TABLE I. 6-Mercaptopurine Dosage and Homograft Survival in Young Male Rats.

Drug dosage (3× per wk), mg/kg	No. of rats	Inj. period in days before grafting	Graft survival in days
Controls	20		11 (10–16)*
20	24	16 (10– 18)*	11 (10–15)
30	42	121 (119–125)	12 (10–16)
40	39	140 (88–174)	12 (10–16)
60	14	29 —	12 (10–16)
80	14	30 —	13 (11–17)
100	14	17 —	12 (10–16)
150	16	10 —	16 (11–39)
200	16	10 —	15 (11–23)
250	31	5 (3–6)	16 (11–46)
300	15	4 —	15 (11–25)
350	16	0 0	— —

* Mean and (range).

350 mg/kg 3 times per week. Full thickness, suprapannicular skin grafts, 1.2 cm in diameter, were taken with a skin punch especially adapted for rats(6). Duration of skin homograft survival was determined by its gross and microscopic appearance. Onset of the homograft reaction was detectable by the appearance of edema, slight dessication with brownish discoloration and diminution of graft size. Shrinkage of the graft without edema or dessication was usually associated with mild reactions and required several days for completion.

Results. The longest mean prolongation of homograft survival beyond that of the control grafts was only 5 days. Neither long term administration nor high doses of 6-MP were effective in suppressing the homograft reaction. Rats are remarkably tolerant to 6-MP given for long periods or in high dosages. To exceed this tolerance required a progressive increase in dosage of 6-MP to 350 mg/kg 3 times a week. At this dosage mean survival of the animals was only 5 days, less than the 11-day survival of the control grafts. The animals receiving 300 mg/kg of 6-MP 3 times per week survived long enough to permit evaluation of the homografts (Table I).

Conclusion and summary. There was an apparent, brief prolongation of the mean skin homograft survival in 241 rats which were given 6-MP. However, neither long term administration nor high doses of this drug consistently prolonged survival time. A few of the treated animals in the higher dosage groups had unusually long graft survivals which accounted for the over-all increase of mean graft survival. Most of the animals in all groups sloughed off their grafts at or near the expected time.

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Synthesis and Electrophoretic Distribution of Antibodies in the Amphibian, *Bufo Marinus.** (26538)

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In spite of considerable research on tissue transplantation in the amphibia(1), there is relatively little published information on agglutinating and precipitating antibody in this class or in fishes and reptiles. From a phylogenetic standpoint this is unfortunate because a comparative study might yield much information. Recent work(2) has shown that the desert lizard, *Dipsosaurus dorsalis* can synthesize agglutinating antibodies for *Salmon-*

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ella typhosa and Bisset(3-5) and Cushing(6) demonstrated that frogs and fish can produce agglutinins. Since the lower vertebrates are poikilothermic, they need to be maintained within the temperature range at which they are normally active, to obtain optimal antibody formation. In Bisset's work, for instance, frogs produced antibody when kept at 20°C but not at 8°C.

Although there have been several reports on electrophoretic separation of serum proteins of the lower vertebrate classes(7-15) there is need for further studies. Some of the serum patterns described appear to differ from the normal electrophoretic distribution of serum proteins in man and other mammals. These differences may prove to have phylogenetic significance. It will also be important to determine the location of antibodies in the serum fractions of the lower vertebrates.

In our investigation, we found that the toad *Bufo marinus* would synthesize precipitating antibody to rabbit gamma globulin and agglutinating antibody to *Salmonella typhosa* after a relatively short period of immunization. Details of the methods for production and measurement of antibodies together with a study of the electrophoretic distribution of agglutinins in 10 different antiserums are reported.

Materials and methods. To prepare the antigen used for immunization, a solution of rabbit gamma globulin (RGG) was mixed with *Salmonella typhosa* H vaccine. The RGG (Pentex Co.) was dissolved in saline (20 mg ml) and the *S. typhosa* H antigen(2) diluted to a density of 10% transmittance at 540 mμ. Shortly before use the two were mixed in equal proportions.

