure of the prostate gland and *L. acuminata* had been included in the synonomy of *L. auricularia rufescens*. The electrophoretic results showed that *L. luteola* and *L. auricularia* were so similar that they could not be distinguished while *L. acuminata* was distinct from them and all three of these species were clearly different from *L. stagnalis*. The distinction between *L. stagnalis* and the other three species was upheld by chromosome studies.

In a major morphological and biological review of the relationships between the hydrobiid genera *Oncomelania* and *Pomatiopsis* Davis (1967) included characteristics of the foot muscle proteins. All *Oncomelania hupensis* subspecies had one or two dense, fast-moving fractions beyond Rf 0·75 and these were lacking in *Pomatiopsis* species. The problem of the relationships of the snail from Taiwan originally described as *Tricula chiui* was partly resolved by study of the foot muscle proteins. The species had been transferred from *Tricula* to *Oncomelania* and Davis (1968a) subsequently showed that the electrophoretic pattern of its foot muscle proteins was identical with that of some populations of *O. hupensis formosana*. Immunological studies supported the conclusion that *O. chiui* is no more than a local race of *O. h. formosana* (see p. 375). Comparison by disc electrophoresis of foot muscle proteins from field-collected and laboratory-bred populations of the Japanese *Oncomelania hupensis nosophora* from the same locality showed some variation in two of 24 major fractions. Of these two fractions one was never found in laboratory-bred snails and it was suggested that this might represent a genetic difference (Davis and Takada, 1969).

Some snails of the pleurocerid genus *Semisulcospira* in the Far East act as hosts for the lung fluke *Paragonimus*. In Japan various species groups of *Semisulcospira* have been established on the basis of chromosome numbers. Two species belonging to the same cytological group (n = 18), *S. libertina* and *S. trachea* are distinguished by shell characters, the first being smooth and living in rivers while the other is lacustrine and has prominent ribs. No significant biological characters could be found to distinguish between the two, and the foot muscle proteins differed only in that eight out of the 24 fractions resolved had slightly higher Rf values in *S. trachea* than their counterparts in *S. libertina*. Differences of this order are only considered significant at the population level

and immunological work (see p. 376) supported the treatment of *S. trachea* as a synonym of *S. libertina*, the few differences found not justifying subspecific status (Davis 1968b). Davis (1969) also used foot muscle proteins to help in establishing the identity of a Japanese population of *Semisulcospira* found to be naturally transmitting *Paragonimus westermani*. The lack of significant differences between this population and topotype *S. libertina* led to the inclusion of the host population in that species and the prediction that the topotype population would probably also be capable of transmitting the parasite. Davis' most recent contribution (1971) in this field concerns another host for *Paragonimus*, the thiarid *Brotia costula episcopalis* from Malaysia. Two populations from separate drainage systems were compared and found to agree with respect to 25 reproducible foot muscle protein fractions but three bands of low reproducibility were found in only one of the populations. When 12 key fractions were compared with the pattern given by *Semisulcospira libertina* it was found that only three peaks were common to both patterns. The most fruitful part of this study is the discussion of relationships between the families to which these two species belong. However, that discussion depends in a large part on the immunological results and will be dealt with later in this review (see p. 376).

To conclude this section on electrophoresis of largely unspecified proteins extracted from molluscan tissues an interesting study on species of the intertidal marine prosobranch genus *Littorina* provides a change from the rather intense air of medical malacology. Wium-Andersen (1970) has used polyacrylamide gel electrophoresis to compare the proteins extracted from the buccal mass of three species, *L. littorea* from Denmark, *L. striata* from Tenerife and *L. rudis* from Denmark and Greenland. The patterns for *L. striata* and *L. littorea* were constant, three bands in *L. striata* of which the two outer are haemoglobin fractions and five bands in *L. littorea*, the two outer fractions again being haemoglobin but considerably faster moving than those of *L. striata*. *L. rudis* by contrast showed great variability both between individuals and populations. A total of 14 bands were found in this species of which eight were common and six rare. Of the eight common bands two are haemoglobin with similar mobility to the two fractions in *L. littorea* but in *L. rudis* they may occur together or separately. Wium-Andersen suggests that these two haemoglobin fractions are determined by two allelic genes and that the individuals in which both fractions occur are therefore heterozygotes. The actual occurrence of the various phenotypes agrees closely with the predicted frequency on this hypothesis and the occurrence of the fast moving HbI on the west coast of Jutland suggests the existence of a gene frequency cline. The rest of the protein bands in *L. rudis* present a confusing picture with high levels of individual variation within populations with the exception of *L. r. groenlandica* from Heligoland and Greenland. From both of these localities the patterns are constant with four bands in the Heligoland population and five in that from Greenland. It is suggested that this may justify consideration of *L. r. groenlandica* as a distinct species. The high levels of variation in *L. rudis* are explained by the

fact that it is a brackish-water species and such habitats in Denmark are geologically young so that it may perhaps be a relatively recent colonizer. In contrast to the other two species, both of which have pelagic larvae, *L. rudis* is ovo-viviparous and its populations are likely to be somewhat parochial while in *L. littorea* and *L. striata* there is a much greater chance of gene exchange throughout the species' range.

