11. Hughes, B. P., Clin. Chim. Acta, 1962, v7, 597.
12. Dewey, M. M., Conklin, J. L., Proc. Soc. Exp. Biol. and Med., 1960, v105, 492.
13. Nowy, H., Frings, H. D., Hohenbleicher, R. E., Arzn. Forsch., 1965, v15, 1253.
14. West, W. T., Murphy, E. D., Anat. Rec., 1960, v137, 279.
15. Lauryssens, M. G., Lauryssens, M. J., Zondag, H. A., Clin. Chim. Acta, 1964, v9, 276.
16. Kruh, J., Dreyfus, J. C., Schapira, G., Gey, G. O., J. Clin. Invest., 1960, v39, 1180.

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Comparative Immunology: Hemolytic Complement in Elasmobranchs.* (31659)

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Considerable interest has been displayed recently in the phylogeny of the immune response, particularly with regard to antibody synthesis in lower vertebrates(1-6). The present report deals with the related area of serum complement as studied in 3 species of elasmobranchs.

It was previously observed that amphibians possess measurable hemolytic complement (C′) levels comparable to those of mammalian sera(7). Titers were much lower than those found in guinea pig sera, however. Sera from each of 3 species of *Amphibia* were capable of reaction in a rabbit antibodysheep erythrocyte hemolytic system; although certain mammalian complements lack this ability(8). Whole complement has also been demonstrated in snakes and certain fish(9-11).

Many species of fish have been shown to elicit a specific immune response(2-6). By using such parameters as response to antigenic stimulation, homograft rejection, and the presence of a thymus and organized lymphopoietic tissues, a progressively decreasing capacity for immune response was suggested as the phylogenetic scale was descended. Minimal responses were noted in certain elasmobranchs and the lamprey, with immunologic activity reaching a virtual null state in the hagfish(12,13). The purpose of the present investigation was to determine whether complement levels decreased in similar fashion in the lower vertebrates.

Materials and methods. Specimens of lemon shark, *Negaparion brevirostris*, and nurse shark, *Ginglymostoma cirratum*, weighing 1000-3000 g, were captured in the waters surrounding Bimini, Bahamas and maintained in outdoor seawater pens in natural environmental conditions at the Lerner Marine Laboratory. The animals were bled from the hemal arch, anterior to the caudal fin, under tricaine methane sulfonate (MS-222) anesthesia. Blood was allowed to clot for one hour at room temperature followed by 2 hours at 10°C. The sera were centrifuged at 3 hours post-bleeding to minimize loss of activity and maintained in an ice bath. Quantitative complement titrations were initiated within 4 hours after bleeding, and all other procedures were begun within 24 hours. Blood samples from the sting ray, *Dasyatis americana*, were obtained by cardiac puncture from mature specimens captured at Bimini. Certain of the sting ray sera, otherwise treated as described above, were maintained in an ice bath for periods up to 24 hours before use.

The natural hemolytic activity of pooled sera from each species of elasmobranch was evaluated by combining serum dilutions with constant amounts of erythrocyte suspensions from several foreign species. A standard pH 7.4 barbital buffer, containing optimal calcium and magnesium ions, was used for all dilutions(14). Each tube contained a final volume of 0.4 ml which included 0.1 ml

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TABLE I. Natural Hemolytic Activity of Pooled Elasmobranch Sera and Modification by Sensitizers.*

Species of elasmobranch	Species of erythrocyte in hemolytic system						Sheep erythrocytes plus		
	Goldfish	Turtle	Chicken	Dog	Rabbit	Sheep	Rabbit ab	Turtle ab	4% carbowax
Lemon shark	128	16	512	256	512	2048	2048	4096	4096
Nurse "	256	16	256	256	1024	256	256	1024	256
Sting ray	8	4	8		8	4	4	4	4
Buffer control	0†	0	0	0	0	0	0	0	0

* Values are expressed in reciprocal of final dilution of serum.
† 0 = no hemolysis at lowest final dilution tested of 1:4.

serum dilution, 0.1 ml erythrocyte suspension (4×10^8 cells/ml), and 0.2 ml buffer. This scheme was varied by substituting 0.2 ml of the appropriate antibody dilution or Carbowax 4000‡ for buffer, in order to evaluate the reactivity of these sera with heterologous sensitizers. An optimal incubation period of 2 hours at 28°C was chosen. This temperature approximated the natural environmental temperature. Trial incubations at 15°, 25°, 30°, and 37°C confirmed that temperature optima lay in the 25°-30°C range. Tubes were then centrifuged and the degree of hemolysis scored visually.

