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THE RELATIONSHIPS OF SOME COMMON AMPHIBIA AS DETERMINED BY SEROLOGICAL STUDY[1]

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It frequently happens in systematic zoölogy that after all the known facts on the anatomy, development, and distribution of a species have been brought together no definite conclusion can be reached as to the immediate relationships of a form. In the class Amphibia there are notable examples of this state of affairs. Among the salamanders, the mud-puppy, *Necturus*, represents such a case, and another is afforded by the eel-like *Siren* which has been well known in both anatomical and herpetological literature for over a century. There is one method of determining relationships that has not been applied to these or to the majority of the Amphibia: this is the precipitin reaction discovered in 1897 by R. Kraus (10[2]). Soon thereafter, it was applied to the study of animal proteins. Chiefly to Nuttall (18) belongs credit for the discovery that the intensity of the reaction is proportional to the degree of relationship of the species tested. The results of 16,000 precipitin tests, involving chiefly the Vertebrata, were described in Nuttall's book, 'Blood Immunity and Blood Relationship,' published in 1904. Recently one of us has devised new methods for the analysis of the precipitin tests on the sera of mammals (2). In the present study the tests have been applied to the Amphibia and especially to those salamanders whose relationships have remained in doubt. This work has involved the testing of other species of known relationship in order to secure a basis for comparison. Before turning to the tests a brief summary may be given of the relationships of these salamanders as at present understood.

THE SYSTEMATIC POSITION OF *SIREN* AND *PSEUDOBRANCHUS*

The systematic relationships of the various families of salamanders have been summarized recently by one of us, in the form of a diagram (13). In only two of the eight families were the relationships so obscure that no connecting line of affinity could be drawn. The first of these

[1]Contributions from the Zoölogical Laboratory, Rutgers University, and from the Laboratory of Experimental Biology, The American Museum of Natural History.

[2]See Bibliography, p. 24.

families, the Sirenidæ, includes *Siren* and the related *Pseudobranchus*. These genera embrace three species of salamanders that retain throughout life most of the structural features of very young larvæ (13*a*). Oddly enough, the skin of *Siren* undergoes a metamorphosis typical of that of land salamanders. This metamorphosis may readily be induced by thyroid solutions (16). The chief difficulty in determining the relationships of *Siren* lies in the fact that most of the structural organization of this salamander remains that of a larva. Since the larvæ of salamanders are very much alike, few consistent family differences having been demonstrated, the number of clues as to the relationships of *Siren* is extremely limited. Moreover, the systematic value to be assigned to some of these characters frequently is a matter of opinion.

One of the most important clues as to the relationships of *Siren* apparently is found in the nasal region. Mesial to the dorsal spines of the premaxillaries there is found on either side a narrow splint of bone that has been homologized with the nasal of other salamanders. In the mature larva of *Hynobius chinensis*, as shown in our dissections, a part of the nasal bone forms a long splint mesial to the spine of the premaxillary very much as in the case of *Siren*. However, the nasal of *H. chinensis* has also a portion lateral to the premaxillary spine that is not found in *Siren* or *Pseudobranchus*. The two nasal bones of *Siren* and *Pseudobranchus* make contact in the midline. The only other salamanders in which the nasals meet without overlapping the premaxillary spines are the Hynobiidæ and the derived Cryptobranchidæ. In one plethodontid and in a few salamandrids, including the common newt, *Triturus viridescens*, the nasals make a contact by overlapping these spines. It might be argued that the condition in *Siren* and *Pseudobranchus* has been derived from that of these salamandrids by a loss of the lateral portions of the nasals. However, the premaxillary spines of *Siren* and *Pseudobranchus* are well separated and it would require less modification to derive the sirenid arrangement from that of *Hynobius*.

Siren and *Pseudobranchus* differ fundamentally from all hynobiids and cryptobranchids in that the angular and prearticular are fused to form a single bone that shows no evidence of its duplex origin even in early stages of development. However, there is little evidence to show that the sirenids are related more to one than to another higher family. Reed (20) has considered that the sound transmitting apparatus of *Siren* resembles that of the newts more than it does that of other salamanders. Unfortunately the development of the otic apparatus of *Siren* is incompletely known. The palatoquadrate bar divides and the

posterior section undergoes degeneration at a time when the skin remains that of a typical larva. Hence it has been questioned as to whether this can be considered a metamorphosis of the palatal region homologous to that of caducibranchs (12). Whether or not the otic apparatus of *Siren* undergoes a transformation is not known, but the final form is very different from that of *Cryptobranchus* as described by Reed (*op. cit.*).

Recently the mode of life history has been found to shed light on the relationships of Amphibia (11). Neither *Siren* (15) nor *Pseudobranchus* (17) lay their eggs in the manner of hynobiids and cryptobranchids. They are deposited either in small groups or singly and not enclosed within the common envelope of jelly so characteristic of the Hynobiidæ. Although the eggs resemble those of some newts more than they do those of any other salamander, the larvæ early develop horny mouth-plates, which have been compared with the predentary sheath of some larval hynobiids and ambystomids. The latter structure is not formed in any salamandrid. The sharp claws on the feet of the sirenids might be compared with the claws of the larval *Onychodactylus*, a hynobiid. The tips of the toes of other salamanders may be covered with horn, but this does not form the pointed claws like those of *Onychodactylus* and the sirenids. Again, the sirenids apparently practice external fertilization, to judge from the structure of their cloacæ, and this is found elsewhere among salamanders only in the hynobiids and cryptobranchids. However, it might be argued that the sirenids in most of their organization have not advanced beyond the condition of very young larvæ and hence the cloacal glands have been suppressed, although present in their immediate ancestors. The evidence of life history can not be considered as giving a decisive answer to the question of the relationships of the Sirenidæ. If one lays emphasis on the structure of the egg-mass the sirenids cannot be closely allied to the hynobiids. On the other hand the horny jaws, clawed toes, and external fertilization appear to indicate affinity to this group.

