III. Gamma Globulin (Serum)

100 μl of serum are added to a Unopette reservoir containing 3.0 ml of 15.5% sodium sulphate. It is allowed to incubate at 37° C for at least one hour, and then centrifuged at full speed for 10 minutes. The supernatant is discarded, touching off the last drop with tissue. 2.0 ml of Biuret reagent (or the contents of a #2710) are added, and mixed with the sedimented protein by shaking. After standing for 30 minutes, the Absorbance is read at 550 mμ. This is an adaptation of a method described by Caraway. (2)

IV. Fibrinogen (Plasma)

In our Unopette Manual of Clinical Laboratory Procedures (7) we have proposed two methods for plasma fibrinogen. One uses Parfentjev's reagent (9) in the reservoir, to which are added 0.25 ml of plasma. After five minutes, the solution is transferred to a cuvette and the Absorbance is read at 510 mμ. We are partial, however, to the fibrinogen method proposed by Stirland. (11) While Stirland originally specified 1% saline, other investigators (9) have modified his method to use 0.85% saline, provided the concentration is accurately made up. In the Unopette adaptation 0.25 ml of plasma are added to 2.6 ml of 0.85% saline (#2705), which is incubated at 56° C for fifteen minutes. The solution is then transferred to a cuvette, and the Absorbance is read at 650 mμ.

Summary

A new system for collection and dilution of fluids, and its application to biochemical analytical procedures is described. The components are precise, accurate, easy to operate, and disposable. Some typical adaptations are given.

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Some Precipitin Tests and Preliminary Remarks on the Systematic Relationships of Four South American Families of Frogs*

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The relation between serological affinities and genetic relationships is supported by many reports in recent years, above all by means of precipitin systems analysis which gives results of interest to the taxonomist. Even first approximation methods can be, in some cases, very useful because specific antigens appear to remain constant in ontogeny, once they are formed, and they are not likely to undergo remarkable morphological and adaptive differentiation. How much the serological properties of the plasmas can be phylogenetically conservative, extending over many branches or taxa, as orders, classes, or phyla, has been repeatedly pointed out (Wilhelmi 1942, 1944; Boyden 1953).

Relatively few studies have been made by precipitin tests on Batrachians, perhaps because of their difficult serological harvest, in terms of available serum samples. In the present work some general results of comparative serological observations on representatives of four South American families of Anurans are presented.

Methods and Materials

Sera of the following families and species, from adult native specimens, just captured, were utilized:

RANIDAE
- *Rana palmipes* Spix (Iquitos, Amazonia, Peru)
- *Rana pipiens* Schreber (Costa Rica, San Jose)

HYLIDAE
- *Hyla faber* Wied (Sao Paulo, Brazil)
- *Phyllomedusa sauvagii* Boulenger (Tucumán, Argentina)

LEPTODACTYLIDAE
- *Leptodactylus ocellatus* (Linné) (Mendoza, Argentina)
- *Pleurodema cinerea* (Cope) (Tucumán, Argentina)

BUFONIDAE
- *Bufo spinulosus* Weigmann (Mendoza, Argentina)

The blood, collected by cardiac puncture, was allowed to clot, and the expressed sera, filtered under sterile conditions, were kept in a refrigerator at 5°C. Antisera were obtained in the usual manner, by a simple injection series. A first dose containing 2 mg of protein per kg. body weight of the rabbit was followed after two weeks by a second equivalent injection. A third injection was given eight days later. The immunizing power of antigens was increased by adding to the serum an equivalent quantity of coadjuvant (Twen 80). The rabbit was completely bled eight days after the last dose injected. The antisera showed a good discriminating power and covered a sufficient range of reaction for comparative observations; they were Seitz filtered, stored in sterile vials, and maintained in refrigerator.

Titration of precipitin reactions was made by the classical quantitative Photronreflectometric technique, as reported in other works.

Antigen dilution was begun at 1:2.5 (Evans Buffer), because of the small quantity of protein in the serum. The prozone could be fully reached in many cases only with a more concentrated antigen, but, as shown by the graphs, the general comparative significance of the here-reported turbidity curves does not change fundamentally. Serological affinities due to common antigen properties are so indicated by the percentage value of the ratios between heterologous/homologous summated turbidities.

