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Received November 29, 1965. P.S.E.B.M., 1966, v121.

Comparative Immunology. Hemolytic Complement in Amphibia.* (30994)

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The participation of complement in immune reactions results from the sequential interaction of a number of components. Complement activity has been studied and characterized in a variety of mammalian and avian systems, but detailed evidence for its presence in lower vertebrates has been fragmentary. A notable exception was the work of Cushing(1,2) which described the hemolytic complement of the carp and bullfrog in some detail, and included a comprehensive review of the earlier literature. More recently, an investigation of complement components of certain bony fish, elasmobranchs and cyclostomes has been reported(3).

The present study was undertaken to investigate various aspects of the phylogeny of the complement system as observed in representative species of amphibia. Certain physical and chemical properties of these amphibian sera were compared with those of a reference guinea pig complement system.

Materials and methods. Three species of amphibia were studied: *Bufo marinus* (marine toad), *Rana pipiens* (leopard frog), and *Necturus maculosus* (mud puppy). Ten specimens from each group were maintained for a minimum of 7 days prior to bleeding in a 25°C controlled temperature environment. Bleeding was accomplished by cardiac puncture, and the individual samples were allowed to clot for one hour at room temperature, followed by two hours at 10°C. After centrifugation at 0-5°C, the sera were maintained in an ice bath. All quantitative procedures were initiated within 4 hours of time of bleeding, since it has been observed that a marked lability to storage, even at low temperatures, characterizes certain of these lower vertebrate sera.

Quantitative estimates of complement levels were determined graphically by plotting the C'H50 for each individual serum, as well as for pooled sera, utilizing the standard technique outlined in Kabat and Mayer(4). The standardized erythrocyte suspension was optimally sensitized with an appropriate dilution of rabbit antibody, and optical densities of the cell lysates and controls were recorded in a Beckman DU spectrophotometer at a wave length of 541 mμ. Duplicate dilutions were carried out for test samples as well as positive and negative controls, using pH 7.4 isotonic veronal buffer containing 0.00015 M calcium ions and 0.005 M magnesium ions. Samples were incubated for one hour at 30°C, followed by one hour at 35°C. This incubation procedure was found to be optimal for amphibian sera, and was used for all experiments unless otherwise noted.

Qualitative assays for natural hemolytic activity were performed with washed erythrocyte suspensions from a variety of species without addition of heterologous antibody. Degree of hemolysis was scored visually. Each tube contained: 0.1 ml serum dilution, 0.1 ml erythrocyte suspension in a concentra-

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* Supported by USPHS Service Grant AI-02693.
† Conducted during tenure of a fellowship on Grant DE-7 from Nat. Inst. of Dental Research.

TABLE I. Natural Hemolytic Titers of Pooled Amphibian Sera.*

Species of amphibia	Species of erythrocyte in hemolytic system							
	Sheep	Rabbit	Dog	Human A	Chicken	Duck	Turtle	Goldfish
Bufo marinus	8	32	0	8	32	0	0	32
Rana pipiens	32	32	0	8	32	0	16	32
Necturus maculosus	8	64	16	32	16	16	32	64
Saline control	0	0	0	0	0	0	0	0

* Values expressed in reciprocal of final dilution of serum.

tion of approximately 4 × 10^8 erythrocytes/ml, and buffer to yield a total volume of 0.4 ml. Variations from this scheme substituted 0.1 ml dilution of rabbit antibody, turtle antibody, or Carbowax 4000 for 0.1 ml of buffer in order to evaluate the reactivity of amphibian sera with heterologous sensitizers.

Inactivation procedures involved treatment of pooled sera with heat, ethylene-diamine tetraacetic acid (EDTA), hydrazine, carrageenin, and preformed antigen-antibody precipitate. The effect of heat was determined by exposure of a series of undiluted serum samples to various incubator temperatures for a period of 20 minutes. A 1:2 dilution of each heated sample was then reacted in a qualitative hemolytic system to determine the degree of inactivation. Since guinea pig complement is inactivated by $-NH_2$ groups(5,6), one ml aliquots of serum from each species were incubated with 0.2 ml of 0.1 M hydrazine to determine whether a similar effect would result with amphibian serum. A 90-minute incubation at 34°C was found to be optimal for this procedure. Carrageenin, a sulfated polysaccharide, was recently shown to be an inhibitor of guinea pig complement activity through inactivation of the C'_1 component(7). The effect of carrageenin was studied on samples of amphibian sera, using previously described procedures(7).