Although some of the distinctive features of the sirenids may be a consequence of a great increase in length, the majority represent a retention of larval structures. Thus the peculiar tail vertebræ have been commented upon (4). We find that the paired hæmapophyses resemble closely those of a young *Amphiuma*, 110 mm. in total length. The paired elements of *Amphiuma* and other salamanders have failed in *Siren* to differentiate beyond this early stage of development. The hyobranchial apparatus of the sirenids is obviously larval but it undergoes an early

ossification not found in caducibranchs. Similarly the hypertrophy of the Jacobson's organ does not occur in early larvæ of other salamanders.

To summarize, it may be said that while *Siren* and *Pseudobranchus* are obviously species that have failed to differentiate most of their structures beyond that of the early larval stage, they are peculiar in that a few structures in the same species are free from this developmental restriction. The skin of *Siren* undergoes a typical metamorphosis, while the palates of *Siren* and *Pseudobranchus* become modified in a manner resembling metamorphosis. Very few structures of the metamorphosed adult ever appear in the sirenids and hence these salamanders must be compared with the larvæ of other families. From the anatomical and life-history data available it is impossible to determine the nearest relatives of the sirenids. Arguments may be found for assuming either a hynobiid or a salamandrid ancestry. Moreover, neither the cryptobranchids nor the ambystomids can be ruled entirely out of the picture. It is very desirable, therefore, to attack the problem of the affinities of the Sirenidæ on some entirely new basis. The serological approach affords such a new angle of attack.

THE SYSTEMATIC POSITION OF *NECTURUS*

Necturus and its European relative *Proteus* have been separated from other salamanders as a distinct suborder, Proteida, not because of any marked structural difference but because they, like *Siren*, are larval types that fail to metamorphose and thus develop the characters usually employed in the identification of families. However, *Necturus* cannot be considered as great a puzzle as *Siren*, because it possesses the elaborate set of cloacal glands found in salamandrid, ambystomid, and all higher families. Like salamanders of these families it has the fused angular and prearticular. These features show that *Necturus* is a higher type than either the Hynobiidæ or the Cryptobranchidæ. The presence of lungs excludes it from close relationship with the Plethodontidæ. *Amphiuma*, the sole member of the Amphiumidæ, is too specialized in its elongate body and reduced hyobranchial apparatus to form the ancestral stock from which *Necturus* evolved. This leaves only the Salamandridæ and the Ambystomidæ among existing families in which to seek its relationships.

To be sure, Reed (20) found that the ear ossicles of *Necturus* showed a greater resemblance to those of Plethodontidæ and Amphiumidæ than to those of other families of salamanders. But these two families are specialized off-shoots of a salamandroid stock. The absence of horny

predentary plates in *Necturus* might be considered a salamandroid feature. *Necturus* lays its eggs attached singly to the under surface of stones or other submerged objects like some salamandrids and plethodontids do, but unlike any ambystomid. The Proteida agree with the Salamandridæ in being found today in both the Old and New World, while the Ambystomidæ are restricted to North America. The fossil record indicates that the salamandrids flourished in Europe from at least the Oligocene, but it gives no clue as to the origin of either *Necturus* or the ambystomids. Nevertheless, the anatomical and life-history data indicated above suggest that *Necturus* has closer affinities with the salamandrids than with the ambystomids. The existing evidence is by no means conclusive and consequently the new approach to the problem of relationships afforded by the serological tests should be of interest.

PREVIOUS SEROLOGICAL STUDIES ON AMPHIBIA

The literature of serological research on animals has recently been summarized by Erhardt (5). The Amphibia have received but little attention. Nuttall (18*a*) tested two weak anti-frog sera (against *Rana temporaria*) and found them to react only with *R. temporaria* and *R. ridibunda.*[1] Philippson (19) tested one weak anti-frog serum (against *R. esculenta*) which reacted only with *R. esculenta* and *R. temporaria* (cited by Nuttall).

These are all the precipitin investigations known to us that deal primarily with amphibian relationships. There are, however, two other studies involving precipitin tests with Amphibia that deal indirectly with the problem of relationships in this class of Vertebrata. The earliest of these is a study of Braus (3) in which it is reported that antisera against adult tissue extracts of *Bombina variegata* failed to react with tissue extracts of the larvæ of the same species. The conclusion drawn was that some important biochemical differences existed between the larval and adult stages of this species. Obviously such differences, if proved to exist, would be reflected in any serological classification of this species, for the larval and adult stages would be expected to react differently to antisera of other species.

The results of Braus have been flatly contradicted by Wilkoewitz and Ziegenspeck (21) who claim that they were unable to distinguish between the larval and adult stages of *Rana esculenta.* In other words there was complete identity, so far as the precipitin technique they employed could determine, in the larval and adult stages of this species.

[1]Modern terminology for these species is used throughout this paper.

Of the two investigations the work of Wilkoewitz and Ziegenspeck is probably most nearly correct, though further refinements in precipitin technique may be able to disclose slight differences in serological reactions between the larval and adult stages of such organisms.

Wilkoewitz and Ziegenspeck further tested antisera against *Rana esculenta* larvæ and against *R. esculenta* adults with *Bufo bufo* and *Triturus* extracts and found them all to react very similarly. They also used a "Kunstserum" against *Bufo bufo* with the other Amphibia mentioned, with similar results.