Quantitative C′ titrations were done according to the standard technique outlined in Kabat and Mayer with modifications as noted (14). Natural antibody or turtle antibody, prepared by injecting Gulf Coast box turtles with sheep erythrocytes, were used to sensitize sheep erythrocytes. The results were plotted graphically in C′H50 units. Routine efforts to absorb out natural antibody from the shark sera with sheep erythrocytes were unsuccessful since hemolysis occurred even at 0-2°C. Complete removal of natural antibody could be effected by incubation of sera with trypsin treated, washed erythrocyte stromata.

Inactivation procedures included treatment of pooled elasmobranch sera with heat, ethylenediamine tetraacetic acid (EDTA), hydrazine, carrageenin,§ and preformed ovalbumin-rabbit antiovalbumin precipitate. Normal serum without added heterologous antibody was used in all inactivation procedures, except for guinea pig controls optimally sensitized with rabbit antibody. The details of these procedures have been described(7). Carrageenin was used in each of 3 concentrations: 0.06 mg/ml, 0.25 mg/ml, and 0.50 mg/ml. In experiments concerned with the effects of EDTA, the test sera and other reactants in the hemolytic system were incubated with increasing concentrations of EDTA of 0.5 mM, 1.0 mM, 2.0 mM, and 4.0 mM. Ions were replaced by adding 0.62 mM, 1.3 mM, 2.6 mM, and 5.0 mM each of calcium and magnesium ions to the corresponding inactivated sera. Positive and negative controls were included.

Results. Sera from each species of elasmobranch hemolyzed erythrocytes from several foreign species (Table I). The degree of lysis was dependent upon the erythrocyte system, which suggested specificity characteristic of a complement-dependent antibody system. Lysis of sheep erythrocytes by shark sera was potentiated by turtle antibody and Carbowax 4000, but sting ray serum did not react with these heterologous sensitizers. None of the elasmobranch sera were potentiated by rabbit antibody. Hemolytic titers of shark sera, even in the absence of added antibody, were extremely high as contrasted to the low titers obtained from sting ray sera.

Quantitative hemolytic complement titers are shown in Table II. Values of shark sera compare favorably with high-titer guinea pig serum. In the lemon shark group 6 specimens had individual titers within the 300-400 C′H50 units/ml range, with one specimen exhibiting a titer of 964 C′H50 units/ml. Values for the nurse shark were of similar magnitude. For reference purposes, a pooled guinea pig serum control contained 246 C′H50 units/ml

‡ Courtesy of Union Carbide and Chemicals Corp., New York.

§ Courtesy of Marine Colloids, Inc., Springfield, N. J.

TABLE II. Quantitative Hemolytic Complement Titers of Elasmobranch Sera Expressed in C'H50 Units.*

Species	No. in group	Individual titers Mean	Range	S.D.†	Pooled titers
Lemon shark	9	458	328-964	134	347 (312)‡
Nurse shark	8	597	304-901	237	420 (363)
Sting ray	5	<1	—	—	<1
Guinea pig	Pool	—	—	—	246

* Sheep erythrocyte-turtle antibody system with unabsorbed sera. Rabbit antibody used with guinea pig serum.

† S.D.=Standard deviation.

‡ The first figure represents the system with added turtle antibody; figure in parentheses represents the system with natural antibody only.

when titrated with the same buffer and standardized sheep erythrocyte suspension. The hemolytic activity of sting ray serum was less than 1 C'H50 unit/ml in all samples.

The hemolytic activities of elasmobranch sera were observed to be extremely labile to dilution, slow freezing and maintenance at —20°C, or rapid dry ice-alcohol freezing and storage at —70°C. A complete loss of activity occurred in some frozen samples within 2 weeks. Despite an initial rapid drop in activity within 8-12 hours post-bleeding, an ice bath was found to be the best method of preservation for periods up to 5 days.

