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EXPERIMENTAL TAXONOMY OF *BIOMPHALARIA* (GASTROPODA: PLANORBIDAE)

I. METHODS FOR EXPERIMENTAL TAXONOMIC STUDIES ON *BIOMPHALARIA* CARRIED OUT BY HORIZONTAL STARCH GEL ELECTROPHORESIS AND STAINING OF TWELVE ENZYMES

ULF BLANGSTED HENRIKSEN and JENS ERIK JELNES

Danish Bilharziasis Laboratory, Jaegersborg Alle 1 D, DK-2920 Charlottenlund (Denmark)

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SUMMARY

Methods for obtaining single individual zymograms of the enzymes esterase, glutamate–oxaloacetate transaminase, leucine aminopeptidase, 3-hydroxybutyrate dehydrogenase, α-glycerophosphate dehydrogenase, mannose-6-phosphate isomerase, phosphoglucose isomerase, phosphoglucomutase, isocitrate dehydrogenase, adenylate kinase, 6-phosphogluconate dehydrogenase and nucleoside phosphorylase on species of *Biomphalaria* are described. Previous electrophoretic work on the genus is mentioned, and the relation of the electrophoretic method to *Biomphalaria* taxonomy, phylogeny and identification is discussed.

INTRODUCTION

An easy and reliable method for species identification is important in efforts to control schistosomiasis, which is transferred to man and his livestock through freshwater snails of the genera *Bulinus* and *Biomphalaria*. These snails show great variability in morphology, anatomy and compatibility to schistosomes, even between closely related species.

During the last decade, an interest in cytological and biochemical characters of schistosome intermediate hosts has been shown by a number of workers. This is not only because morphological characters alone have been unsatisfactory in elucidating the taxonomy of the group, but also because differences in susceptibility to infection have been detected at the population level, even when there are no obvious morphological differences. The cytological studies on *Biomphalaria* have not added information to the taxonomy of the genus, as the chromosome number found is identical in all investigated species ($2n = 36$). Further, the small size of the chromosomes makes a karyotype study difficult.

Electrophoresis of proteins and enzymes has been used in many investigations. *Biomphalaria glabrata* from the neotropics is the species that has been studied most

frequently by electrophoretic methods. Wright and Ross[1] have reported on the blood and egg proteins, and Bair and Etges[2] showed differences in esterase frequencies between five strains of this species (both these studies were carried out on laboratory stocks). With use of partly laboratory-bred material and partly wild material, Malek and File[3] have reported on genetic polymorphism in esterases in the neotropical *Biomphalaria* species *glabrata, tenagophila, peregrina. straminea* and *obstructa.* Narang and Narang[4–6] and Monteiro and Narang[7] have described genetic polymorphism in esterases, glucose-6-phosphate dehydrogenase and alcohol dehydrogenase observed in natural populations of *B. glabrata* and *B. tenagophila.* The African species of *Biomphalaria* have also been subject to intensive studies. Wright *et al.*[8] studied organ specificity of esterases, acid and alkaline phosphatases in *B. sudanica.* Coles[9–11] has undertaken a study of 14 different enzymes in African freshwater snails, including the species *B. pfeifferi, B. sudanica* and *B. choanomphala.* Ukoli[12] and Wium-Andersen[13,14], studying esterases of *Biomphalaria* species, reported a remarkably small amount of geographical variation in laboratory stocks of *B. camerunensis* and *B. pfeifferi.* Wium-Andersen[13,14] showed the relationship of *B. alexandrina wansoni* to *B. camerunensis* on the basis of esterase pattern, and indicated the validity of *B. salinarum* as a distinct species[15]. Egg proteins of various species of *Biomphalaria* from South America and Africa have been studied by Wright and Ross[16], and Pflüger[17], using a different technique, reported on the egg proteins in Malagasy populations of *B. pfeifferi.*