These meager results indicate that the problem of the serological study of amphibian relationships has barely been touched. It has therefore seemed advisable to apply our tests to a number of Amphibia of known relationships before drawing conclusions as to the significance of our work with species of doubtful affinity.

PROCEDURE USED IN THIS STUDY

A.—Preparation of the Antigens (Blood Sera)

The materials first needed in serological work are the proteins to be used in the production of antisera. In this study only blood sera have been so used. The species from which sufficient blood was obtained for the production of antisera are as follows:

Rana catesbeiana
Rana pipiens
Amphiuma means
Necturus maculosus
Cryptobranchus alleganiensis
Siren lacertina

These animals were bled from the heart, usually after slight ether anæsthesia. The anæsthetized animals were tied with their backs to a board. A slight cut was then made in the ventral body-wall exposing the heart, under the tip of which a centrifuge tube was placed. The animals were then placed vertically and the blood collected. It clotted quickly and was put in the ice-box overnight. The clot was then cut with a scalpel and the clear serum centrifuged off. This serum was then passed through sterile Seitz filters and bottled in sterile 5 ml. vaccine ampules with rubber stoppers. The last vial to be filled was left at a room temperature to test for contamination; the rest were stored in the ice box. If the test vial remained clear the serum was considered sterile.

In addition to the six species of Amphibia named above, from each of which 35 cc., or more, of serum were obtained, smaller amounts of serum were obtained from the following species:

Rana clamitans
Hyla septentrionalis
Ambystoma opacum
Desmognathus fuscus
Gyrinophilus porphyriticus
Triturus viridescens
Plethodon glutinosus

There were thus available seven more species for titration. In the case of the last four species, the animals were bled after decapitation, the blood being wiped off on filter paper. While still wet this paper was cut in strips and extracted in 0.9 per cent sterile saline. After extraction overnight in the ice box the diluted serum was filtered, tested, and stored in the usual manner.

B.—Preparation of the Antisera

The first attempts at producing precipitin antisera in rabbits resulted in the death of the injected animals, due apparently to the inherent toxicity of the amphibian sera. In the next attempt fowls were used. Here, too, there were several fatalities until care was taken to get tough healthy cocks and to keep them in an outdoor pen during the course of the injections.

A common method of injection was followed. Each bird was given a series of increasing doses of one antigen, twice weekly, the first three or four injections being intraperitoneal, the following ones being intravenous (wing-veins). After five or six injections a rest period of seven days was allowed at the end of which time a small sample of blood (3–4 cc.) was withdrawn from a wing-vein for a preliminary titration. If this titration showed the antiserum to be too weak for use (titer of less than 3200) further injections were given, the first of these being small and intraperitoneal.

This second series of injections was often accompanied by symptoms of sickness on the part of the bird, diarrhœa, wobbling, weakness, and going to roost in midday, which in extreme cases led to prostration and death. Usually the bird recovered completely in the course of a half hour. After the last injection the bird was given a rest period of ten days, the last twenty-four hours being without food so as to clear the blood. It was then bled completely after decapitation, the blood being collected in a large pan. As soon as the blood had clotted it was cut in thin strips with a knife and these strips were put into flasks and placed in the ice-box overnight. The clear serum was poured off the clot and centrifuged. This antiserum was then passed through a sterile Seitz filter, bottled, tested, and stored in the same way as for the antigens. Eleven antisera were so prepared, their specificity as follows:

C_4, C_5, C_8, C_9	anti-*Rana catesbeiana*
C_{16}	anti-*R. pipiens*
C_6, C_{10}	anti-*Amphiuma means*
C_7	anti-*Necturus maculosus*
C_{12}, C_{14}	anti-*Cryptobranchus alleganiensis*
C_{15}	anti-*Siren lacertina*

C.—Making the Tests

The general procedure in a serological study of biological relationships is to test each antiserum with the particular antigen used in its formation, and then follow with the testing of all the other available antigens. But it is of the first importance in comparative work that all the antigens used be OF THE SAME STRENGTH. This has been emphasized in previous work (1) dealing with Mammalia. In order to equalize the concentrations of the different antigens it was necessary to determine the protein nitrogen and calculate the content of protein with the usual factor 6.25. The method for determining the protein nitrogen was the Folin-Wright modification of the Macro-Kjeldahl (7). The results are given in Table I.

TABLE I.—Protein content of amphibian sera

SPECIES	Protein in gms. per 100 cc. of serum or 100 cc. of extract
[1]A.—*Rana catesbeiana 7a*	(serum).....2.66
R. pipiens a	(serum).....1.80
Amphiuma means 5b	(serum).....3.39
Necturus maculosus 1	(serum).....1.58
Cryptobranchus alleganiensis A	(serum).....2.56
Siren lacertina	(serum).....2.71
[2]B.—*Rana clamitans*	(serum).....1.58
Hyla septentrionalis	(serum dil. 1:50).....0.025
Ambystoma opacum	(serum dil. 1:10).....0.25
Plethodon glutinosus	(extract).....0.013
Desmognathus fuscus	(extract).....0.094
Gyrinophilus porphyriticus	(extract).....0.033
Triturus viridescens	(extract).....0.18

The values given in Table I are fairly consistent for the sera, but quite variable, as is to be expected, for the filter-paper extracts of whole blood. The B series of values is in excess of the true value of protein to the amount of the non-protein nitrogen present. The average amount of non-protein nitrogen present in the A series was 5.4 per cent. The quantities of antigen available in the B series were too small to allow the determination of the non-protein N, but the error due to this is probably insignificant in the secondary data based on the non-reciprocal tests in this series, which gave results to be accepted tentatively only.