Results

Rana palmipes, the only known Ranid frog south of the Panamanian Isthmus, evidenced the greatest serological distance from almost all the neotropical Batrachians here considered (Table I). The percent average of its crossed tests was 17.9% with a range of 15.0%-19.9%. A test between *Rana palmipes* and *Rana pipiens* from Costa Rica (where it is sympatric with *palmipes*) gave a value of 51.0%. The only remarkably high test values lie between *Rana palmipes* and the peculiar South American genus *Phyllomedusa* (20.8%-35.0%).

Analyzing all the other tests, carried out between South American representatives of *Hylidae*, *Leptodactylidae*, and *Bufonidae*, the following summarized data can be expressed:

Between *Leptodactylus ocellatus*, *Pleurodema cinerea*, and *Bufo spinulosus* the average of the percent values is 36.0%, with a range of 31.2%-40.2%.

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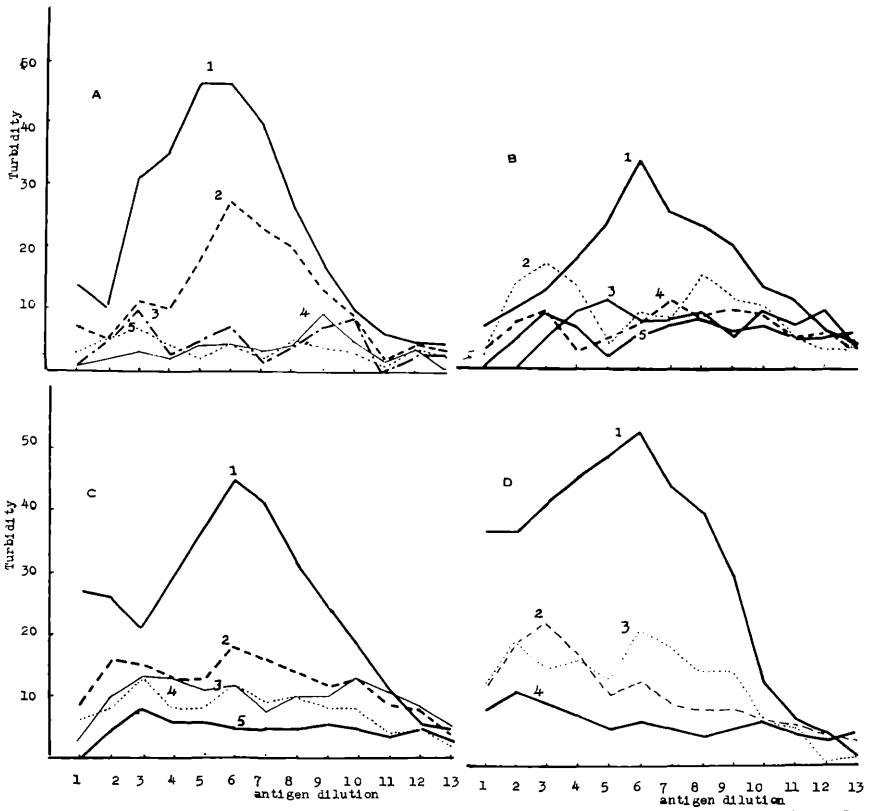

Figure A. The relationships between South American families of frogs and toads with anti-*Rana palmipes* serum made by single injection series (coadj.: Twen 80).

Antiserum		*Antigen*		*Curve No.*	*% Area*
anti-*Rana palmipes*					
CB-22	X	*Rana palmipes*	A-0190	1	100.0
		Rana pipiens	A-0195	2	51.0
		Leptodactylus ocellatus	A-0134	3	19.8
		Hyla faber	A-0125	4	15.0
		Bufo spinulosus	A-0163	5	15.7

Figure B. The relationships between South American families of frogs and toads with anti-*Phyllomedusa sauvagii* serum made by single injection series (coadj.: Twen 80).