Comparison of the different isoenzyme studies carried out on *Biomphalaria* species is difficult, as many techniques, media and enzymes have been used for the studies and, mostly, geographically distant populations are compared. With the aim of simplifying and improving the identification of *Biomphalaria* species we proposed to use electrophoresis as a possible method of characterisation. At the same time, we hoped to define the genetic (allozymic) structure of various populations of *Biomphalaria* species with respect to (1) the amount of variability in various populations, (2) genetic similarities and differences among populations, and (3) a possible correlation between specific genes and the compatibility to infection with *Schistosoma mansoni.*

In electrophoretic studies, the development of standard procedures is necessary in order to obtain comparative data on snails examined at different times. For use in the taxonomy and identification of *Bulinus*, Jelnes[18] has described standard electrophoretic procedures for 10 enzymes. Since usable electrophoretic techniques differ, even when closely related genera are considered, we describe here methods through which any single *Biomphalaria* specimen with a shell diameter of more than 4 mm can be surveyed for 12 enzymes; we also describe our system of internal standards.

MATERIAL AND METHODS

To perform the electrophoretic investigation of all 12 enzymes on one snail, its shell diameter should be at least 4 mm. The snails to be analysed are blotted with filter paper to remove adhering water, then each snail is placed in a separate micro test-tube. With the aid of a rotating glass rod, each snail is homogenized; centrifugation of the homogenate at 18,000 *g* for 2 min gives a clear supernatant liquid, which is ready for application to the gel.

Preparation of the gel

A 400-ml portion of the gel buffer is divided in 2 halves. One half is poured into a round-bottomed flask, where boiling of the gel is to take place, and the other half is poured into a 250-ml beaker containing 40 g of Connaught hydrolysed starch. Both the starch and the gel buffer are thoroughly mixed, then the gel buffer is heated to boiling over a low gas flame. When the gel buffer is boiling, the starch is stirred thoroughly once more and then slowly added to the boiling gel buffer. Heating is continued over the low flame, with vigorous stirring or shaking, until the mixture is boiling again. The gel is then allowed to cool for 5 min at room temperature with the opening of the flask covered; the gel is stirred three times during this cooling period. With a water-suction pump, the gel is now de-gassed, the de-gassing being stopped when large bubbles of air rise in the gel; by this time, all dissolved air has been boiled off. The gel is then poured in a rectangular Perspex frame (0.6 × 15 × 24 cm, inside measurements) placed on a horizontal surface. The amount used produces a small surplus of gel, but the top of the gel should be higher than the height of the frame (making use of the surface tension of the gel). The gel is now left to cool until it is opaque and is stiff; then it is covered by a sheet of plastic and left to cool for 1 h in a refrigerator.

Electrophoresis

From each snail, a sample of the supernatant liquid is sucked up in a 6 × 3 mm piece of Whatman No. 4 chromatography paper, and the papers (one from each snail) are placed in a slit cut 4 cm from the long edge of the cold gel. With a distance of about 3 mm between the samples, each gel can accomodate 36 samples. Three pieces of Whatman No. 1 chromatography paper serve as bridge between the gel and the buffer compartments, each containing 900 ml of bridge buffer. The compositions of the gel and bridge buffers and the electrophoretic conditions used are given in Table I. During electrophoresis, the gel is kept at 2° by circulating water through cooling plates in close contact with the gel. At the end of electrophoresis, the application papers are removed, and the gel is sliced horizontally with a stretched piano wire. After careful separation of the two halves, detection of enzyme activity can be made on the cut surface of each half of the gel.

TABLE I

BUFFERS USED FOR ELECTROPHORESIS OF *BIOMPHALARIA*

Amounts are given in grams per litre.