D. ISOENZYMES

Although a few minor reports of molluscan enzyme systems had appeared before 1964 it is the work of Norris and Morrill (1964) which can be regarded as the basis for subsequent detailed studies. In this work they examined the organ specificity and embryological development of nine hydrolytic enzymes in *Lymnaea palustris*. In all they resolved 42 fractions, 16 aromatic esterases, 6 alkaline phosphatases, 4 acid phosphatases, 4 leucine aminopeptidases, 4 alanine aminopeptidases, 4 β-galactosidases, 2 α-glucosidases and 1 each of β-glucosidase and β-glucuronidase. The maximum number of fractions found in one organ was 32 in the digestive gland and the minimum was 10 in the muciparous and oothecal glands. In the first two days of development five bands are present in extracts of the embryo, 2 acid phosphatases and 1 each of leucine and alanine aminopeptidase and galactosidase. Esterases do not appear in the embryo until the third day and at the time of hatching 29 of the total of 42 adult fractions can be resolved. In a comparable study on hydrolytic enzymes of developing embryos of the marine prosobranch *Ilyanassa obsoleta* the same authors (Morrill and Norris, 1965) found that some of the bands present in the earliest stages disappear between the second and fourth days of embryonic development while others persist throughout and still others do not appear until the third to the seventh days. Similar observations on the sequential development of lactic dehydrogenase enzymes in the marine prosobranch *Argobuccinum oregonense* were reported by Goldberg and Cather (1963).

As a prelude to taxonomic application of isoenzyme studies Wright *et al.* (1966) carried out a comparative survey of esterases and acid and alkaline phosphatases in the organs of one species each in the planorbid genera *Bulinus* and *Biomphalaria*. Alkaline phosphatases proved to be weak and difficult to resolve, the acid phosphatases showed relatively little variation either between the species or in different organs with the exception of the digestive gland but the esterases were more abundant and gave more fractions, especially in the kidney and digestive gland. In the light of these results it was decided to concentrate for taxonomic purposes on the α-napthyl esterases of digestive gland extracts. Burch and Lindsay (1967) used disc electrophoresis to compare the esterases of foot muscle extracts in 17 populations of *Bulinus* belonging to the *B. tropicus* and *B. truncatus* groups. They found 4–10 fractions and although there was some variation in the slow-moving bands there were always two heavily-staining fast bands in all diploid samples but only one in polyploids.

FIG. 3. Digestive gland esterase isoenzymes of a random sample of ten adult *B. tophicus* from a laboratory-bred population derived from Lake Ibbini near Arusha, Tanzania. Most populations of *Bulinus* show fairly uniform patterns of esterase isoenzymes with seldom more than two bands varying within a population. The colony from which this sample was taken has been bred in the laboratory for over nine years and with the exception of the fourth and fifth specimens in this series no two out of the ten give the same pattern.

FIG. 4. Egg proteins and digestive gland esterase isoenzymes of octoploid *Bulinus* from Ethiopia. a: 'b' type egg protein. b: 'd' type egg protein. c and d: two of the three esterase isoenzyme patterns found in octoploid populations. 'b' type egg proteins were found in 17 octoploid populations and 'd' type in three. Both types were found together in a single sample but no intermediate patterns were seen and isolated individuals of each type gave rise only to progeny with the same type. The esterase pattern shown in c was found in seven populations and that in d, in four, in neither case did the esterase pattern appear to segregate with the egg protein pattern.

Wright and File (1968) pursuing their earlier work described the digestive gland esterases from about 50 populations of 17 nominal species of *Bulinus* distributed throughout the four species groups into which the genus was at that time divided. The range of variation encountered between populations was so great as to make the characterization of species impossible and even the definition of species groups was difficult. A certain amount of individual heterogeneity within populations was encountered, particularly in two populations of the *B. tropicus* complex. These belong to the assemblage of diploid populations investigated by Burch and Lindsay (1967) and, although the two fast running bands mentioned by them were not found in all of our diploid samples, in those in which they did occur individuals which lacked the faster moving fraction of the pair were present. Wright and File (1968) concluded that for most taxonomic purposes digestive gland esterases are only suitable for comparisons at the population level. Wright (1971) showed that an Aldabran and a Madagascan population of *B. bavayi* differed by only a single slow-moving esterase fraction but the marked differences in both esterases and acid phosphatases served to distinguish *B. obtusispira* clearly from *B. liratus*, two Madagascan species which had in the past been confused. Brown and Wright (1972) found three esterase pattern types among twelve octoploid populations of Ethiopian *Bulinus*. One of the patterns had affinities with a group of tetraploid populations and the other two with two groups of diploids. These differences in esterase patterns were not correlated with the differences in egg proteins already discussed (see p. 364).

Coles in a series of papers (1969a, b; 1970) has studied a wide range of enzyme systems in the digestive glands of a few species of *Bulinus* and *Biomphalaria*, *Lymnaea natalensis* and the African freshwater prosobranch *Pila ovata*. The enzymes studied were acid and alkaline phosphatases, acetyl and butyl esterases, leucine aminopeptidase, β-glucosidase, β-glucuronidase, peroxidase and the following dehydrogenases: lactate, malate, β-hydroxybutyrate, α-glycerophosphate, α-alanine, glutamate, isocitrate, glucose-6-phosphate and 6-phosphogluconate. Unfortunately the range of material covered in this survey is inadequate to assess the taxonomic value of most of the systems. Coles suggests that the leucine aminopeptidases have the greatest potential use. However, although Coles separated four fractions of this enzyme in *Bulinus nasutus* and three in *B. africanus*, Wright and Moule (unpublished) were unable to resolve more than one in several species of *Bulinus*. On the other hand Coles obtained only three acid phosphatase fractions and one β-glucuronidase in these two bulinid species, while Wright and Moule resolved seven acid phosphatases and three β-glucuronidases.