In every case the dilution factor necessary to reduce the most concentrated sera to a standard dilution was calculated. The basic standard dilution chosen was a solution of 1 part of protein to 500 parts of

[1]A. Protein based on Total N minus non-protein N.
[2]B. Protein based on Total N.

saline. From this basic standard solution all higher dilutions were made. This procedure guarantees that all the corresponding dilutions of every antigen are truly comparable in their total protein content.

The series of antigen dilutions used is shown in Table II.

TABLE II.—Standard dilutions used in amphibian tests

Tube 1 = 1 part of protein to 500 parts saline	
Tube 2 = 1:1,000	Tube 8 = 1:64,000
Tube 3 = 1:2,000	Tube 9 = 1:128,000
Tube 4 = 1:4,000	Tube 10 = 1:256,000
Tube 5 = 1:8,000	Tube 11 = 1:512,000
Tube 6 = 1:16,000	Tube 12 = 1:1,024,000
Tube 7 = 1:32,000	Tube 13 = 1:2,048,000

Having determined the dilution factors to be used according to the strength of each available antigen in order to make the basic standard dilutions, one may begin to make the actual titrations. The procedure followed here was the usual ring-test procedure. A series of small clean test tubes of clear wall (heating to a glow in the gas flame clears and thins the wall) is arranged in a rack. Into each one except the first is pipetted 0.5 cc. sterile 1.8 per cent buffered saline of pH 7. The method of buffering was that of Evans (6). Then with a 1 ml. sterile pipette, 0.5 cc. of a 1:500 standard dilution of a particular antigen is put into tube 1 and a similar amount into tube 2. The contents of tube 2 are now thoroughly mixed by repeatedly drawing up the liquid and letting out of the pipette and then 0.5 cc. of the mixture (now 1:1000) is transferred to tube 3 where the mixing process is repeated. Thus each succeeding tube comes to possess half the concentration of the preceding one in the series. The last tube is given no antigen.

Into the bottom of each tube beginning with the last (which serves as a control) is carefully pipetted 0.1 cc. of the particular antiserum to be investigated. As soon as the series is finished the whole rack is placed in the water bath at 37.5°C. At 20, 40, and 60 minutes the reading rack is placed on a special reading stand so lighted that the zone of contact between antiserum and antigen is strongly illuminated. With the aid of a reading-glass the presence of a white layer of precipitate in the region of contact of antigen and antiserum may easily be observed. The precipitate is usually in a fairly thick layer in the tubes containing the lower dilutions and gradually thins out in the tubes of higher antigen dilutions. The last tube containing a distinct ring at the time of reading defines the titer or strength of the reaction. The readings at one hour were chosen for analysis in this investigation.

Experience with precipitating antisera obtained from both rabbits and fowls enables us to say that the fowl antisera are the most difficult to read. They tend to be slightly opalescent, though this may decrease on ripening in the ice-box over a period of several months. This opalescence is sometimes great enough to appear as a non-specific precipitation running through a whole series of dilutions and into the control. This appearance may sometimes be prevented by the use of the more concentrated saline (1.8 per cent) as suggested by Hektoen (9). But the real "specific" precipitation, whether homologous or heterologous, is usually sharp and clear-cut and hence readily distinguishable from the so-called non-specific precipitations. We have recorded as the titers of these tests only what appeared to be sharply defined distinct reactions and have disregarded the diffuse hazy appearance which is characteristic of the non-specific reactions.

One more matter should be explained before proceeding to the results of the tests. The relationship between any two species A and B is expressed in per cent. It is the ratio between the heterologous and homologous titers for those species. For example, suppose the homologous titer of antiserum A tested with serum A is 1:10,000; this is called 100 per cent. If the same antiserum tested with serum B gave a titer of 1:5000 the ratio of the heterologous and homologous titers is $\frac{5000}{10000}$ or 50 per cent. The degree of relationship between species A and B is then 50 per cent or, more accurately, the blood proteins of the sera of these two species are 50 per cent similar as tested by this biological reaction. But now it has been pointed out (1) that the relationship between these two species can be determined not only in the above way but also in the contrary way, i.e., by testing antiserum B against serum B and serum A and comparing the heterologous and homologous titers again. Theoretically the two values should check within the limits of error of the tests if there are no disturbing factors, and hence the quantitative measure of the degree of relationship can be doubly checked.

Each test has been repeated one or more times and the variability in the successive readings indicates the amount of error involved. The error of reading is somewhat greater in this investigation than in the previous study of mammalian relationships mentioned above—due probably to the relatively greater opalescence of the antisera—and the reciprocal values do not check as closely as in the mammalian work. For the amphibian work one can only say that the reciprocal tests of the same two species are of the same order of magnitude.

THE PRIMARY DATA (RECIPROCAL TESTS)

Now let us proceed to the primary data resulting from the reciprocal tests. Table III gives the results of the reciprocal tests as tube numbers. By referring to Table II the actual titers in every test may quickly be determined. Note that the homologous titers are never exceeded by heterologous titers beyond the limits of error of the reaction. Note that the heterologous titers may sometimes be equal to the homologous titers, which indicates a close blood relationship. More often the heterologous titers are considerably less than the homologous titers. In some cases the heterologous reactions are very weak or negative, which indicates a remote degree of relationship. Note the general parallelism where two or more antisera of the same kind are tested with the other antigens. The reactions are all consistent enough, bearing in mind the errors involved in the tests, with one exception: this is the surprisingly high value of the C_{15} (anti-*Siren*) antiserum when tested with *R. pipiens* serum. A much lower reaction was expected in view of the weak reaction with *R. catesbeiana* serum. This marked difference in the behavior of the two *Rana* species is consistent with the relatively low value for the reciprocal tests between these two *Ranas* species, which indicates a not very close relationship. But let us not put too much emphasis upon such an unusual reaction; it cannot be fully explained at present.