Antiserum		*Antigen*		*Curve No.*	*% Area*
anti-*Phyllomedusa sauvagii*					
CB-21	X	*Phyllomedusa sauvagii*	A-0170	1	100.0
		Bufo spinulosus	A-0163	2	54.8
		Hyla faber	A-0125	3	39.9
		Leptodactylus ocellatus	A-0134	4	40.3
		Rana palmipes	A-0190	5	35.0

Figure C. The relationships between South American families of frogs and toads with anti-*Bufo spinulosus* serum made by single injection series (coadj.: Twen 80).

Antiserum		*Antigen*		*Curve No.*	*% Area*
anti-*Bufo spinulosus*					
CB-2	X	*Bufo spinulosus*	A-0163	1	100.0
		Phyllomedusa sauvagii	A-0170	2	49.5
		Pleurodema cinerea	A-0168	3	40.2
		Leptodactylus ocellatus	A-0134	4	31.2
		Rana palmipes	A-0190	5	19.5

Figure D. The relationships between South American families of frogs and toads with anti-*Leptodactylus ocellatus* serum made by single injection series (coadj.: Twen 80).

Antiserum		*Antigen*		*Curve No.*	*% Area*
anti-*Leptodactylus ocellatus*					
CB-9	X	*Leptodactylus ocellatus*	A-0134	1	100.0
		Phyllomedusa sauvagii	A-0170	2	33.4
		Bufo spinulosus	A-0163	3	36.8
		Rana palmipes	A-0190	4	19.9

Between *Leptodactylus ocellatus*, *Bufo spinulosus*, and *Phyllomedusa sauvagii* the average of the percent values increases to 44.5% (range 33.4%-54%).

The only reading made between *Phyllomedusa sauvagii* and *Hyla faber* gives a percentage of 39.9%.

The graphs of Figures A, B, C, D show characteristic precipitin curves plotted to express the above-mentioned homologous/heterologous reactions.

Discussion

Phyletic divergence of the Ranid diplasiocoel stock from the procoels South American families is easily pointed out by this first attempt at a serological approach to some of their representatives. A general morphotaxonomic analysis can be thus supported by a preliminary serological study. The only relatively high precipitin reaction was given with *Rana palmipes* by an anti-*Phyllomedusa* serum (CB-21). Some remarks on this peculiar neotropical hylid group shall be presented later.

Relationships between the tested *Bufonidae* and *Leptodactylidae* seem to be at the same level as the relationships between both these families and *Phyllomedusa*. and also between *Phyllomedusa* and *Hyla*. In the present, and other not yet published, studies we observed that with the standardized kind of antisera used in our tests, percent areas above 40%-50% generally belong to the inter-specific precipitin reactions; relationships between genera and close-related families being normally expressed by 20%-40% values. While the values of reciprocal tests between *Leptodactylus* or *Pleurodema* and *Bufo* (as in other unpublished data) range from 31.2% to 40.2%, the values of the tests between *Leptodactylus*. *Bufo*. and *Phyllomedusa* also spread out from 33.4% to 54.8%, the last value (anti-*Phyllomedusa* serum, CB-21, per *Bufo spinulosus*) being strikingly the highest obtained also in comparison with the *Bufo* × *Leptodactylus* reactions. The low turbidity percent (39.9%) registered in the *Phyllomedusa* × *Hyla faber* reaction, must be pointed out. Thus my tentative approach to a first "two-dimensional" approximation, as in Boyden's definition (1962), for a serological comparison between typical elements of the neotropical batrachofauna, calls attention to the remarkable position of the *Phyllomedusa* "hylid" stock.

Serological affinities between *Phyllomedusa* and *Bufo* were strengthened by these preliminary precipitin tests. This finding needs, without any doubt, a further examination. The systematic and phyletic position of the *Phyllomedusa-Agalychnis* neotropical group, as pointed out in her review by Funkhouser (1957), is based on the state-