In the experiment employing EDTA, all dilutions were carried out in buffer free of divalent cations. The reaction tubes contained EDTA in a final dilution of 0.5 mM with other reactants of the hemolytic system added to yield final volume of 5 ml. After 30 minutes' incubation at 35°C, during which all positive controls exhibited complete hemolysis, a solution containing 0.62 mM each of calcium and magnesium ions was added to the EDTA-containing tubes. A second series of EDTA-containing tubes was included as a control to which no cations were added.

The decomplementation procedure employed a saline suspension of ovalbumin-rabbit antiovalbumin precipitate, prepared at equivalence, containing 160 μg N per ml. One ml of this suspension was incubated with one ml of test serum for 2 hours at 30°C with constant stirring. A 1:2 control dilution of serum was similarly treated. At the end of the incubation period the precipitate was removed by centrifugation, and the hemolytic activity of the test sample compared to that of the control by quantitative photometric methods.

Results. As indicated in Table I, serum from each of the amphibia exerted a natural hemolytic effect on erythrocytes from a broad spectrum of foreign species. This effect was not uniformly directed against all cell systems. *B. marinus* and *R. pipiens* serum, for example, exerted no hemolytic effect on dog or duck erythrocytes, but readily hemolyzed rabbit and goldfish red cells. Table II shows potentiation of the natural hemolytic activity of amphibian sera by both specific and nonspecific sensitizers. Data reported in Table II reflect activities of different serum pools from those used in Table I, which explains the difference in lysis of sheep erythrocytes by normal *B. marinus* serum.

TABLE II. Hemolytic Titers of Pooled Amphibian Sera with Addition of Heterologous Sensitizers.*

Species of amphibia	No sensitizer	Rabbit Ab	Turtle Ab	Carbowax
Bufo marinus	32	128	32	64
Rana pipiens	32	256	64	64
Necturus maculosus	8	16	32	8
Saline control	0	0	0	0

* Sheep erythrocyte hemolytic system. Values expressed in reciprocal of final dilution of serum.

TABLE III. Quantitative Complement Levels Expressed in C'H50 Units.

Species of amphibia	No. in group	Avg wt (g)	C'H50 units Mean*	 Range	 S.D.†	 Pooled serum
			(a)	(b)	(c)	(d)
Bufo marinus	10	134	13	2.0-24	6.9	13
Rana pipiens	10	82	44	26 -89	17	40
Necturus maculosus	10	149	21	17 -27	3.7	21

* Arithmetic mean of individual titers.

† S.D. = standard deviation.

TABLE IV. Effects of Inactivation Procedures on Amphibian Sera.

Species	Positive control	(a) 0.005 M EDTA added	(b) 0.005 M EDTA + 0.0062 M Ca & Mg	(c) Carrageenin	(d) Hydrazine	(e) Heat inactivation*	(d) + (e)†
B. marinus	++++	—	++++	—	—	48	+++
R. pipiens	++++	—	++++	++++	—	48	++++
N. maculosus	++++	—	+++	+	—	45	++
Guinea pig	++++	—	++++	—	—	(56)	+++

++++ indicates maximal hemolysis, — indicates no hemolysis.

* Minimum inactivation temperature in °C at 20 min incubation time as determined by series of incubations at varying temperatures. Standard 56°C temperature used for guinea pig serum.

† Incubation of heat-inactivated serum with hydrazine-inactivated serum.