To bring out more clearly the various degrees of relationships of these species to each other, the results have been calculated as per cents of the homologous titers, and these corresponding per cent values are given in Table IV. These per cent values are average values, based on all the titers of the repeated tests with the same antigen and antiserum.

Now, to get a final simple quantitative expression for the degree of relationship of these species, all the corresponding reciprocal per cent values for antisera and antigens of the same two kinds have been averaged and the probable errors of the resulting means (M) calculated. The results are given in Table V. Most of the M values are significant statistically. The exceptions are two of the *Siren* values: viz., *Rana pipiens* vs. *Siren lacertina*, and *Cryptobranchus alleganiensis* vs. *Siren lacertina*. These exceptional values are to be accepted subject to verification. As to the *Cryptobranchus-Siren* value, it is very low (2.61) and could not be much less than it actually is (0 being the lower limit). It might be somewhat greater, but not much greater without disturbing the other values whose reliability is more certain, and therefore we may accept it as being approximately correct.

TABLE III.—Results of the reciprocal tests given as tube numbers corresponding to the titers of the reactions. *a*, *b*, *c* are the three series of readings made. Series *a* was practically completed in June, 1931; series *b* in October, 1931; and series *c* in March, 1932. Homologous titers are italicized

Antisera \ Antigens	*Rana catesbeiana* 7*a*			*Rana pipiens* *a*			*Amphiuma means* 5*b*			*Necturus maculosus* 1			*Cryptobranchus alleganiensis* A			*Siren lacertina*		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
C_4 *Rana catesbeiana*	*12*	*12*	*13*	12	12	11	4 3	5	4	2	3	4	2 2	4	3	4	5	4
C_5 *Rana catesbeiana*	*12*	*12*	*12*	11	12	11	1	2	3 3	3	2	3	2	3	3	3 3	3	4
C_8 *Rana catesbeiana*	*8*	*8*	*8*	6	4	5	0	0	2	0	0	2	1	0	2	1	0	0
C_9 *Rana catesbeiana*	*10*	*11*	*11*	9	10	11	1	3	1	2	3	2	0	2	1	2	3	2

C_{16} *Rana pipiens*	8	7	6	*13*	*10*	*10*	2	0	3	3	2	3	0	0	0	0	1	0
C_6 *Amphiuma means*	3	3	3	0	0	0	*11* *10*	*11*	*11*	11	11	10	9	9	8	11 11	11	11
C_{10} *Amphiuma means*	3	3	3	2	1	3	*13* *11*	*12*	*12*	12	12	10	10	11	10	11	12	13
C_7 *Necturus maculosus*	2	1	1	2	2	3	10	9	10	*13*	*13*	*14*	10	11	10	13	13	13
C_{12} *Cryptobranchus alleganiensis*	3	3	4	2	3	3	8	9	8	11	11	11	*13*	*13*	*13*	7	5	5
C_{14} *Cryptobranchus alleganiensis*	3	2	3	5	2	4	7	4 5	5 3	9	9	9	*10*	*11*	*12*	4	5	5
C_{15} *Siren lacertina*	2	2	2	7	9	9	10	10	9	12	12	12	9	10	10	*13*	*14*	*13*

TABLE IV.—Results of the reciprocal tests given as per cent values corresponding to the titers of the reactions. The homologous reactions are recorded as 100 per cent and italicized

Antisera \ Antigens	*Rana catesbeiana* 7a	*Rana pipiens* a	*Amphiuma means* 5b	*Necturus maculosus* 1	*Cryptobranchus alleganiensis* A	*Siren lacertina*
C_4 *Rana catesbeiana*	*100*	62.5	0.33	0.17	0.15	0.39
C_5 *Rana catesbeiana*	*100*	66.7	0.13	0.23	0.16	0.24
C_8 *Rana catesbeiana*	*100*	14.6	0.52	0.52	0.78	0.26
C_9 *Rana catesbeiana*	*100*	70.0	0.23	0.31	0.12	0.31
C_{16} *Rana pipiens*	5.3	*100*	0.14	0.24	0.0	0.024

C_6 *Amphiuma means*	0.44	0.0	*100*	95.2	23.8	114.3
C_{10} *Amphiuma means*	0.17	0.10	*100*	66.7	29.6	103.7
C_7 *Necturus maculosus*	.024	0.05	7.8	*100*	12.5	75.0
C_{12} *Cryptobranchus alleganiensis*	0.13	0.08	4.16	25.0	*100*	0.008
C_{14} *Cryptobranchus alleganiensis*	0.17	0.45	1.13	13.3	*100*	0.007
C_{15} *Siren lacertina*	0.04	3.5	7.8	37.5	7.8	*100*

TABLE V.—Mean values of reciprocals and their probable errors

Rana catesbeiana × *Rana pipiens* = 43.82 ± 8.4
Rana catesbeiana × *Amphiuma means* = 0.30 ± .039
Rana catesbeiana × *Necturus maculosus* = 0.25 ± .050
Rana catesbeiana × *Cryptobranchus alleganiensis* = 0.25 ± .065
Rana catesbeiana × *Siren lacertina* = 0.249 ± .035
Rana pipiens × *Amphiuma means* = 0.121 ± .01
Rana pipiens × *Necturus maculosus* = 0.143 ± .045
Rana pipiens × *Cryptobranchus alleganiensis* = 0.266 ± .088[1]
Rana pipiens × *Siren lacertina* = 1.77 ± .82[2]
Amphiuma means × *Necturus maculosus* = 56.6 ± 14.2
Amphiuma means × *Cryptobranchus alleganiensis* = 14.67 ± 4.12
Amphiuma means × *Siren lacertina* = 75.3 ± 18.6
Necturus maculosus × *Cryptobranchus alleganiensis* = 16.94 ± 2.22
Necturus maculosus × *Siren lacertina* = 56.3 ± 8.9
Cryptobranchus alleganiensis × *Siren lacertina* = 2.61 ± 1.43

To express these quantitative measures of relationship graphically the following procedure is used (2). Since a high value for M means close relationship and this should be expressed graphically by a short distance between the species, it is a simple matter to take the value 100-M as the map or "tree" distance apart of the species. The values of 100-M for the species of Caudata tested reciprocally are given in Table VI.