Table III shows the effect of absorption procedures on shark serum titers. Antibody could be removed by incubation of these sera with sheep erythrocyte stromata, but even with repeated 20-minute absorptions, slight hemolysis (less than 50%) remained in the 1:2 dilution. Neither was natural antibody totally removed from the control guinea pig serum using stromata at 0°-2°C, despite its comparatively low level of natural antibody. Turtle antibody was used for recombination with C' in the absorbed shark sera, but the C'H50 levels produced were lower than those recorded using natural antibody. A second source of antibody for recombination with absorbed shark sera was provided by using a 1:10 dilution of the corresponding heat-inactivated shark serum, which was presumed to contain an excess of antibody while absent in C'1, C'2, and perhaps other of the C' components. As indicated in Table III, the addition of either shark or turtle antibody to absorbed shark sera resulted in a considerable increase of titer. The replacement of natural antibody in absorbed leopard frog and guinea pig serum by rabbit antibody is shown as a reference. The drop in titer of these sera after absorption reflects simultaneous fixation of C' even at the low temperature. Since leopard frog and shark sera have lower temperature optima for hemolysis than those of mammalian sera, it seems probable that a greater amount of C' fixation occurs during absorption of these sera than would occur in absorption of guinea pig serum. A consideration of the absorption times versus the pre- and post-absorption titers of guinea pig and leopard frog sera tend to affirm this conclusion.

Reactions of elasmobranch sera to inactivation by EDTA, carrageenin, hydrazine, and heat were similar to reactions observed in control guinea pig serum. Sting ray and nurse shark sera were inactivated by heat at 48°C for 20 minutes, while lemon shark

TABLE III. Absorption of Natural Antibody From Selected Sera and Replacement of Antibody.*

Species	Pre-absorption titer Natural serum	Natural serum plus added ab†	Absorption time (min)	Post absorption titer Absorbed serum	Absorbed serum plus added ab
Nurse shark	126	268	40	2	18, (80)‡
Lemon "	141	160	60	2	21, (46)
Leopard frog	2	133	10	1	80
Guinea pig	3	200	40	1	110

* Results expressed in C'H50 units.

† Rabbit antibody added except in case of sharks (turtle antibody).

‡ Titer in parentheses obtained with shark Ab (1:10 dilution of heated shark serum).

TABLE IV. Effects of Inactivation Procedures on Elasmobranch Sera.

Species	Positive control	(a) Ag-Ab ppt	(b) EDTA	(c) EDTA plus Ca^{++}, Mg^{++}	(d) Carrageenin	Hydrazine	Heat†	(e) + (f)‡
Lemon shark	+++	+++	—	—	—	—	50°	+++
Nurse "	+++	+++	—	—	+	—	48°	+++
Sting ray	+++	+++	—	—	—		48°	
Guinea pig	+++	—	—	+++	—	—	56°	+++

+++ indicates maximal hemolysis, — indicates no hemolysis, N. Para. † minimum inactivation temperature in degrees C. at 20 min incubation time as determined by a series of incubations at varying temperatures. Standard 56°C temperature used for guinea pig serum. NP ‡ Incubation of heat inactivated serum with hydrazine-inactivated serum.

serum required a 50°C exposure for complete inactivation. When judged by mammalian standards this would normally indicate the destruction of C'1 and C'2. A final dilution of 0.02 M EDTA, in a total volume of 5 ml, prevented hemolysis by lemon shark and sting ray sera and a 0.04 M EDTA solution inactivated nurse shark serum indicating divalent cation dependent systems. None of the sera, so treated, could be reactivated by addition of excess calcium or magnesium ions, in contrast to control guinea pig serum which was inactivated by 0.5 mM EDTA and reactivated by divalent cations. Lemon shark and sting ray sera were completely inactivated by carrageenin, with about 90% inactivation occurring in serum of the nurse shark, which suggested the presence of a C'1 component (15). Treatment of shark sera with hydrazine produced inactive sera through the probable destruction of C'4 and C'3 components(16, 17). Combination of the heat treated and hydrazine treated shark sera resulted in reactivation of hemolytic activity in serum from both species. Similar recombination was noted in control guinea pig sera. Exposure of shark sera to an ovalbumin-rabbit antiovalbumin precipitate failed to decrease hemolytic activity. Shark C' appeared incapable of binding to antigen-rabbit antibody complexes, a species specific problem previously described in fish sera(10).

Discussion. The investigation of hemolytic C' activity in primitive vertebrates necessitated some modifications of traditional methodology. These changes involved principally the time and temperature of incubation, and the method of sensitization of erythrocytes used in the indicator system. High C'H50 titers were recorded from shark sera even when sensitization was dependent solely on the natural antibody present. Since experience with other animal sera indicates that sensitization by natural antibody usually affords minimal hemolytic titers, the natural hemolytic titers of 312 C'H50 units/ml for lemon shark and 363 C'H50 units/ml for the nurse shark may represent only minimal titers in each case.