Values obtained from quantitative complement titrations are expressed in Table III. The *R. pipiens* group possessed the highest mean complement titer of 44 C'H50 units/ml, with one specimen exhibiting a maximum titer of 89 C'H50 units/ml. Ranges and standard deviations within the *R. pipiens* and *B. marinus* groups were large, but individual titers within the *N. maculosus* group were oriented closely around the mean of 21 C'H50 units/ml. The *B. marinus* group possessed the lowest mean titer of 13 C'H50 units/ml, and also the lowest individual titer recorded of 2 C'H50 units/ml. For reference purposes, titrations were done on pooled human and pooled guinea pig sera using the same hemolytic system and plotting procedures, but with incubation at 37°C. Values so obtained of 36 C'H50 units/ml for human serum, and 264 C'H50 units/ml for guinea pig serum compare favorably with those reported in the literature(8).

Inactivation procedures, as summarized in Table IV, indicate that amphibian complements react similarly to guinea pig complement. Inactivation by heat shows heat labile components with critical temperatures lower than those required for mammalian sera. *N. maculosus* serum, deactivated after 20 minutes' incubation at 45°C, was most labile to heat. Incubation temperatures used throughout this study were lower than the customary 37°C in order to minimize destruction of complement components. Inactivation of amphibian sera by EDTA and subsequent reactivation by calcium and magnesium ions suggest a dependency on divalent cations identical to that of mammalian systems(9). Carrageenin effectively inhibited the hemolytic reaction in all sera except *R. pipiens*. Hydrazine treatment produced a hemolytically inactive serum in all instances, presumably by inactivation of the C'4 and C'3 components(5,6). When combined with heat inactivated serum which furnished these components, reactivity of hydrazine-treated serum was restored.

Fig. 1 illustrates the successful decomplementation of *B. marinus, R. pipiens,* and control guinea pig sera by exposure to antigen-antibody precipitate. This was somewhat predictable since these sera were observed to react with rabbit antibody in the hemolytic system. There was a total lack of fixation of *N. maculosus* complement by the ovalbumin-antiovalbumin system.

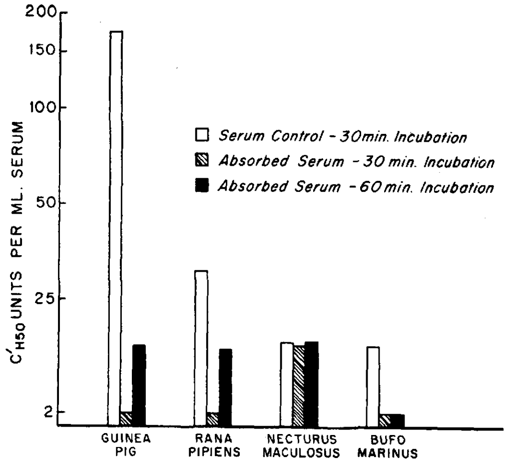

FIG. 1. Decomplementation of sera by exposure to antigen-antibody precipitate.

Discussion. The natural lytic action of amphibian sera on heterologous erythrocytes could be due to non-specific lysins as described in the lamprey(3), or related to specific "natural antibody"-complement systems. The evidence presented here supports the assumption that lysis in each case was due to a complement dependent antibody system. The varied hemolytic effect (Table I), dependent in degree on the species of foreign erythrocyte, also pointed toward such a specific system. Further evidence in this regard was indicated in Table II by the potentiation of natural hemolysis with the non-specific erythrocyte sensitizer, Carbowax 4000, or by addition of heterologous antibody. Significantly, serum from each of the 3 species was capable of reaction with heterologous antibody.

The traditional sheep erythrocyte-rabbit antibody system was used for quantitative titrations reported in Table III, in order to afford a more meaningful comparison with reported values for other species. Evidence obtained in this laboratory suggested that the use of a rabbit erythrocyte system, or use of a buffer of varied ionic strength and pH, may have produced higher complement titers. Comparative interpretation under such conditions would have been more difficult. Results of the quantitative determinations showed each of the 3 species to possess measurable complement levels comparable to those reported for many mammalian sera(8); although none possessed titers approaching that of guinea pig controls. The low complement titers for the *B. marinus* group might reflect the fact that animals were maintained at a constant temperature of 25°C. Variations in the rate of antibody synthesis at different temperatures have been reported for *B. marinus*(10-12); however, the effect of body temperature on complement levels remains to be investigated.

