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A Serological Technique for Gastropod Taxonomy[1]

James N. Cather[2]

Serological techniques have been used in systematic studies for about fifty years. At first their use was confined to those groups of animals offering plentiful supplies of blood, since blood was the tissue most widely used. Recent modifications that employ other tissues have now made possible similar study of groups of smaller animals.

Serological work in the invertebrates has in general been limited, but includes outstanding studies on the Crustacea by Boyden ('36, '39) and Leone ('49). Blood was the tissue used by these workers. Wilhelmi ('44) used other tissues of several invertebrates, including *Busycon;* desiccating them after freezing and then preparing a saline extract.

Boyden points out ('42, '53) that serological data do not offer complete answers to taxonomic problems. They can, however, aid when morphological differentiation is inconclusive, and thus help in establishing a more natural classification of families and genera. Serologists interested in comparative morphology and physiology assume that animal proteins are less rapidly changed in evolution than are most morphological features. Proteins, we should expect, suffer less change from environmental influences than do morphological features. The value of comparative serology in taxonomy has been proved in many instances, especially where its results have comported with those got by conventional comparative morphological methods. Such methods are often difficult to use at taxonomic levels above the genus, because of degeneracy, convergence, and parallelism. The quantitative and objective nature of comparative serology makes the method highly useful, when used with data obtained from comparative morphology.

The Gastropoda notably require such study, since so much of our classification of them rests upon characteris-

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tics that show wide variation. Some taxonomists rely primarily upon reproductive structures as a basis for classification; others depend upon shell characters which are more obvious to the average student and require less skill in their use. Shell characters have been used to advantage in many groups, even at the species level; but in a genus such as *Physa,* intergradation seems universal. A key based upon shell characters would be the only practical kind for

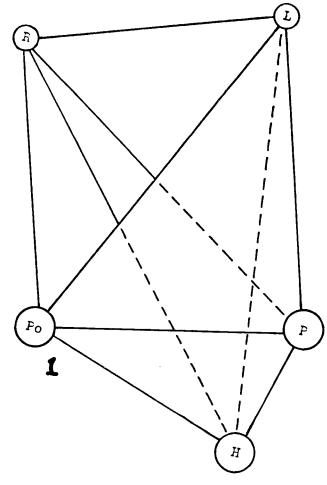

Figure 1. Relationships of the Gastropods studied. (Po, *Pomacea;* P, *Physa;* H, *Helisoma;* R, *Rumina;* L, *Limax.*)

persons interested in this taxonomic group. Yet such keys may lead to confusion in certain families and genera, because of wide variation in shell characteristics. There may be no solution of this problem of shell variation within a genus or species, when dealing with a plastic group like the gastropods, that reflect so much their environment-background.

General Methods and Materials

Most of the previous work in comparative serology, in which precipitin tests were used, employed whole sera as antigens. Martin & Leone ('52) and Stallcup ('54) showed that tissue extracts are satisfactory as antigens in serological testing with birds.

Wilhelmi ('44) stated that lipids must be removed for accurate serology, but Stallcup's ('54) work showed this not

always necessary. In my work, removal of lipids was not attempted.

The Gastropoda in these tests (exclusive of *Pomacea caliginosa*) were collected in Dallas County, Texas, in March, 1955, and included the following:

Subclass Prosobranchia
Order Monotocardia
Ampullariidae
Pomacea caliginosa
Subclass Pulmonata
Order Bassommatophora
Physidae
Physa anatina Lea
Planorbidae
Helisoma trivolvis Say
Order Stylommatophora
Suborder Sigmurethra
Holopoda
Order Stylommatophora
Suborder Sigmurethra
Holopoda
Achatinidae
Rumina decollata (L.)
Aulacopoda
Limacidae
Limax flavus L.

Antigen Preparation.—From 6 to 30 grams of living snails were placed in a Waring blender and minced with 100 ml. of unbuffered 8 percent NaCl solution. The mixtures were refrigerated for 48 hours at 2° C. to permit extraction of proteins. The tissue residues were then centrifuged out, and the extracts stored at 2° C. Protein content of the extract was determined by the biuret method, using the Bausch & Lomb "Spectronic 20" colorimeter.

Antisera Preparation.—All antisera were produced in domestic rabbits. A series of 4 injections were given each rabbit, with an interval of 48 hours between injections. The following methods of injection were used:

(1) 0.5 ml. intravenously,
(2) 0.5 ml. intravenously; 0.5 ml. subcutaneously,
(3) 1.0 ml. intravenously; 1.0 subcutaneously,
(4) 1.0 ml. intravenously; 3.0 ml. subcutaneously.