For this reason, turtle antibody was used additively in an attempt to more closely approximate the true C'H50 titer. As previously noted, shark sera will not react with rabbit antibody, and attempts to substitute antibody from channel catfish, margate, and iguana for shark antibody were equally unsuccessful.

Long absorption times were necessary for the removal of natural antibody from shark sera due to the high antibody level and, perhaps also, poor avidity of the antibody for the erythrocyte antigen. Considering this long absorption period and the demonstrated ability of these sera to hemolyze erythrocytes at low temperatures, it seems probable that a considerable amount of shark C' is fixed during the absorption process. The use of absorbed shark serum for quantitative titrations would yield an erroneously low approximation of the true C' titer of the serum.

Because of the difficulty experienced initially in separating elasmobranch complement from the natural hemolysin, consideration was given to the adoption of the term H50 to describe the level of hemolytic activity in these animals. Evidence from inactivation procedures, however, indicated that a C' system does exist in these species which resembles

that of mammalian sera, and the usual terminology seemed valid under the experimental conditions outlined above.

Previous work with amphibians(7) and studies in progress on teleost fishes have shown that these groups generally possess lower levels of complement than the nurse and lemon sharks. Present evidence indicates, therefore, that no orderly decrease in complement levels occurs as the phylogenetic scale is descended through the *Elasmobranchii.*

Summary. Sera from 2 species of sharks exhibited a high degree of hemolytic activity for erythrocytes of several foreign species. Natural antibody was absorbed by trypsinized erythrocyte stromata and replaced by shark or turtle antibody. Potentiation by heterologous sensitizers and inactivation by heat, hydrazine, carrageenin, and EDTA suggested that these primitive vertebrates possess C′ systems similar to those of mammals. Quantitative C′ titers were comparable to those of guinea pig sera. Sting ray sera displayed extremely low levels of hemolytic activity which was not potentiated by heterologous sensitizers. Responses to inactivation procedures indicated that this activity might also be C′ mediated. An extreme lability to freezing was characteristic of elasmobranch sera.

Results suggested that no orderly decrease in C′ forming ability occurs as the phylogenetic scale is descended through the elasmobranch level, as has been suggested for other parameters of the immune response.

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1. Evans, E. E., Fed. Proc., 1963, v22, 1132.
2. Clem. L. W., Sigel, M., ibid., 1963, v22, 1138.
3. Hildemann, W. H., Cooper, E. L., ibid., 1963, v22, 1145.
4. Papermaster, B. W., Condie, R. M., Finstad, J., Good, R. A., Gabrielson, A., ibid., 1963, v22, 1152.
5. Evans, E. E., Kent, S. P., Attleberger, M. H., Seibert, C., Bryant, R. E., Booth, R., Ann. N. Y. Acad. Sci., 1965, v126, 629.
7. Legler, D. W., Evans, E. E., Proc. Soc. Exp. Biol. and Med., 1966, v121, 1158.
8. Rice, E. E., Crowson, C. N., J. Immunol., 1950, v65, 201.
9. Bond, G. C., Sherwood, N. P., ibid., 1939, v36, 11.
10. Cushing, J. E., Jr., ibid., 1945, v50, 61.
11. Gewurz, H., Finstad, J., Muschel, L. H., Good R. A., in Phylogenetic Approaches to Immunity, R. T. Smith, R. A. Good, P. A. Miescher, Eds., U. of Florida Press, 1966, 105.
12. Papermaster, B. W., Condie, R. M., Finstad, J., Good, R. A., J. Exp. Med., 1964, v119, 105.
13. Finstad, J., Papermaster, B. W., Good, R. A., Lab. Invest., 1964, v13, 490.
14. Kabat, E. A., Mayer, M. M., Experimental Immunochemistry, 2d. ed., C. C Thomas, Springfield, Ill., 1961, 133.
15. Davies, G. E., Immunology, 1965, v8, 291.
16. Pillemer, L., Ecker, E. E., J. Immunol., 1941, v40, 89.
17. Nishioka, K., Linscott, W. D., J. Exp. Med., 1963, v118, 767.

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Differentiation of Trachoma Bedsoniae *in vitro.** (31660)

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Immunologic typing of trachoma-inclusion conjuctivitis (TRIC) bedsoniae has been accomplished by immunizing mice followed by lethal toxic challenge (1,2,3,) or by immunofluorescence using absorbed human serum(4). Both of these tests have proven impractical for general use, the former because it is labo-

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