Buffer system	*Boric acid*	*Tris*	*Titriplex II*	*Trisodium citrate·2H₂O*	*Histidine hydrochloride*	*pH*	*Electrophoresis*
A: Bridge	4.64	12.72	0.06	—	—	8.6	400V/45mA
Gel	0.680	1.817	0.585	—	—	8.1	in 3 h
A: Bridge	—	—	—	29.40	—	7.5	120V/90mA
Gel	—	—	—	—	2.09	Adjust with NaOH to 8.0	3 h

Enzyme staining

The utility of the buffer systems related to the enzymes examined is shown in Table II. All enzyme staining took place at 37° in the dark.

TABLE II

POSSIBLE ENZYME DEVELOPMENTS IN *BIOMPHALARIA*, DEPENDING ON THE BUFFER SYSTEM USED

	Est	*Got*	*Lap*	*Hbdh*	*α-Gpdh*	*Mpi*	*Pgi*	*Pgm*	*Idh*	*Ak*	*6-Pgd*	*Nsp*
Buffer system A	+	+	+	+	+	+	+	+	+			
Buffer system B				+	+	+	+	+	+	+	+	+

Esterases (Est). The stain used contained 100 mg of Fast Blue Salt BB, 2 ml of 1% α-naphthyl acetate solution in acetone and 250 ml of 0.06 *M* Tris–HCl buffer of pH 8.0.

Glutamate–oxaloacetate transaminase (Got). The stain contained 3.8 g of K_2HPO_4, 1 g of polyvinylpyrrolidone, 100 mg of ethylenediaminetetra acetic acid, 74 mg of 2-oxoglutaric acid, 266 mg of L-aspartic acid and 100 ml of distilled water; just before use, 250 mg of Fast Violet B Salt was added.

Leucine aminopeptidase (Lap). This stain was prepared according to Shaw and Prasad[19]; it contained 50 ml of solution A, 10 ml of solution B, 35 mg L-leucyl-β-

TABLE III

STAINING PROCEDURES FOR ENZYMES OF *BIOMPHALARIA* WITH THE AGAROSE-OVERLAY TECHNIQUE

The chemicals are dissolved in 25 ml of 0.06 *M* Tris hydrochloride, pH = 8.0. To each staining solution is added 2.5 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 1.25 mg of phenazine methosulphate and 25 ml of 2% agarose solution in 0.025 *M* $MgCl_2$ at 60° just before use.

Enzyme	*Substrate*	*G-6-pd* 7U*	*Pgi 7U*	*ADP 100 mg*	*Hk* 7U*	*Xod* 14U*	*NAD 2.5 mg*	*NADP 2.5 mg*
Hbdh	3-Hydroxybutyrate, 100 mg						+	
α-Gpdh	α-Glycerophosphate, 100 mg						+	
Mpi	Mannose 6-phosphate, 20 mg	+	+					+
Pgi	Fructose 6-phosphate, 10 mg	+						+
Pgm	Glucose-1-phosphate–1% 1,6-diphosphate, 40 mg	+						+
Idh	DL-Isocitrate, 100 mg							+
Ak	Glucose, 100 mg	+		+	+			+
6-Pgd	6-Phosphogluconate 20 mg							+
Nsp	Inosine, 50 mg					+		

* G-6-pd = Glucose-6-phosphate dehydrogenase; Hk = hexokinase; Xod = xanthine oxidase. U = international enzyme unit.

naphthylamide and 30 mg of Fast Black K Salt. (Solution A: 8 g of NaOH and 19.6 g of maleic anhydride, in 1 l of H_2O. Solution B: 12.0 g of NaOH in 1 l of H_2O).