The work of Manwell and Baker (1968) on two populations of the helicid land snail genus *Cepaea* has provided some interesting background ideas for molluscan isoenzyme studies. Working with two population samples from localities in the south-west of England they compared *iso*citrate, malate and 6-phosphogluconate dehydrogenases. 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*. All three of these enzyme systems (and others mentioned in less detail) showed considerable polymorphism. The NADP-dependent *iso*citrate dehydrogenases form two distinct groups of fractions, one slow-moving and the other fast. The fast-moving fractions in *C. hortensis* show a triallelic variation and the six possible phenotypes, three homozygous and three heterozygous were found, the heterozygotes showing both parental fractions together with a usually more intense 'hybrid' zone of intermediate mobility. Polymorphism of the slow NADP-dependent *iso*citrate dehydrogenases was also found but the variation was less easily analysed and did not coincide with that of the fast-moving enzyme. In *C. nemoralis* only a single fast NADP-*iso*citrate dehydrogenase was found in the pure population and thus corresponded to one of the *C. hortensis* forms 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 *nemoralis* sample. The most striking example of this was shown by the glucose-6-phosphate dehydrogenase which occurred as a single fraction in both species. However, in the pure population of *C. nemoralis* from Tintagel this fraction has a mobility 10–12% faster than that found in the mixed population from Erme valley, where the enzyme appears to be identical in both *C. nemoralis* and *C. hortensis*. The authors mention that other samples of *C. nemoralis* from Devon also have the slower moving fraction but they do not make it clear whether these specimens were from pure or mixed populations. In discussing their results Manwell and Baker offer four explanations.

1. The presence of variants in common between closely related species may be the result of retention of variation present in the ancestral form.
2. The common variants may actually represent different mutations.
3. Similar selection pressures on sympatric individuals of the two species may preserve variants which are lost in populations subject to different ecological conditions.
4. Introgressive hybridization which may allow the occasional transfer of some genes from one species to the other.

On the whole the authors appear to favour the theory of introgressive hybridization but detailed discussion on the basis of results from only two populations must remain highly speculative.

In the marine field taxonomic enzyme studies are so far limited. Reid (1968) compared the distribution of esterases and endopeptidases in the bivalve genera *Lima*, *Mya*, *Chlamys*, *Pecten*, *Glycymeris*, *Modiolus*, *Crassostrea*, *Arcopagia*, *Tresus*, *Arctica* and *Cardium* and showed differences between related genera and between two species of *Mya*, both in extracts of the digestive diverticula

and in the stomach fluid. The patterns for three groups of these lamellibranchs showed characteristic features which could be related to gut morphology and methods of digestion. Changes due to starvation were observed in some species and these included an increase in several of the esterase fractions. In a subsequent study of the esterases of eight species of the bivalve genus *Macoma* Reid and Dunnill (1969) examined 40 specimens of each of two species and 10 each of the other six. In some species they found a high level of individual variation but this was less marked in other species. In *M. secta* the gastric contents and diverticular extracts gave identical patterns but in the other species there were differences in the esterases from these two sources. Multiple arylesterases were present, there was a single aliesterase common to all of the species but no cholinesterases were found. Differences in diet surprisingly did not appear to affect the distribution of these digestive system esterases. The authors were cautious in the taxonomic conclusions which they drew in view of the relatively limited amount of material examined.

IV. Immunological Methods

The application of immunological techniques to problems of molluscan systematics antedates the use of all other experimental methods. A preliminary contribution was made by Makino (1934) and ten years later Wilhelmi (1944) published a study on the relationships of the Mollusca to other phyla. Wilhelmi prepared antisera to tissue extracts of an annelid (*Nereis virens*), an arthropod (*Limulus polyphemus*), an echinoderm (*Asterias forbesi*), two gastropod molluscs (*Busycon carica* and *B. canaliculatum*) and a bivalve (*Pecten irradians*), using both 'whole' and lipid-free antigens. Comparisons were made by the interfacial ring-test method and the conclusion was reached that the molluscs had some ancestral affinity with the annelids since this was the only reciprocal interphylar reaction which was obtained. Cross reactions between the molluscs used were rather weak, the heterologous titres between the two species of *Busycon* being only 36 and 27% of their respective homologous titres and between the gastropods and the bivalve the heterologous reactions were at only about 2% of the homologous titres. Wilhelmi found that the reactions obtained with antisera made from 'whole' extracts gave unreliable results and advocated the use of lipid-free antigens for future work. Hiscock (1949) tried to overcome this problem by using the mucins extracted from chopped foot tissue of a number of Australian marine prosobranchs. It seems likely that these extracts contained more than just mucins and in the only preliminary results which were published the homologous titre for an antiserum to *Austrocochlea torri* was 1/1,024 and the heterologous titres with *Bembicium melanostoma* and *Cellana tramoserica* were respectively 1/8 and 1/2 using a ring-test procedure. From this time onwards the progress of immunological taxonomy of molluscs follows the familiar pattern of domination by studies on the freshwater snails of medical importance.

In a preliminary note Wright (1959b) mentioned attempts to immunize rabbits with foot muscle proteins of *Bulinus*. This project was unsuccessful, in part because shortage of material led to inadequate immunization and very weak antisera, also because the test method employed was densitometric, using the photron-reflectometer, and it proved to be impossible to obtain sufficiently clear test antigen solutions from the tissue extracts. Tran Van Ky *et al.* (1962) made antisera to *Biomphalaria glabrata*, *Planorbarius corneus*,