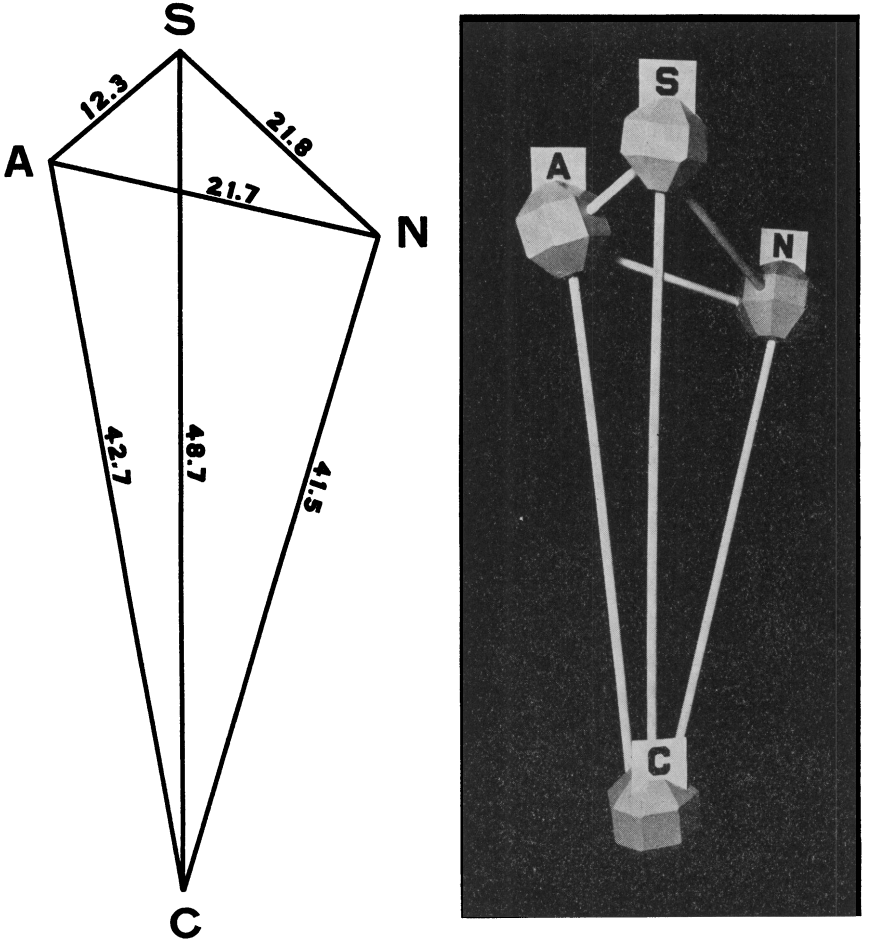
TABLE VI.—100-M values for the Caudata tested reciprocally

Amphiuma means vs. *Necturus maculosus*	43.4
Amphiuma means vs. *Cryptobranchus alleganiensis*	85.3
Amphiuma means vs. *Siren lacertina*	24.7
Necturus maculosus vs. *Cryptobranchus alleganiensis*	83.1
Necturus maculosus vs. *Siren lacertina*	43.7
Cryptobranchus alleganiensis vs. *Siren lacertina*	97.4

When it came to locating the loci of these species on a "tree" expressing their present relationships, it was found that the values would not fit on a plane surface without breaking them into segments, but that they would fit very well in three dimensions, as was the case in the Mammalia (2). Taking *Cryptobranchus*, the most primitive form among the Caudata tested, as the starting point, the other species are located with reference to *Cryptobranchus* and to each other by their corresponding 100-M values. (Actually the values $\frac{100\text{-}M}{2}$ in centimeters were taken, simply to give a figure of convenient size.) A projection on to a plane surface of the caudate loci, with their corresponding tree distances, is shown in Figure 1*a*, and an actual photograph of the model itself is

[1]Really not a reciprocal as the test can be calculated only one way.
[2]The *Siren* antiserum × *R. pipiens* antigen reaction was high, nearly as great as for the caudate antigens, but not so with *R. catesbeiana* antigen.

shown in Fig. 1*b*. These figures show, in a new way, the relationships of these Caudata. It may be of especial significance that the mathematical values for their interrelationship, based on quantitative precipitin tests, do fit together in a remarkable way. Furthermore, unless the relationship values are broken into segments, the old manner of expressing phylogenetic relationships on a plane surface will no longer suffice, for



a *b*

Fig. 1. The relationships of *Cryptobranchus* (C), *Amphiuma* (A), *Siren* (S), and *Necturus* (N).

a. Projection onto a plane surface of the model pictured in *b*. The numerical values are the actual dimensions of the model in cm. equivalent to the $\frac{100-M}{2}$ relationship values.

b. Photograph of a model of the phylogenetic polyhedron of these salamanders.

these values will fit together only in three dimensions. May it not be of some unusual meaning that these values require three dimensions for their expression? Real trees grow in three dimensions, and this new method of picturing relationships seems therefore more natural than previous methods. The fact that this method is applicable to Mammalia also (2) suggests that it may be of some general significance.