Table I

Antiserum	Kind	Homolog. area	Heterol. area	Kind	Percent Heterol. area / Homol. area
CB-22	*Rana palmipes* Amazonia	292	149	*Rana pipiens* Costa Rica—0195	51.0
" "	"	292	46	*Bufo spinulosus* Mendoza—0163	15.7
" "	"	292	58	*Leptodactylus ocellatus* Mendoza—0134	19.8
" "	"	292	61	*Phyllomedusa sauvagii* Tucuman—0170	20.8
" "	"	292	44	*Hyla faber* Brazil—0125	15.0
CB-9	*Leptodactylus ocellatus* Mendoza	451	90	*Rana palmipes* Amazonia—0190	19.9
" "	"	451	166	*Bufo spinulosus* Mendoza—0163	36.8
" "	"	451	151	*Phyllomedusa sauvagii* Tucuman—0170	33.4
CB-2	*Bufo spinulosus* Mendoza	323	63	*Rana palmipes* Amazonia—0190	19.5
" "	"	323	101	*Leptodactylus ocellatus* Mendoza—0134	31.2
" "	"	323	130	*Pleurodema cinerea* Tucuman—0168	40.2
" "	"	323	160	*Phyllomedusa sauvagii* Tucuman—0170	49.5
CB-21	*Phyllomedusa sauvagii* Tucuman	208	73	*Rana palmipes* Amazonia—0190	35.0
" "	"	208	83	*Hyla faber* Brazil—0125	39.9
" "	"	208	84	*Leptodactylus ocellatus* Mendoza—0134	40.3
" "	"	208	114	*Bufo spinulosus* Mendoza—0163	54.8

ment of Noble (1931), who considers that these genera arose from *Hyla* through the less specialized *Agalychnis* types.

As properly stated by Funkhouser, the knowledge of these frogs is incomplete and it is very difficult to establish the exact course of phylogenesis, the original links being long since gone. It is also correct to say that the characters used to differentiate them are surely only a fraction of those available. Phyllomedusids are undoubtedly related with Hylids, but to what extent they could have arisen from hypothetical *Hyla* forms is now difficult to establish. Resemblances in breeding habits could be adaptive trends with convergence.

If Phyllomedusids are a proper phyletic branch arising from some undifferentiated Hylid-Bufonid stock, to be erected now as a proper family intermediate between *Hylidae* and *Bufonidae*, as *Pseudidae* Savage and Carvalho (1953) lie between *Hylidae* and *Leptodactylidae*, is a question that cannot be solved at the level of the present non-morphological observations. But in calling attention to the real herpetological interest of the problem I wish to add that recent researches (Erspamer, Bertaccini, and Cei 1962; Erspamer and Cei 1963) demonstrate the existence of characteristic specific patterns of bradykinin-like polypeptides in the skin of all the studied *Phyllomedusa* forms *(moreleti-rohdei-sauvagii-hypochondrialis)*.

This biochemical category of functional substances is lacking in all the actually studied Hylid representatives *(Hyla, Sphoenorynchus, Trachycephalus, Corythomantis, Osteocephalus, Gastrotheca,* and *Phrynohyas)*, but it is quite similar to the "physalaemin" polypeptides, also found in some specialized leptodactylid genera *(Physalaemus, Eupemphix)*, and in all the yet examined *Rana* species, including *Rana palmipes* and *Rana pipiens* from the neotropical realm.

Summary

Phyletic divergences between neotropical Ranids and other South American Anurans *(Leptodactylidae, Hylidae, Bufonidae)* were indicated by precipitin tests. Remarkable serological affinities between *Phyllomedusa* and Bufonids and Leptodactylids were discovered. The results warrant further studies on the problem of relationships between the true Hylids and the very specialized forms of the neotropical *Phyllomedusa-Agalychnis* stock.

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Electrophoretic Patterns and Systematic Relations in South American Toads[1]

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The utility of paper electrophoresis as a discriminating method in systematic studies was pointed out in the papers by Dessauer and Fox (6) and Dessauer, Fox, and Ramirez (7). It may be useless or play only a secondary role in studies of species from different phylogenetic stocks. Many recent studies support its value in taxonomic studies of closely related forms, especially those in their incipient stages of speciation.

Since 1959 our interest has been addressed to the study and control of the specificity and characteristics of the seroproteinic patterns of neotropical batrachians belonging to the local bufonid and leptodactylid stocks. We have observed a remarkable and specific constancy in their electrophoretic patterns, under standard conditions of comparison (*i.e.* Buffer pH

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