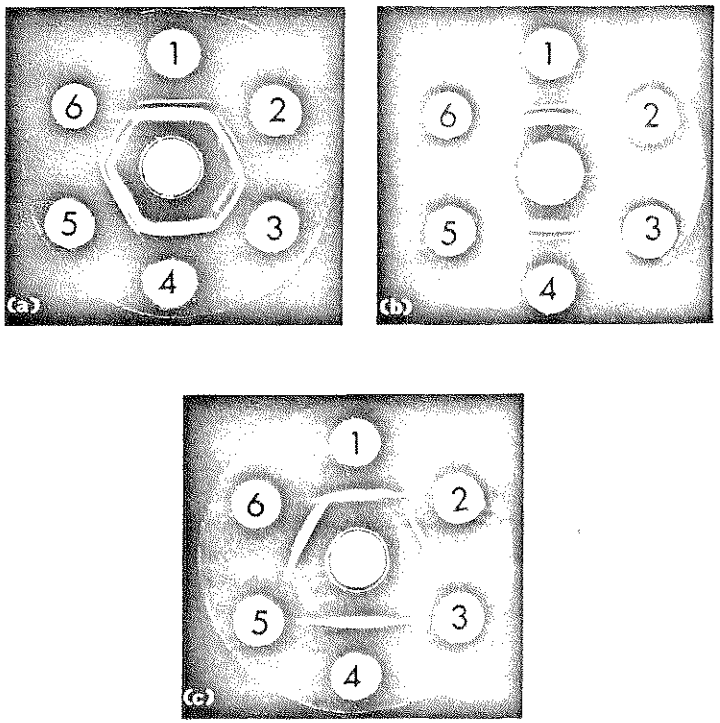


FIG. 5. Ouchterlony gel-diffusion plates showing the immunological relationships of the egg proteins of certain *Bulinus* species. (a) Antiserum to egg proteins of *B. wrighti* in centre well. Wells 1 and 4, egg proteins of *B. wrighti*; Well 2, egg proteins of *B. forskali*; Well 3, egg proteins of *B. natalensis* (*B. tropicus* group); Well 5, egg proteins of *B. truncatus*; Well 6, egg proteins of *B. globosus* (*B. africanus* group). (b) Arrangement as in (a) but antiserum in centre well absorbed by egg proteins of *B. forskali*. (c) Antiserum to egg proteins of *B. obtusispira* in centre well. (Wells 1 and 4 egg proteins of *B. obtusispira*; Wells 2, 3, 5 and 6 as in a).

In (a) the representatives of the four species groups of *Bulinus* are shown reacting with antiserum to *B. wrighti* and *B. truncatus* appears to give the strongest cross-reaction. (b) shows that after the antiserum is absorbed by *B. forskali* antigen the homologous reaction is still strong, the cross-reactions with the *africanus* and *forskali* groups are eliminated and the residual cross-reactions of the *tropicus* and *truncatus* groups are about equal. This result suggests that the affinities of *B. wrighti* are a little closer to the *tropicus truncatus* complex than to the *forskali* group in which it was originally included. (c) shows unequivocaly the close relationship of *B. obtusispira* to the *africanus* group.

Lymnaea palustris and *L. stagnalis* using saline extracts of whole freeze-dried snails as antigens and giving subcutaneous injections with Freund's adjuvant to rabbits over a long period of time (fortnightly injections for 16 weeks). Using immunoelectrophoresis they reported about 15 precipitin lines in each homologous reaction and the heterologous cross reactions were said to accord with the existing systematic position of the species concerned.

Credit for the first production of a reasonably selective antiserum for taxonomic purposes undoubtedly goes to Morrill *et al.* (1964) who employed the egg albumen of *L. palustris* as their basic antigen. The purpose of their study was to compare the antigenic composition of various organs within the homologous species and their taxonomic interests were secondary. Using immunoelectrophoresis they were able to demonstrate the presence of 19 antigens in the egg albumen and all of these were also found in extracts of the albumen gland. Thirteen of these antigens were unique to the albumen gland and egg contents, three were found in all the organs tested and of the other three, one was present in both the prostate and seminal vesicle, one was found only in the prostate and the other only in the seminal vesicle. In heterologous tests against the egg proteins of *L. columella* and *L. stagnalis* Morrill *et al.* found nine antigens in common between *L. palustris* and *L. columella* and only six between *L. palustris* and *L. stagnalis*. Egg proteins were also adopted as the most useful antigens for work on planorbid snails by Wright and Klein (1967) who used Ouchterlony plate double diffusion methods as the test procedure. Comparisons between antisera to 'whole snail' (all organs except digestive gland and gonad), foot muscle, albumen gland and egg proteins of various species of *Biomphalaria* and *Bulinus* showed that the last two antigens produced much more selective antibodies capable of discrimination at species-group and species level. Egg proteins again have the obvious advantage that they may be obtained without damage to the snails producing them (see p. 362). The antigens in egg proteins were found to be shared fully by the albumen gland but scarcely any cross reaction was obtained with other adult organs and although newly hatched embryos also gave a strong reaction with antisera to egg proteins this was lost two days after emergence from the eggs. The Ouchterlony plate technique was used in preference to immunoelectrophoresis because the pH needed to obtain satisfactory separations of planorbid egg proteins (see p. 364) is too high for the formation of precipitin arcs. Laborious transfer procedures involving cutting the cellulose acetate strips on which egg proteins have been separated and placing these on agar diffusion slides have been tried (Wright *et al.*, 1967) but the results are somewhat unsatisfactory.

A. BLOOD PROTEINS

Michelson (1966a, b) used blood proteins of planorbids (*Biomphalaria glabrata*, *Helisoma anceps* and *Bulinus globosus*) as antigens and found that using interfacial ring-tests and a micro-ouchterlony diffusion methods his antisera were only able to discriminate at the generic level. Immunoelectrophoresis gave only five arcs in the homologous reaction of *Biomphalaria glabrata*, all of these corresponding to the slow moving benzidine-positive fractions identified as haemoglobins by disc electrophoresis (see p. 361). Absorption of the *B. glabrata* antiserum by blood of *Helisoma anceps* removed all but one of the precipitin arcs, the one remaining corresponding to the fourth fraction. These results tend to lend some weight to the arguments against the use of molluscan blood in taxonomic studies (see p. 361). Burch (1967, 1968) described a micro-ouchterlony absorption method using foot muscle tissue antigens. In a preliminary study on the polyploid complex of *Bulinus* he reported identity reactions between populations having the same chromosome number and non-identity between snails of different chromosome numbers. These results were confirmed in greater detail by Burch and Lindsay (1970), but only three antisera were used, one to a population of diploids, one to a tetraploid sample and the third to an octoploid population.