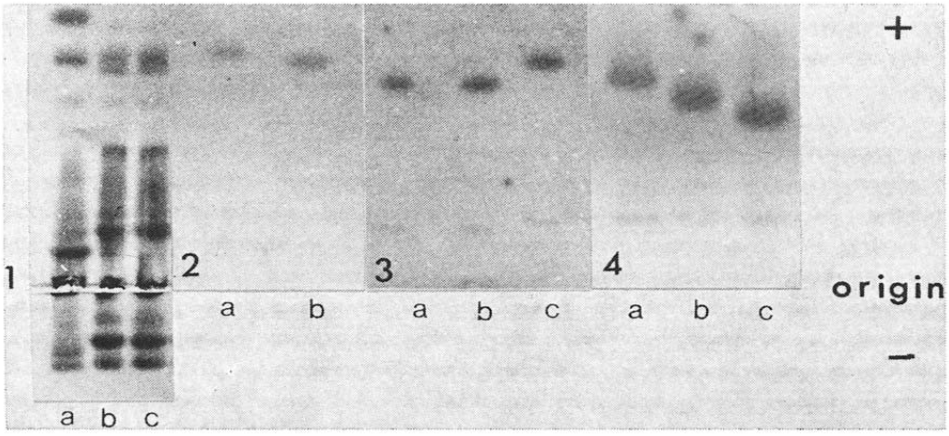
The staining procedures for 3-hydroxybutyrate dehydrogenase (Hbdh), α-glycerophosphate dehydrogenase (α-Gpdh), mannose-6-phosphate isomerase (Mpi), phosphoglucose isomerase (Pgi), phosphoglucomutase (Pgm), isocitrate dehydrogenase (Idh), adenylate kinase (Ak), 6-phosphogluconate dehydrogenase (6-Pgd) and nucleoside phosphorylase (Nsp) are given in Table III.

Standards

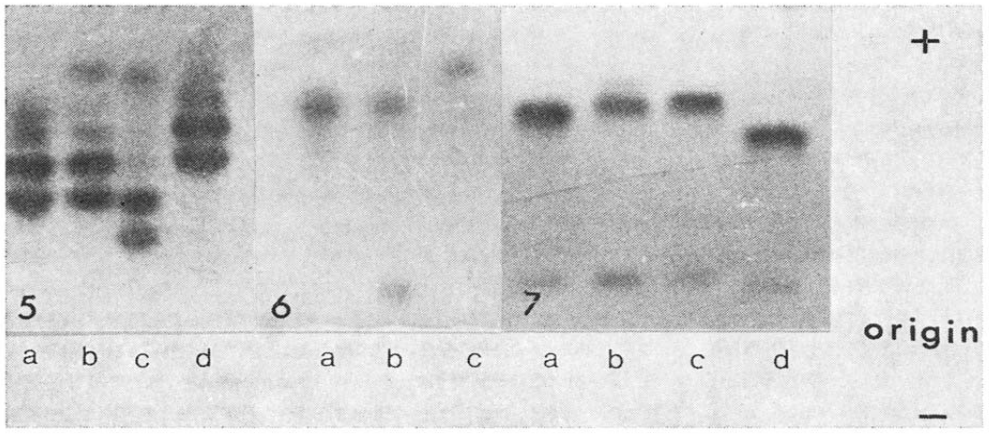
In order to compare electrophoretic results, it is desirable to use internal standards with known electrophoretic mobility. Very little isoenzyme variation is found in *B. camerunensis*, which makes this species suitable as a reference snail. At the Danish Bilharziasis Laboratory *B. camerunensis* collected in July, 1972, in Kinshasa, Zaire, is used as internal standard, and the electrophoretic mobilities of the enzymes in all other *Biomphalaria* species are expressed as mobilities relative to this stock (*rm* values).