Inactivation of amphibian complements by standard procedures further suggests component structures similar to that of guinea pig complement. The exception was the failure of carrageenin, in any of 3 dilutions, to inhibit the activity of *R. pipiens* serum. This may indicate some variation in the sub-structure of the C'_1 component. Failure of fixation of *N. maculosus* complement may be partially explained on the basis of a difference in species specificity. As previously noted, there was minimum potentiation of the natural hemolytic activity of *N. maculosus* serum by addition of rabbit antibody. It has been shown that the ability of an antibody to bind a heterologous complement is influenced by the structure of the antigen (13). Poorer binding, in this case, results with rabbit antibodies directed against ovalbumin, as compared to those against sheep erythrocytes. Decomplementation of *B. marinus* and *R. pipiens* sera was achieved in varying degree, and their reaction to this procedure may be equated to that of the control guinea pig serum.

Summary. Representative specimens from 3 orders of amphibia were investigated for the presence of serum complement, using the hemolytic assay system as an indicator. All 3 groups exhibited hemolytic activity capable of potentiation by heterologous antibody, with complement levels within the range of those reported for mammalian species. With certain exceptions, amphibian complement systems appeared comparable to guinea pig complement in their reactions to heat, EDTA, hydrazine, carrageenin, and antigen-antibody precipitate.

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Received November 29, 1965. P.S.E.B.M., 1966, v121.

Effect of Benzyl N-Benzyl Carbethoxyhydroxamate on Cholesterol Metabolism in the Rat.* (30995)

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Berger and his coworkers(1) have reported that benzyl N-benzyl carbethoxyhydroxamate (W-398) will reduce serum and liver lipids and degree and extent of atherosclerosis when administered to cholesterol-fed rabbits at a level of 2% of the diet. This compound (0.25-0.50%) has also been found to lower serum cholesterol levels of weanling rats fed a hypercholesteremic diet. We have investigated the effect of W-398 upon cholesterol metabolism in normocholesteremic rats fed a sterol-free semi-synthetic diet. The results of our experiments are reported below.

Methods. All rats used were males of the Wistar strain and their average weight at the beginning of each experiment was 185 ± 5 g. The rats were maintained on a diet consisting of infant cereal (Pablum, Mead Johnson Co.) (70), wheat germ (7) skim milk powder (21) and vitamin mix (2). The diet provides 20% protein, 11% fat and 62% carbohydrate, and is readily accepted by the rats. The experimental compound was added to the diets at the expense of the cereal.

The rats were kept on the diets for 3 weeks, at which time they were weighed, injected with either sodium acetate-1-^{14}C (1 μc/100 g or mevalonic acid-2-^{14}C (0.5 μc/100 g) and killed by exsanguination 4 hours after administration of the radioactive substrate. The livers were homogenized in chloroform-methanol 2:1 and an aliquot of the dried extract was taken for cholesterol determination. Another aliquot of each extract was taken for saponification and separation of the cholesterol, whose radioactivity was determined by liquid scintillation spectrometry (2). Serum and liver cholesterol levels were determined by the method of Mann(3). In the experiment in which cholesterol biosynthesis was studied *in vitro*, liver slices (0.5 g) were incubated for 3 hours under 100% oxygen in 5 ml phosphate buffer, pH 7 containing 0.006 M $MgCl_2$ and 0.03 M nicotinamide and 1 μc of either sodium acetate-1-^{14}C or mevalonic acid-2-^{14}C. The reaction was stopped by addition of hot alcoholic KOH and the cholesterol isolated and analyzed for radioactivity. In this experiment a one gram aliquot of each liver was taken for cholesterol analysis. Radioactive substrates were purchased from New England Nuclear Corp., Boston, Mass. Benzyl N-ben-

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* Supported, in part, by USPHS Research Grant HE-03299 and Research Career Award 5-K6-HE-734 from Nat. Heart Inst.