B. FOOT MUSCLE PROTEINS

The widest use of micro-ouchterlony absorption methods and immunoelectrophoresis has been made by Davis in his series of works on freshwater prosobranchs in the Far East. As in his electrophoretic studies the source of the protein used as antigen has always been foot muscle. In the first of these contributions Davis (1968a) drew attention to one of the most important technical points in the use of immunological methods, the variability in quality of antisera. His objective was to determine the precise relationships of *Oncomelania hupensis chiui*, a snail found in an isolated region of the northern part of Taiwan and originally described as *Tricula chiui*. Five antisera were prepared to *O. h. formosana*, the common form of *Oncomelania* on Taiwan and comparisons were made with other subspecies of *O. hupensis* from Japan and the Phillipines. One of the five antisera was capable of discriminating between these geographically isolated subspecies but despite absorption by heterologous antigens the other four were not able to do this. Even with the highly specific antiserum *O. h. chiui* proved to be indistinguishable from *O. h. formosana* thus providing support for the morphological and electrophoretic evidence (see p. 365) which suggested that it is no more than an isolated race of the more widely distributed species. This variation in quality of antisera is a common problem and one which leads many people to have doubts about the practicability of immuno-taxonomic work. The ratio of one good discriminating antiserum to four of lower specificity is by no means unusual and in cases where supplies of antigen are limited this can represent an enormous wastage, not only of material but also time. Davis and Suzuki (1971) point out that it may require several thousand individuals of one of the small *Oncomelania* species to yield enough foot muscle tissue to immunize a single rabbit. Simi-

larly, in this laboratory, we have found that we need a packed volume of at least 5 ml of bulinid egg masses to prepare antigen for the injection of one rabbit. In some of the smaller species which do not breed easily this may represent at least six months collection from a laboratory colony. With this problem in mind Davis and Suzuki (1971) have described a method requiring small quantities of antigen innoculated intraperitoneally into mice together with Sarcoma 180 cells and Freund's adjuvant. Ascitic fluid with high antibody content, often comparable to the most discriminating rabbit antisera, can thus be produced in relatively short periods of time with small amounts of antigen. However, this technique has not been widely used and most of the work so far reported has been done with traditional rabbit antisera.

In both of Davis' studies on *Semisulcospira* (1968b, 1969) he was concerned with relationships at the specific level, in the first instance with the precise identity of *S. trachea* and in the second with the affinities of a population known to act as host to the lung-fluke *Paragonimus westermani*. In both cases the material under investigation proved to be no more than a local race of *S. libertina*, a species to which several antisera were available. On micro-ouchterlony tests reactions of identity were obtained between *S. libertina* and the other forms but with immunoelectrophoresis some unabsorbed antisera appeared to show minor differences between the homologous and heterologous antigens. However, absorption of the antisera failed to confirm these fine distinctions and the immunological results thus supported the findings based upon morphology and straight protein electrophoresis.

The electrophoretic data from Davis' (1971) study on *Brotia costula* established that two populations of this species from separate river drainage systems were virtually identical. Also, comparison of 12 key fractions in the foot muscle protein patterns of this species and *Semisulcospira libertina* showed that only three of the major peaks appeared to be common to both species (see p. 366). Micro-ouchterlony gel diffusion tests using both rabbit antisera and ascitic fluid from mice immunized with antigens to *S. libertina* showed from 4–7 precipitin lines in the homologous system. Absorption of the immune sera and ascitic fluids with antigens to *Brotia costula* still left 2–4 precipitin lines in the homologous reactions. Taking these lines as representing the part of the total homologous reaction which is unique to *S. libertina* and expressing their number as a percentage of the total for each of the eight immune fluids tested gives an average figure of 48%. The percentage of precipitin arcs peculiar to *S. libertina* on immunoelectrophoresis with unabsorbed antisera was 50–55%, a reasonably close figure to that established by gel diffusion absorption methods (Davis and Suzuki, 1971). The total of major electrophoretic peaks and Ouchterlony precipitin lines by which *B. costula differs* from *S. libertina* is 12 out of 18, and Davis suggests that, using these data, the degree of *relationship* between the two species is 34%. This level of similarity, Davis argues, indicates a closer affinity between the two families (Thiaridae and Pleuroceridae) to which these species belong than that which had previously been suggested on

the basis of morphological data. One must have some misgivings about the validity of this index of relationship. In the first place only 12 out of a total of about 24 protein fractions were selected for comparison in the electrophoretic separations and their identity or non-identity with those of the other species was determined solely by a somewhat arbitrary standard of their relative mobility. Thus the three out of 12 'key' fractions common to the two species represent only 25% of the fractions selected for comparison and yet the immunoelectrophoretic results suggest that approximately 50% of the *total* foot muscle proteins are common to both. Further the same complex of unidentified proteins was used for both the electrophoretic and immuno-diffusion techniques and it is questionable as to whether the summation of these two sets of data is justified since they are merely different methods of comparing the same material. Davis himself, in discussing these results, draws attention to a further need for caution based upon experience with two species of *Semisulcospira*, *S. libertina* and *S. niponica*. Despite the fact that these are distinct species with haploid chromosome numbers of 18 and 12 respectively no differences could be found between them, either in the electrophoretic separations of their foot muscle proteins or in immunological tests using the same antigens. With these results, therefore, the index of relatedness between the two species (as applied to the *B. costula*–*S. libertina* system) would be 100%! The whole question of the quantification of degrees of affinity based upon the physical attributes of unidentified proteins is very much open to debate. Even in the field of isoenzyme studies, where it is possible to characterize more precisely the fractions separated, anomalous results are frequently encountered.