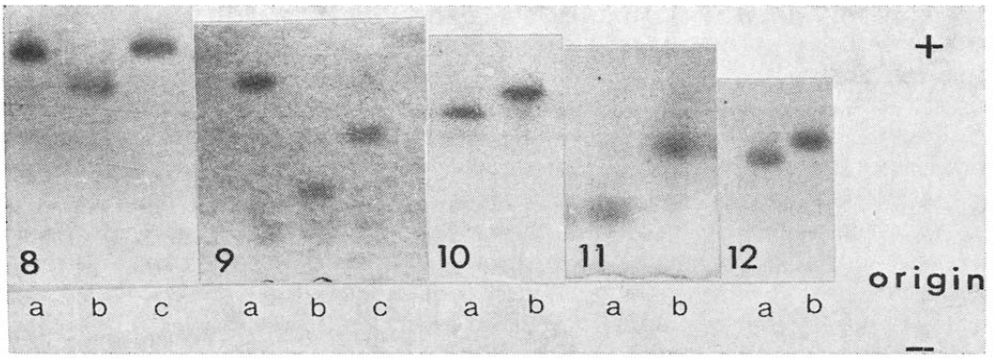
RESULTS AND DISCUSSION

The methods described above were devised by staining for about 25 enzymes in 10 different buffer combinations; they are the most reproducible methods so far found on electrophoresis of *Biomphalaria*. In Figs. 1–12, an example of a zymograms for each of the 12 enzymes is shown. These figures are deliberately chosen to show the variation between different stocks and species of *Biomphalaria*, and, whenever possible, differences between closely related species are shown. The zymograms are meant to demonstrate the possibilities for species identification by electrophoresis, and a series of papers presenting the results of 6800 zymogram readings is in preparation. On the basis of Figs. 1–12, it would be premature to draw conclusions regarding characters for species identification. Our full set of data strongly support the utility of electrophoresis in identification of *Biomphalaria* species and indicate that reliable species identification is possible from the *rm* values of four enzymes; often, esterase pattern alone can provide good tentative identification.



Figs. 1–4.





Figs. 1–12. Starch gel zymograms of whole animal extracts of *Biomphalaria*. Fig. 1. Esterases in buffer A: (a), *B. camerunensis*, Kinshasa, Zaire; (b), *B. alexandrina*, Khartoum, Sudan; (c), *B. alexandrina*, Khartoum, Sudan. Fig. 2, α-Glycerophosphate dehydrogenase in buffer A: (a) *B. pfeifferi*, Kakonde, Zaire; (b), *B. camerunensis*, Basoko, Zaire. Fig. 3. Nucleoside phosphorylase in buffer E: (a), *B. alexandrina*, Ismalia, Egypt; (b) *B. camerunensis*, Kinshasa, Zaire; (c), *B. pfeifferi*, Yaoundé, Cameroun. Fig. 4. Adenylate kinase in buffer B: (a), *B. pfeifferi*, Kano Plain, Kenya; (b), *B. camerunensis*, Kabondo, Zaire; (c) *B. choanomphala*, Pansiansi, Tanzania. Fig. 5. Phosphoglucomutase in buffer A: (a), *B. pfeifferi*, Kakonde, Zaire; (b) *B. pfeifferi*, Shepeteri, Nigeria; (c) *B. glabrata*, Puerto Rico; (d) *B. straminea*, Sydney, Australia. Fig. 6. Leucine aminopeptidase in buffer A: (a), *B. camerunensis*, Basoko, Zaire; (b), *B. sudanica*, Kiloleri, Tanzania; (c), *B. alexandrina*, Qualyub, Egypt. Fig. 7. 6-Phosphogluconate dehydrogenase in buffer B: (a), *B. camerunensis*, Kinshasa, Zaire; (b), *B. salinarum*, Grootfontein, Namibia; (c) *B. pfeifferi*, Accra, Ghana; (d), *B. pfeifferi*, Hedjo, Togo. Fig. 8. Isocitrate dehydrogenase in buffer A: (a), *B. pfeifferi*, Kakonde, Zaire; (b), *B. choanomphala*, Pansiansi, Tanzania; (c), *B. camerunensis*, Kabondc, Zaire. Fig. 9. 3-Hydroxybutyrate dehydrogenase in buffer A: (a), *B. camerunensis*, Kinshasa, Zaire; (b), *B. pfeifferi*, Kakonde, Zaire; (c), *B. pfeifferi*, Shepeteri, Nigeria. Fig. 10. Mannose-6-phosphate isomerase in buffer A: (a), *B. sudanica*, Mwanza, Tanzania; (b), *B. camerunensis*, Kinshasa, Zaire. Fig. 11. Glutamate-oxaloacetate transaminase in buffer A: (a), *B. pfeifferi*, Shepeteri, Nigeria; (b), *B. pfeifferi*, Kakonde, Zaire. Fig. 12. Phosphoglucose isomerase in buffer A: (a), *B. pfeifferi*, Malumfashi, Nigeria; (b), *B. glabrata*, St. Lucia.