The data shown in Tables III, IV, and V then give us a mathematical expression of the degrees of similarity in the blood proteins of the species studied. Insofar as similarity in blood proteins means genetic relationship, they may serve as a measure of the degree of present relationships among these forms. Considering especially the per cent values of Table IV and the reciprocals in Table V, it is seen that the relationship between Caudata and Salientia is very distant. On the other hand the Caudata all show definite relationships among themselves, and so do the Salientia.

As to the relation between *Rana catesbeiana* and *Rana pipiens*, it is (by the precipitin test) less close than the relation between *Amphiuma* and *Necturus*, or between *Amphiuma* and *Siren*, or between *Necturus* and *Siren*. These facts suggest that these two *Rana* species are not so close as to justify their being placed in the same genus. But it must be admitted that at the present time we have no anatomical basis for isolating them in separate genera. On the other hand, Hadley (8) describes a marked incompatibility of the skins of *Rana pipiens* and *Rana clamitans* in grafts. So far as this goes it suggests a lack of close relationship between these two species. This is what would be expected on the basis of the precipitin tests which put *Rana clamitans* and *R. catesbeiana* close together and both fairly distant from *R. pipiens*, and therefore to some degree supports the serological findings.

Our main problem, however, was to determine the mutual relations of the salamanders, especially those of the genera *Siren* and *Necturus*, whose exact systematic position has been long in doubt. *Cryptobranchus* is now known to be a large hynobiid salamander that normally metamorphoses its skin (14), but retains most of the organization of a larval or partly metamorphosed form. Because of its hynobiid affinities it may be placed with confidence at the base of our phylogenetic tree. It is of great interest that *Siren*, *Necturus*, and *Amphiuma* stand closer to one another than any one of them does to *Cryptobranchus*. *Siren*, which the anatomical evidence suggested was perhaps equally near hynobiids and salamandrids, is shown by the serological tests to be more remotely related to *Cryptobranchus* than is either *Necturus* or *Amphiuma*. We shall refer to the matter again after considering the secondary data.

No attempt was made to locate the Salientia on the "tree" because of their remoteness from all the Caudata tested. Nor were there reciprocal data sufficient to justify making a tree for the Salientia alone. Further work is indicated for these Amphibia.

It should be emphasized that the phylogenetic polyhedron shown is not a simple guide to ancestry. It does express PRESENT RELATIONSHIPS. It also suggests ancestry insofar as the base-form, *Cryptobranchus*, is really primitive, but it should not be interpreted literally as meaning that *Amphiuma*, *Necturus*, and *Siren* diverged from the *Cryptobranchus* we know today exactly along the lines connecting the loci of these species.

THE SECONDARY DATA (NON-RECIPROCAL TESTS)

These data were obtained for the species in which the tests could be made in only one way, because of the lack of sufficient antigen to serve for antiserum production. The results therefore lack the double check available for the primary data (reciprocal tests) and are to be considered tentative only. They are shown in Table VII as tube numbers and as per cent values.

The results shown in Table VII may be summarized briefly, the average degree of resemblance between each species and all the others in the series being as follows:

Rana catesbeiana 100 per cent; *Rana clamitans* 151 per cent; *Hyla* 2.7 per cent. *R. pipiens* 100 per cent; *R. clamitans* 0.43 per cent; *Hyla*<0.57 per cent.

Amphiuma 100 per cent; *Triturus* 18.5 per cent; *Desmognathus* 7.3 per cent; *Gyrinophilus* 1.4 per cent; *Plethodon* 1.24 per cent; *Hyla* 1.1 per cent; *R. clamitans* 0.57 per cent; *Ambystoma* 0.43 per cent.

Necturus 100 per cent; *Triturus* 9.4 per cent; *Plethodon*, *Ambystoma*, and *Hyla* 0.88 per cent; *Gyrinophilus* 0.43 per cent; *R. clamitans* 0.29 per cent; *Desmognathus* 0.15 per cent.

Cryptobranchus 100 per cent; *Triturus* 4.1 per cent; *Plethodon* 2.4 per cent; *Hyla* 0.60 per cent; *R. clamitans* 0.25 per cent; *Gyrinophilus*, *Desmognathus*, and *Ambystoma* 0.17 per cent.

Siren 100 per cent; *Triturus* 7.0 per cent; *Ambystoma* 1.8 per cent; *Plethodon* 0.88 per cent; *Desmognathus*, *R. clamitans*, and *Hyla* 0.44 per cent; *Gyrinophilus* 0.40 per cent.

The results indicate tentatively that *Rana catesbeiana* stands very close to *R. clamitans*,[1] and quite a distance from *Hyla*. On the other hand, *R. pipiens* is rather distantly related to both *R. clamitans* and *Hyla*.

[1]The 240 per cent heterologous reaction between C_9 and *R. clamitans* serum is an extreme error. The average value of all the tests between these two species is 151 per cent, which represents more clearly the error expected in the readings. It may be that although *R. catesbeiana* and *R. clamitans* sera were equal in total protein, the latter contained a larger proportion of serologically active protein.