C. EGG PROTEINS

Recent applications of immunological techniques in the taxonomy of bulinid snails have been described by Wright (1971) and Brown and Wright (1972). In both of these contributions egg proteins were used as antigens in ouchterlony gel-diffusion plates, in the first instance to determine the species-group relationships of two species of uncertain affinities and in the second to attempt to differentiate between members of the polyploid series of *Bulinus* in Ethiopia. *B. obtusispira* was a little-known species from Madagascar usually considered to be no more than a form of the somewhat polymorphic *B. liratus*, which in turn is regarded simply as the Madagascan representative of the *B. tropicus* complex. However, discovery of snails identified as *B. obtusispira* carrying natural infections of *Schistosoma haematobium* called for a re-assessment of the situation because no member of the *B. tropicus* group is known to act as a host for this parasite. Morphological and cytological studies provided few distinctive characters, nor did electrophoresis of egg proteins. Chromatography of body surface mucus and isoenzyme electrophoresis (see p. 370) showed that *B. obtusispira* is distinct from *B. liratus* but neither technique gave

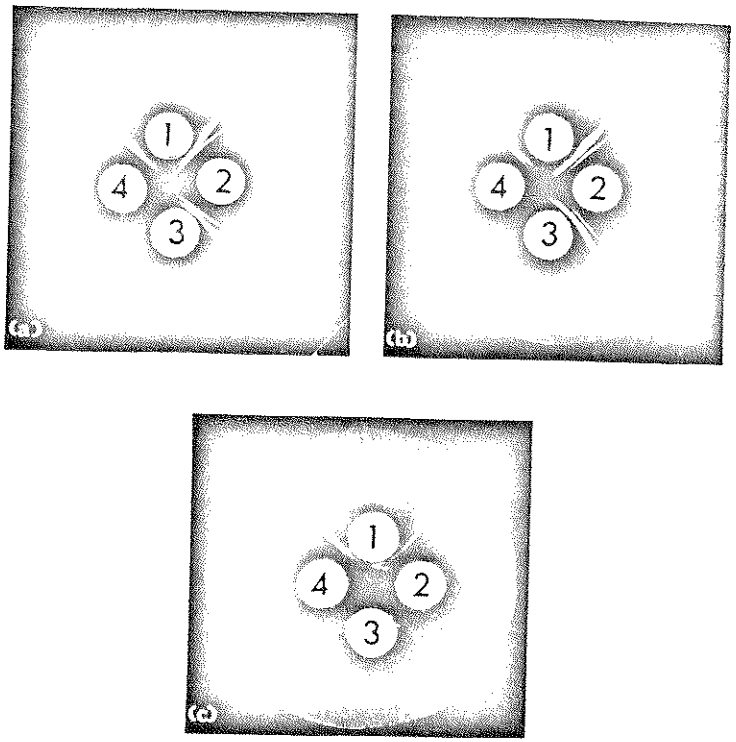


FIG. 6. Ouchterlony gel-diffusion plates using an absorption technique to show the relationships between pairs of species (based on the technique used by Davis 1968b, 1969 and 1971). a, Well 1, antiserum to egg proteins of *B. wrighti*; Well 2, egg proteins of *B. wrighti*; Well 3, antiserum to *B. wrighti* absorbed by egg proteins of *B. scalaris*; Well 4, egg proteins of *B. scalaris*. b, Well 1, antiserum to egg proteins of *B. wrighti*; Well 2, egg proteins of *B. wrighti*; Well 3, antiserum to *B. wrighti* absorbed by egg proteins of *B. forskali*; Well 4, egg proteins of *B. forskali*. c, Well 1, antiserum to egg proteins of *B. scalaris*; Well 2, egg proteins of *B. scalaris*; Well 3, antiserum to *B. scalaris* absorbed by egg proteins of *B. forskali*; Well 4, egg proteins of *B. forskali*.

The precipitin lines between Wells 1 and 2 show the homologous reaction in the system, those between Wells 1 and 4 the heterologous reaction. The absence of precipitin lines between Wells 3 and 4 indicates that absorption of the antiserum in Well 3 by the heterologous antigen is complete and the presence of lines between Wells 2 and 3 gives an indication of the antigens as common to the two species. In (a) and (b), it is apparent that *B. wrighti* has substantial antigens not present in either *B. scalaris* or *B. forskali* and is therefore not closely related to either species. The antiserum to *B. scalaris* used in (c) is considerably weaker than that to *B. wrighti* used in (a) and (b). This is shown by the relatively poor homologous reaction between Wells 1 and 2. A very weak line between Wells 2 and 3 (scarcely visible in the photograph) shows that *B. scalaris* and *B. forskali* can be distinguished immunologically despite their much closer relationship to one another than either has to *B. wrighti*.