On the first day of immunization, 10 large (3½ inch to 5 inch) adult *Bufo marinus* were injected with 0.2 ml of antigen. On the second and third days, they received 0.6 ml. After a 4-day rest period, 3 more injections of 0.6 ml each were given on consecutive days. All injections were made with a 23 gauge needle into the subcutaneous space of the sacral region. These animals plus 10 additional non-immunized controls were maintained in covered jars containing a layer of damp sphagnum moss. Ambient temperature was maintained at 25°C ± 1°. All animals were fed, *ad lib.*, on Tenebrio larvae. Seven days after the last injection the animals were bled by cardiac puncture and the blood allowed to clot. The serum was harvested and used to determine the titer of agglutinating antibody for *S. typhosa* H antigen as in(2). Precipitins were measured by layering various dilutions of the RGG antigen over undiluted antiserum in capillary tubes.

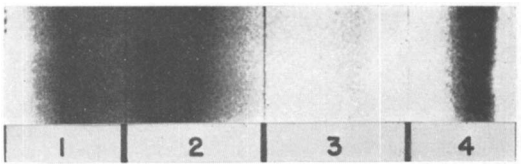

FIG. 1. Paper electrophoresis strip stained with bromphenol blue to show the 4 major fractions of serum from *B. marinus*. Point of origin was near center of fraction 1.

To determine which electrophoretic fraction contained the antibody, the serums were analyzed in a Beckman Spinco electrophoresis cell with a Duostat power supply and B-2 veronal buffer, pH 8.6. Amperage was held constant at 2.5 milliamperes (8 strips). After preliminary determinations had disclosed that there were 4 well-defined components in the serum of *B. marinus* (Fig. 1), each antiserum was fractionated in the following way:

Each of 7 filter paper strips (Spinco no. 300-028) was spotted with 40 μl of serum (4 × 10 μl) and placed in the Spinco cell. Strip no. 8 was also inserted, but was left blank since the serum fractions tended to migrate more slowly on this strip. After 24 to 32 hours of migration, the strips were removed and one strip was quickly dried with a hair dryer and rapidly transferred through methanol, bromphenol blue solution and 5% acetic acid to develop the protein bands. While still wet, the remaining 6 strips were cut along lines between each fraction (Fig. 1) using the stained strip as a guide. The 6 pieces of filter paper from Fraction no. 1 were placed in a 40 ml centrifuge tube and macerated in 15 ml of saline solution at 37°C. This process was repeated with each of the other fractions. After 30 minutes incubation, the tubes were centrifuged, the supernate was

TABLE I. Results of Precipitation and Agglutination Tests on Serums of 10 Non-immunized and 10 Immunized *Bufo marinus*.

No. of animals with:	Group (10 animals each) Immunized	Non-immunized
Precipitating antibody (RGG)	10/10*	0/10
Agglutinating antibody (Typhoid H)	10/10	1/10
Titers: 1:10	0/10	1/10
1:320	1/10	0/10
1:640	4/10	0/10
1:1280	5/10	0/10

* No. positive/total.

saved and the pulp again rinsed with 15 ml of saline. The rinsing was repeated with 10 ml of saline, and the 3 eluates combined to give a total volume of 40 ml for each fraction. This eluate was dialyzed for 24 hours against 0.1% sodium chloride, then reduced to 1/10 volume by perevaporation before an electric fan at room temperature. This fluid was adjusted to 1:20 in terms of the 240 μl of serum applied to the 6 strips and used to set up agglutination tests as in(2). When precipitin tests with RGG were found to be negative at a 1:20 dilution of serum, the fractions were again concentrated by a similar procedure to equal a dilution of 1:2 in terms of the whole serum.

Results. Agglutination tests with *S. typhosa* H antigen failed to reveal any agglutinins in the non-immunized group except for one animal which had a titer of 1:10. None of these serums precipitated with RGG (Table I).

In the immunized group, *S. typhosa* H titers ranged from 1:320 to 1:1280 and all serums produced positive ring precipitin tests with RGG. Although not shown in the table, positive ring tests were obtained with the following concentrations of RGG: 10, 1, and 0.1 mg/ml. Agglutination tests were read after 2 hours incubation in a 37°C water bath and ring tests after 2 hours at room temperature.