TABLE VII.—The results of the non-reciprocal tests, the titers being indicated by the corresponding tube numbers and per cent values. Readings were made in two series, *a* and *b*, during May, 1932

Antigens / Antisera	*Rana clamitans*		*Hyla septentrionalis*		*Ambystoma opacum*		*Gyrinophilus porphyriticus*		*Triturus viridescens*		*Desmognathus fuscus*		*Plethodon glutinosus*	
	Tube	%	Tube	%	Tube	%	Tube	%	Tube	%	Tube	%	Tube	%
C_4 *Rana catesbeiana*	*a* 12 *b* 13	112	8 8	4.7	—		—		—		—		—	
C_5 *Rana catesbeiana*	*a* 12 *b* 12	100	5 5	0.78	—		—		—		—		—	
C_9 *Rana catesbeiana*	*a* 12 *b* 12	240	5 5	1.9	—		—		—		—		—	
C_{16} *Rana pipiens*	*a* 3 *b* 4	0.43	<4	<0.57	—		—		—		—		—	

C_6	*a*	3		<4		1		3600		9		8		5		
Amphiuma			0.45		<0.90		0.17		0.80		28.6		14.3		1.8	
means	*b*	3		<4		2		3600		9		8		5		
C_{10}	*a*	5		6		5		28800		9		4		5		
Amphiuma			0.70		1.4		0.69		1.96		8.3		0.35		0.70	
means	*b*	5		6		5		14400		8		4		5		
C_7	*a*	5		7		6		7200		10		4		6		
Necturus			0.29		0.88		0.89	14400	0.43		9.4		0.15		0.88	
maculosus	*b*	5		6		7		14400		10		4		7		
C_{12}	*a*	4		6		4		7200		8		4		7		
Cryptobranchus			0.29		0.78		0.29		0.17		3.1		0.20		2.3	
alleganiensis	*b*	5		6		5		3600		8		4		8		
C_{14}	*a*	3		<4		0		<3600		7		2		6		
Cryptobranchus			0.21		<0.42		0.05		<0.38		5.0		0.14		2.5	
alleganiensis	*b*	3		<4		2				8		2		7		
C_{15}	*a*	5		5		7		7200		9		6		6		
Siren			0.44		0.44		1.76		0.39		7.0		0.44		0.88	
lacertina	*b*	6		6		8		14400		10		5		7		

As for the Caudata, *Necturus* is related to *Triturus* but distant from all the others; *Cryptobranchus* is quite distant from all of the Caudata tested; *Siren* is related to *Triturus* but distant from the others. These results on the whole are corroborative of the reciprocals in indicating that *Siren* stands fairly close to *Necturus* and *Amphiuma* but not to *Cryptobranchus*. *Triturus* seems to behave as a stem-form, bridging the gap between *Cryptobranchus* and the other species. *Triturus* is a primitive salamandrid and hence the serological tests lend strong support to one of the views expressed above, that on the basis of anatomical and life-history data both *Siren* and *Necturus* have sprung from a primitive salamandrid stock.

The reactions with the Salientia are all very weak and subject to great errors of reading, as are all weak reactions. The same is true for the Caudata with values under 2 per cent. Because of these greater errors and the lack of reciprocal checks, no significance is to be attached to the difference between the per cent values that are themselves under 2 per cent.

DISCUSSION

We have taken a problem in phylogeny that, though a considerable amount of anatomical and life-history data had been brought together, still remained unsolved. After examining the evidence both pro and con for the phylogenetic placing of the species, we have applied serological tests and have secured evidence of relationship to support one of the views. Neither *Siren* nor *Necturus* is a primitive salamander, but both are allied to *Amphiuma* and to *Triturus*. Very probably they represent an off-shoot from a salamandroid stock that made its way to America early in the Tertiary.

Nuttall showed conclusively that the precipitin reaction gave results that paralleled the systematic positions based on the older methods of phylogenetic investigation. With improvements in technique this test can now give a quantitative measure of present relationships, a measure whose reliability can be determined by the accepted biometric methods. We have then a means of studying present relationships independently of the older methods.

Already it appears that there are some important advantages in the serological method of attack. It is an objective test requiring a minimum of interpretation. It can become quantitative when carefully performed and its reliability determined by the usual methods. Moreover, there is the double check available in the reciprocal tests. Finally, it is likely

that the serological method is capable of differentiating forms whose structures are convergent, and of distinguishing at times between specialization toward simplicity in structure and real primitiveness.

It is interesting also that the data from this investigation support previous work which led to a new type of graphical expression of phylogenetic relationships: viz., a three-dimensional "tree" or phylogenetic polyhedron. Surely a family tree, like any other, should grow in three dimensions.

But this is just a beginning. Much additional work must be done before the final evaluation of the serological attack on problems of relationships and phylogeny can be made. In the case of *Siren* and *Necturus* it apparently has given us a satisfactory solution of a problem of long standing. In the case of *Rana*, further tests on some of the many other species in the genus are desirable before any final statement may be made as to number of subgroups that exist within this genus.

CONCLUSIONS

The results of 300 precipitin tests on the blood sera of thirteen species of Amphibia are recorded quantitatively. The data suggest the following conclusions.

1.—The serological relationships of Caudata and Salientia are relatively remote.

2.—Within the Salientia, *Rana catesbeiana* and *Rana clamitans* are very close together, while *Rana pipiens* is not very closely related to either. *Hyla septentrionalis* is remote from all the species of *Rana*.

3.—Within the Caudata, *Cryptobranchus*, an acknowledged primitive form, is not very closely related to *Siren*, nor to *Amphiuma* nor *Necturus*, the last three standing fairly close to each other.

4.—*Siren* and *Necturus* are related to *Triturus*. Apparently they both evolved from a salamandroid stock and not from the Hynobiidæ, as some anatomical and life-history evidence indicated.

5.—Three dimensions are required to express graphically the present per cent relationships of the Caudata tested (unless the per cent values are broken into segments).

6.—The principle of reciprocal relationships generally holds to the extent that the per cent values of relationship of any two species are of the same order of magnitude, whichever way the test is made.

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