definite guidance as to its species-group affinities. Ouchterlony gel-diffusion tests using an antiserum to *B. tropicus* from the high veldt of South Africa emphasized that *B. obtusispira* has little in common with *B. tropicus* while tests with an antiserum to *B. obtusispira* showed clearly that the only strong cross-reactions are obtained with members of the *B. africanus* group. Further immunological tests using antisera to members of the *africanus* complex confirmed that while *B. obtusispira* is definitely correctly placed in this group it is a very distinctive species. The general conclusion is that *B. obtusispira* is something of a 'relic' which has survived in isolation on Madagascar in company with many other elements of the early African fauna. The affinities of *B. wrighti*, a species from South Arabia provided similar problems. Morphology indicated close similarity to *B. reticulatus*, an uncommon species occurring in isolated populations in East and Central Africa. *B. reticulus* was originally assigned to the *B. forskali* complex because of general similarities in the structure of the radular teeth. However, the principal distinction between *B. reticulatus* and *B. wrighti* is the form of the radular teeth which are quite exceptional in the Arabian species. Mucus chromatography showed *B. wrighti* to be lacking in the fluorescent greenish-blue band at high Rf which is characteristic for the *forskali* group (see p. 357). Electrophoresis of egg proteins gave an undistinguished pattern for *B. wrighti* but showed two complex patterns in *B. reticulatus*, samples from East Africa being quite distinct from those of southern Central Africa (see p. 364). Immunological tests using antisera to members of *africanus*, *forskali*, *tropicus* and *truncatus* groups showed that *B. wrighti* was consistently the odd one out in plates which included members of the homologous species complex. Finally, antisera to *B. wrighti* itself indicated a lack of very close affinity with members of any of the other groups, the *tropicus* complex being perhaps the closest. As a result *B. wrighti* and *B. reticulatus* were removed from the *forskali* group and united in a new separate group, the *reticulatus* group (Wright, 1971).

The immunological results of Burch and Lindsay's (1970) study of the polyploid series in Ethiopian *Bulinus* have already been mentioned (see p. 375). They found that members of populations having the same chromosome number gave reactions of identity using foot muscle antigens in a micro-ouchterlony system. Non-identity reactions were obtained when populations of different chromosome number were compared. Brown and Wright (1972) used both unabsorbed and absorbed antisera on normal ouchterlony plates and their findings proved to be more complex than those of Burch and Lindsay (1970). Ethiopian tetraploids proved to be identical with the tetraploid *B. truncatus* from North Africa and the Middle East. Octoploid populations from the Ethiopian highlands gave reactions closely similar to those of tetraploids when tested against unabsorbed tetraploid antisera but absorption of these antisera with some diploid antigens reduced the octoploid reactions much more severely than those of tetraploids. The early reactions of both tetraploids and octoploids to some unabsorbed diploid antisera were very

similar but when these plates were developed for periods in excess of eight days strong spurs were formed by homologous diploid reactions against adjacent tetraploids but the spurs formed against heterologous octoploids were either much smaller or absent. These results suggested that octoploid populations have more antigens in common with some diploids than have tetraploids. Nevertheless, tetraploids tested against octoploid antiserum gave stronger cross reactions than did diploids suggesting that tetraploids have more antigens in common with octoploids than they have with diploids. Thus the octoploids appear to have more common antigens with diploids and tetraploids than either of these groups have with one another. The most interesting point raised by this study, both from the biological and the technical aspects, is the apparent heterogeneity of the diploids. So far no diploid antisera have been produced which are capable of discriminating between the diploid members of the *B. tropicus* complex but when diploid antigens are tested against tetraploid and octoploid antisera their reactions often differ. Using unabsorbed anti-octoploid serum spurs were formed both by the homologous octoploid and a tetraploid against one diploid population (from a stream near Haik village, Ethiopia) but not against the diploid from Lake Awasa, Ethiopia. Absorption of the same octoploid antiserum by the Haik diploid antigen eliminated cross reactions with several diploid populations but still resulted in a trace precipitin line from the Lake Awasa diploid. An unabsorbed antiserum to an Ethiopian population of *B. truncatus* formed spurs between the homologous antigen and those of a Haik diploid as well as *B. tropicus* from South Africa and the diploid *B. natalensis* from Mozambique. However, the spurs against *B. tropicus* were much more strongly marked than those against the Haik diploid and *B. natalensis* in turn formed spurs against *B. tropicus*. Absorption of this antiserum with antigens of the Haik diploid left a residual cross reaction with *B. natalensis* and after the third day of incubation a very faint precipitin line formed against the well containing *B. tropicus* antigen. From these results it would appear that these three diploid populations vary in the number of antigens which they have in common with this particular population of *B. truncatus*. Although tests using diploid antisera have so far failed to reveal differences of this kind it may be that wider use of heterologous (but closely related) antisera may help to sort out taxonomic problems in similar poorly differentiated complexes.

D. HETEROAGGLUTINATION

No account of immunological methods in molluscan taxonomy would be complete without mention of the indirect approaches derived from human blood-group studies. Various substances capable of agglutinating the erythrocytes of vertebrates have been reported in extracts of several snails (Boyd *et al.*, 1966) and some of them show considerable specificity for particular human blood-groups (Boyd and Brown, 1963; Prokop *et al.*, 1965). It is not the general distribution of these agglutinnis which is of particular interest here