On the eighth day after the final injection, 10 ml. of blood were withdrawn from the main artery of the ear, and the antiserum was used in a homologous precipitin test (against the antigen which produced the antiserum) to determine whether antibodies were present in sufficient amounts. If enough were present, the rabbit was bled to death by cardiac puncture, using an 18-gauge needle and a 50 ml. syringe. The whole blood was placed in test tubes and allowed to

clot. The blood was allowed to stand until the serum was expressed. Both serum and clots were then centrifuged, and the serum decanted. This was then sterilized by filtration

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Increasing Dilution	Tube Number	Limax	Physa	Helisoma	Rumina	Pomacea
	1.	5.8	3.0	2.2	1.0	0.0
	2.	6.0	3.0	2.5	1.5	0.0
	3.	6.5	3.0	2.0	2.0	0.2
	4.	3.5	3.0	1.5	1.0	0.2
	5.	2.5	2.2	1.5	1.0	0.2
	6.	1.5	2.0	0.5	1.0	0.1
	7.	1.0	1.0	0.5	0.5	0.0
	8.	0.7	0.5	0.5	0.4	0.0
	9.	0.7	0.5	0.5	0.5	0.0
	10.	1.0	0.5	0.0	0.8	0.0
Total		29.2	18.7	11.7	9.7	0.7

3

Increasing Dilution	Tube Number	Helisoma	Physa	Rumina	Pomacea	Limax
	1.	13.5	9.0	10.5	8.0	2.0
	2.	15.1	8.0	0.3	6.0	3.5
	3.	15.0	7.5	1.5	4.0	4.8
	4.	11.0	6.5	2.0	5.0	4.0
	5.	7.5	5.0	2.0	3.0	2.5
	6.	4.2	2.5	3.0	3.0	1.8
	7.	2.7	2.5	2.0	2.5	0.5
	8.	1.5	2.0	2.2	2.5	0.2
	9.	1.0	2.0	1.5	2.5	0.2
	10.	1.4	1.0	1.5	2.0	0.5
Total		72.9	46.0	26.5	38.5	20.0

4

Increasing Dilution	Tube Number	Rumina	Helisoma	Physa	Pomacea	Limax
	1.	8.2	2.0	7.5	7.0	4.0
	2.	3.7	2.0	7.0	8.5	4.0
	3.	14.4	1.0	6.5	6.5	4.0
	4.	20.5	1.0	5.0	5.0	2.5
	5.	21.1	1.0	4.0	3.0	2.0
	6.	14.1	0.2	3.0	2.3	2.0
	7.	8.0	0.8	2.5	1.2	1.0
	8.	6.6	0.5	2.0	1.2	1.0
	9.	1.8	0.0	1.0	1.0	2.0
	10.	0.7	0.0	1.0	1.0	1.0
Total		99.1	8.5	39.5	36.7	23.5

5

Increasing Dilution	Tube Number	Pomacea	Physa	Helisoma	Rumina	Limax
	1.	20.0	16.2	11.0	18.0	1.5
	2.	23.2	6.7	3.0	0.5	2.5
	3.	9.7	6.0	4.0	1.5	2.5
	4.	8.0	4.0	2.0	2.5	3.0
	5.	7.2	3.0	1.5	1.5	2.0
	6.	4.7	2.5	1.0	2.0	1.0
	7.	3.7	1.5	1.8	1.0	1.0
	8.	2.5	0.5	1.5	0.5	0.5
	9.	1.9	0.2	1.0	0.0	0.5
	10.	0.9	1.0	1.0	0.0	1.0
Total		81.8	41.6	27.6	27.5	15.5

Figures 2-5. Turbidity values of precipitin Tests with antisera of *Limax* (2), *Helisoma* (3), *Rumina* (4), and *Pomacea* (5).

through a Seitz filter. It was then transferred to sterile 5 ml. bottles with sterile pipettes, and stored at 2° C. until used.