When the 4 major electrophoretic fractions (Fig. 1) were eluted from paper strips, the dilution of each in terms of original serum was 1:20. When this was diluted with an equal volume of H antigen in the agglutination test, the dilution was 1:40. All fractions except no. 1 yielded titers of less than 1:40, but fraction no. 1 from each serum agglutinated strongly with titers ranging from 1:160 to 1:640 (Table II). In the case of 2 serums these titers equalled those of the unfractionated serum and in the others were one or 2 tubes lower. When serum from non-immunized animals was separated electrophoretically and eluted by the same process, no agglutinins were found in any fraction.

Despite repeated attempts to demonstrate precipitating antibody in one of the 4 fractions, results were negative even when the serum was concentrated to equal a dilution of 1:2 of the original serum. Further work has been hampered by the small volume of serum available from each animal, but additional studies are contemplated using immunoelectrophoresis and agglutination of RGG-sensitized erythrocytes.

Discussion. Although there is relatively little experimental work on comparative immunology of the lower vertebrate classes the available data suggest that reptiles, amphibia and fishes can form antibodies of the sort that have been widely investigated in mammals and certain birds. The results presented here are in accord with this viewpoint. *B. marinus* was shown to produce typhoid H agglutinins as well as precipitating antibody for rabbit γ-globulin, and the agglutinin was found in the slowest-moving of the 4 major serum components. On the basis of its electrophoretic mobility, this component would be consid-

TABLE II. Reciprocals of *S. typhosa* H Agglutinin Titers with Eluates from Paper Electrophoresis Strips.

Animal No.	Titer of whole serum	Major electrophoretic fraction 1	2	3	4
21	640	320*	<40	<40	<40
22	1280	640	<40	<40	<40
23	1280	640	<40	<40	<40
24	1280	320	<40	<40	<40
25	1280	320	<40	<40	<40
26	640	640	<40	<40	<40
27	320	320	<40	<40	<40
28	640	320	<40	<40	<40
29	640	160	<40	<40	<40
30	1280	640	<40	<40	<40

* Dilutions are expressed in terms of volume of whole serum applied to strips.

ered analogous to the gamma globulin fraction of mammals.

Additional work is required to assess the full phylogenetic significance of these results. It will be of interest to ascertain whether a similar electrophoretic distribution of antibodies is found in the serum of other lower vertebrates and in animals immunized to antigens other than those used here.

Summary. Adult specimens of *B. marinus* maintained at 25°C were immunized with a mixture of *S. typhosa* H antigen and rabbit gamma globulin (RGG). Each animal produced precipitins for the RGG and agglutinins for typhoid H after a short period of immunization. When the antiserums were separated electrophoretically, the agglutinins were found in the slowest-moving of the 4 major serum components. On the basis of electrophoretic mobility, this fraction is analogous to the gamma globulin of mammals.

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Endogenous Cathepsin Activation of Trypsinogen in Extracts of Dog Pancreas.* (26539)

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The pancreas and pancreatic juice normally contain negligible amounts of active trypsin (1). Trypsin, in the form of its zymogen trypsinogen, is secreted into the intestine where the activation of trypsinogen to trypsin is catalyzed by the enzyme enterokinase. Once formed, trypsin may also catalyze the formation of trypsin from trypsinogen. The mechanism by which the extracellular enzymes enterokinase and trypsin activate trypsinogen has been established as the hydrolytic cleavage of a hexapeptide from the amino terminal portion of the trypsinogen molecule(2,3). The only intracellular mammalian enzyme that has been demonstrated to activate trypsinogen is the partially purified enzyme cathepsin B(4,5). Our previous *in vitro* studies have shown that beef spleen cathepsin B purified 200 fold, catalyzes the activation of crystalline trypsinogen to trypsin (or a trypsin-like enzyme) as determined by the action of the active product against synthetic peptide substrates and its inhibition by trypsin-soy bean inhibitor(4,5). These studies demonstrated that optimum pH for this activation occurred at pH 3.6, a pH at which no trypsin activation of trypsinogen occurs. It was further demonstrated that addition of .001 M iodoacetic acid (a known inhibitor of cathepsin B) or omission of cysteine (a known activator of cathepsin B) inhibited the reaction.

In view of the intracellular location of cathepsin B(6), it was of interest to deter-

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