If the taxonomy based on morphological characters is compared with that based on electrophoretic characters, the two sets of data give roughly identical systematic relationships between the species; however, the electrophoretic studies are

providing data indicating the phylogenetic relationships of *Biomphalaria* species from Africa and South America.

The size of the snails had no effect on the zymograms, indicating that even very young specimens can be properly identified. Such specimens are almost impossible to identify by using morphological characters.

In Table IV, the suitabilities of buffer–enzyme combinations for the genera *Bulinus* and *Biomphalaria* are compared. It can be seen that a number of differences exist between the two genera, the most notable being found in the enzymes Mpi and xanthine dehydrogenase (Xdh). Mpi is scored easily in all four buffers on *Biomphalaria* species, whereas this enzyme only can be scored in two buffers on *Bulinus* species. In Xdh the opposite situation is found, the *Bulinus* being scored easily in all four buffers, but only two buffers are useful for scoring *Biomphalaria* species.

TABLE IV

COMPARISON OF BUFFERS USED FOR ELECTROPHORESIS OF *BULINUS* AND *BIOMPHALARIA* WHOLE-SNAIL EXTRACTS

The underlined combinations are used routinely in the survey of wild populations of the two genera. *Bul.* = *Bulinus*, *Biom.* = *Biomphalaria*. 3 = Enzyme bands strong and easy to read; 2 = enzyme bands weak or difficult to read; 1 = enzyme activity present, but bands very difficult to read; —, no enzyme activity; 0 = not tested.

Enzyme	*Buffer A**	*A***	*Buffer B***		*Buffer C***		*Buffer B**	
	Bul.	*Biom.*	*Bul.*	*Biom.*	*Bul.*	*Biom.*	*Bul.*	*Biom.*
Est	3	3	2	2	1	1	0	2
Got	2	3	3	2	2	2	3	2
Lap	—	2	—	—	1	—	0	2
Hdbh	3	3	2	3	3	3	2	3
α-Gpdh	2	3	2	2	3	2	3	3
Mpi	2	3	1	3	1	3	2	3
Pgi	3	3	2	2	2	2	2	3
Pgm	2	3	1	2	1	2	3	2
Idh	1	3	2	3	3	3	3	3
Ak	1	2	1	1	1	2	1	3
6-Pgd	2	2	1	1	3	2	2	3
Nsp	3	2	3	2	2	1	3	3
Fk***	2	2	1	2	2	2	2	3
Xdh‡	3	1	3	1	3	2	3	2

* This paper.
** Jelnes[15].
*** Fructokinase.
‡ Xanthine dehydrogenase.

In conclusion, it must be mentioned that the experimental taxonomy of *Biomphalaria* represents a promising extension of morphological identification methods. The method is still in its infancy, as much more material needs to be surveyed before a full picture of the relationships between the different species of *Biomphalaria* emerges.

ACKNOWLEDGEMENTS

We hope that this paper will encourage scientists to take up this field of taxonomy, and we would be pleased to receive live samples of *Schistosoma* intermediate hosts, so that the taxonomy of the genera *Biomphalaria* and *Bulinus* can be improved. This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and from the Danish Natural Science Research Council. The skillful technical assistance of Mrs. R. Herk-Hansen is gratefully acknowledged. Sincere thanks are due to the numerous persons who have been, or will be, providing the laboratory with live material. Any sample from any area will provide information.

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