Method of Testing. — Antigenic substances introduced into the body of an animal cause the production of antibodies, which in turn cause precipitation of the antigen when the two are mixed. When an antiserum is added to some of the antigen that caused its production (homologous antigen), maximum precipitation occurs. When the antiserum is added to the other antigens (heterologous antigens), a smaller amount of precipitate is formed. The

amount of precipitate produced corresponds to the degree of similarity of the antigen in question, to the homologous antigen. Thus we are able to infer serological relationships. The method of testing follows that used by Leone ('49), and requires the use of the Libby ('39) photronreflectometer. This instrument. measures the turbidities produced by the interaction of antigen and antiserum. Biuret tests were run to determine the amount of protein in each mixture, then enough 8 percent salt solution was added to give an initial dilution of 1 part of protein to 500 parts of salt solution. Tests were run in Kolmer test-tube racks with ten tubes for each test. The first tube contained an initial dilution of 1

Increasing Dilution: Tube Number	Physa	Helisoma	Rumina	Pomacea	Limax
1.	11.2	7.5	0.0	5.0	3.5
2.	9.9	7.5	0.0	5.0	5.0
3.	8.1	6.2	1.0	5.0	4.0
4.	7.0	5.5	1.0	3.5	3.0
5.	6.2	4.2	2.0	2.5	2.0
6.	4.6	2.8	1.0	1.0	2.2
7.	3.3	1.8	1.0	1.0	1.0
8.	2.6	2.0	1.0	0.5	0.5
9.	1.6	1.8	0.5	0.8	1.5
10.	1.2	1.0	0.6	0.2	2.0
Total	56.7	40.3	8.1	24.5	24.7

Figure 6. Turbidity values of precipitin tests with *Physa* antiserum.

part of protein in 500 parts salt solution, and each successive tube contained a protein concentration only half of that of the preceding tube. The photronreflectometer was made to use a volume of 2 ml., and this quantity was used in all tests. The amount of antiserum was held constant at 0.3 ml., but the concentration of antigenic extract was varied. The volume of antigen was always 1.7 ml., but increasing dilutions were made by adding 8 percent saline. Each test to determine the inherent turbidity was read as soon as it was completed. The turbidities were allowed to develope over a 24-hour period, when a second reading was taken. The difference between the two readings is the turbidity caused by antigen-antibody reaction (Figs. 2-6). The tests were carried out at room temperature. "Merthiolate" (Thimerosal,

Lilly) in a final dilution of 1:10,000 was added to prevent bacterial reaction.

Serological Relationships

Serological evidence showed *Helisoma* and *Physa* to be the most closely related genera studied (Figs. 1; 7). This would be expected from the classification given on page 7; and both paleontology and morphology give evidence that the Bassommatophora arose from the Stylommatophora. As these genera have probably undergone similar evolutions from a common ancestor, we should expect similar proteins.

	Pomacea calignosa	Physa anatina	Helisoma trivolvis	Rumina decollata
Pomacea calignosa				
Physa anatina	47			
Helisoma trivolvis	43	68		
Rumina decollata	31	27	22	
Limax flavus	11	55	34	27

Figure 7. Percentage values obtained from analyses of precipitin reactions.

Pomacea shows a more closely related serological kinship to *Physa* and *Helisoma* than to *Rumina;* but *Limax* is apparently closer to *Physa* than to either *Pomacea* or *Rumina.* At the outset this differs from what one would expect, but it may reflect origins. The Monotocardia are believed to be ancestral to the Pulmonata (because of the retention of the single auricle by the Pulmonata) ; the first evolved members of the Pulmonata were the Stylommatophora. If this be true, then *Pomacea* and *Rumina* should be relatively close in serological relationship. While this does not seem to be the case at first sight, our findings indicate that *Pomacea* is closer to *Rumina* than *Rumina* is to any of the other snails tested (Fig. 7). Thus we may infer that *Rumina* lies to one side of the line ancestral to *Physa* and *Helisoma.*

The genus *Pomacea* and the genus *Limax* show the widest divergence in serological relationship of all genera studied. *Rumina* is also more remotely related to *Limax* than are *Helisoma* and *Physa*. This may mean that *Physa* and *Limax* are related, and it is possible that an emergence took place a second time, and that *Limax* descended from some bassommatophoran. The Limacidae show variability within the family, and perhaps are polyphyletic in origin. Whether this is true or not, it is logical to infer that a family that evidences so much evolutionary change in morphology would undergo more extensive serological change than the more conservative snails studied in these investigations.

SUMMARY

Serological techniques which have been applied to vertebrates may be of value in gastropod taxonomy.

The results of the present study appear to support the theory that the Pulmonata arose from the Monotocardia. From the results we can infer that *Rumina* lies to one side of the main path of the evolution of the Bassommatophora, and that *Limax* has undergone considerable protein change since its presumed early origin from the Monotocardia.

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