for their occurrence appears to be rather erratic and not closely associated with taxonomic relationships. What is interesting is their presence or absence in different populations of the same species and the possibility that they may serve as genetic markers. Thus Gilbertson and Etges (1967) found agglutinating activity for the red cells of hamsters, rabbits and man in the blood of *Biomphalaria sudanica* from Mwanza in Tanzania, for rabbit and human cells in two populations of *B. glabrata* from Salvador in Brazil and for man only in *B. glabrata* from Surinam. Three other populations of *B. glabrata* from Puerto Rico, Belo Horizonte in Brazil and Venezuela and samples of *B. straminea* from Brazil and *B. pfeifferi* from Liberia all failed to show agglutinating activity with any of the test cells. Gold *et al.* (1967) obtained agglutination of human red cells of groups A, B and O with extracts of the eggs of *Lymnaea stagnalis* from Bristol in south-west England but extracts of the whole snail failed to react. Lee-Potter (1969) found no agglutinating activity in extracts of *L. stagnalis* eggs from Sussex in south-east England but got a weak positive result with eggs from a Moroccan population. Lee-Potter also investigated the eggs of several other species of *Lymnaea* and *Bulinus* without finding agglutinins but extracts of eggs of a population of *B. globosus* from Zambia proved to be markedly haemolytic while those of a Ghanaian population of the same species showed no activity of any kind. Gold and Thompson (1969a) found that saline extracts of the albumen gland of *Helix aspersa* from the Bristol area were active in reverse passive haemagglutination tests with human A cells, although there was some variation in the activity of extracts from different individual snails. Material from Australian *H. aspersa* proved to be far less active and negative results were given by *H. pomatia*, *Helicella virgata*, *Cepaea nemoralis* and *Otala lactaea*. These results are interesting in that Boyd and Brown (1965) had previously reported strong specific activity in extracts of *O. lactaea* and Prokop *et al.* (1965) found very strong anti-A activity in extracts of the albumen gland of *H. pomatia* and *Cepaea hortensis* from Berlin. Gold and Thompson (1969b) found an extract of the albumen gland of one *H. aspersa* from Bristol that achieved complete haemolysis of human A, B and O cells while another extract from a different individual and diluted egg contents haemolysed only cells of group A. Again extracts from Australian *H. aspersa* were less active and gave only partial haemolysis of A cells.

So far these somewhat isolated reports do little more than draw attention to the existence of possible biochemical markers. In the future they may well serve as a fruitful link between those interested in the population genetics of both man and molluscs.

V. Summary

To summarize what is, in effect, little more than a summary is a difficult and perhaps unnecessary task. However, certain general considerations arising from this review suggest themselves.

The somewhat erratic and, in most cases, rather unsophisticated attempts

which have been made to apply experimental methods to molluscan systematics reflect the present state of taxonomy in the phylum as a whole. This in turn is a reflection of the complex biology of the Mollusca. The reproductive patterns range from those of dioecious forms, some with internal and others with external fertilization, through hermaphrodites with varying potentials for cross- and self-fertilization to parthenogenetic species. Life-spans vary from periods of years to no more than a few months in different groups and the diversity of habitats colonized by molluscs results in an almost limitless variety of population types from marine species with pelagic larvae potentially capable of being carried throughout the geographical range of the species to self-fertilizing hermaphrodites in isolated temporary rainpools. The possible scope of different opportunities for genetic interchange, both between individuals and populations, makes generalizations about patterns of speciation within the Mollusca unrealistic. Add to this a long history of taxonomy based solely on features of the shell, a structure notoriously subject to modification by environmental influences and some of the reasons for the imperfections in molluscan classification become apparent. The demands of medical and veterinary parasitology which have provided such a stimulus to the development of biochemical and immunological methods in the taxonomy of gastropods have in turn contributed an element of confusion to the picture. Concerned less with determining the affinities of the snails than with defining their host-potential for a particular trematode, parasitologists have tended to seek for some precise character which may be linked with susceptibility to infection. No universal, key character determining susceptibility is likely to be discovered because such a concept fails to take proper account of genetic factors within the parasite which determine its infectivity to, and ability to survive in, a given population of snails. Thus there are populations of snails capable of acting as effective hosts to some strains of parasite but not others and there are strains of trematodes which are able to develop in a wide range of snail populations while other strains are more restricted in their host requirements. The only reliable guide to susceptibility on the part of any particular mollusc population is either successful experimental infection or the finding of naturally infected individuals. Determination of the affinities of the snail hosts must still be carried out by normal taxonomic procedures.

Of the range of techniques so far investigated there is no single method which can be designated as the most useful, each has its place depending upon the objective of the enquiry. There is, therefore, an advantage in having available a diversity of methods. Moreover some of the simple techniques can be applied in preliminary investigations before decisions are taken on the adoption of more complicated approaches requiring expensive equipment and specialized knowledge. Some techniques can provide results giving information at different taxonomic levels, for instance chromatography of mucus in *Lymnaea* has provided a method for detecting population differences in *L. peregra*, for differentiating between the closely related *L. peregra* and *L. auricularia* and for showing a group relationship between these species and *L. natalensis*. Similarly cellulose acetate electrophoresis of planorbid egg proteins reveals differences at the population level but also provides an unequivocal character for distinguishing members of the *Bulinus truncatus* species group from the rest of the genus. Broadly speaking the methods of choice at different taxonomic levels are as follows: the individual—isoenzyme electrophoresis; the population—isoenzyme electrophoresis, general protein electrophoresis (foot muscle, egg albumen etc), mucus chromatography; the species—general protein electrophoresis, mucus chromatography, immunoelectrophoresis, ouchterlony gel immunodiffusion with absorbed antisera; the species group—general protein electrophoresis, mucus chromatography, gel immunodiffusion with or without absorption of antisera; above the species group level the only techniques of much value are those of quantitative immunology but, with the exception of Wilhelmi's (1944) study on general relationships of the phylum, these have not been pursued to any appreciable extent in the Mollusca.

In conclusion it cannot be too strongly stressed that the results of any of these experimental procedures are of little value in the absence of appropriate background information on the material studied. Also the material must be adequate both in respect to numbers and condition. Too often the conclusions drawn from elegant biochemical data are marred by the use of insufficient samples and questionable preliminary identification of the material. My earlier injunctions on this theme (Wright, 1959c; 1966b) have been reinforced by Davis and Lindsay (1967) whose concluding remarks bear repetition here 'Within a framework of precise anatomy and cytology, biophysical data have their useful place. Without this framework, however, one is caught in a morass of disjunct molecular populations'.

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