EVOLUTION OF THE IMMUNE RESPONSE

I. The Phylogenetic Development of Adaptive Immunologic Responsiveness in Vertebrates*, ‡

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Plate 1

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Mammalian hosts, as well as simpler organisms, rely on complex mechanisms to provide resistance against infection and invasion. Such protective factors as lysozymes in body fluids, polyelectrolytes, complement, non-specific bactericidal substances, and phagocytosis are among the innate mechanisms of resistance. Although some of these are well developed in invertebrates and may indeed be the effective mechanisms of defense, they have been reviewed heretofore (1) and will not be considered in this paper.

Contact with antigen is an event necessary for the immunologic maturation of lymphoid cells and is capable of inducing specific recognition, proliferation, and directed protein synthesis. This sets the immune response apart from the less specific defense processes. Although clearly involving genetic control by mechanisms as yet obscure, this response is both acquired and induced. The term adaptive immunity thus seems appropriate to connote the capacity for immunologic response and would include anamnesis.

The evolutionary origin of the vertebrates is controversial since fossil records of the earliest ancestors are incomplete. Nevertheless, recourse to study of existing vertebrate species has indicated that they likely have arisen from the tunicates in

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which the larval form is bilaterally symmetrical and contains a dorsal nerve chord, notochord, and gill slits (2). The vertebrate ancestors probably developed in the late Ordovician period in the Paleozoic era from ancestral precursors similar to *Amphioxus* (3). Recent evidence compiled by Robertson (3) indicates that the earliest vertebrates evolved in a marine environment.

Many anatomic features indicate that the cyclostomes are the living representatives of the most primitive vertebrates. Among the most significant are the absence of jaws, spherical gill pouches, only two semicircular canals in the internal ear, persistent notochord, lack of complete vertebra formation, and a cartilaginous skeleton (4). The cyclostomes are divided into two main groups: the hagfishes and the lampreys (4). While the lampreys are found in marine and fresh water, the marine forms spawn in fresh water. The hagfishes, on the other hand, are entirely marine. Hagfishes are considered to be more primitive than lampreys on the basis of anatomic structure, physiologic studies of the heart (5), biochemical studies on the plasma ionic concentration of the hagfish which resembles sea water more closely than that of any other vertebrate (6), and hemoglobin structure and function (7).

The higher vertebrates evolved from cyclostome-like ancestors in the late Silurian and early Devonian seas. Fossil evidence indicates that three distinct groups of fishes evolved from the placoderms or placoderm-like forms: the bony fishes, the cartilaginous fishes, and the dipnoid fishes (8, 9). Modern day representatives of the primitive bony fishes are the chondrosteans represented by the paddlefish (*Polyodon spathula*) and the sturgeon (*Acipenser sp.*) and the holosteans represented by the bowfin or dogfish (*Amia calva*) and the gar (*Lepisosteus sp.*). The cartilaginous fish are represented by the sharks, skates and rays, and chimeroids; and the dipnoids by the African and Australian lungfish.

Immunity in invertebrates has been reviewed previously (10–13). Invertebrate and vertebrate immune responses are discussed to emphasize the significant difference in the manner with which these groups deal with foreign substances. The concept of cellular immunity and the role of phagocytosis was developed by Metchinkoff (14, 15). Cellular responses to foreign materials were characteristic and consisted of proliferation, phagocytosis, and digestion. With non-digestable material encapsulation was observed (16, 17).

Bactericidal substances in coelomic fluids and hemolymph of *Lepidoptera* have been studied (18). These factors appeared within hours after injection of live and killed bacteria and in no instance was definitive evidence presented relating these factors to antibody globulins (19). Non-specific agglutinins present in hemolymph were found to be heat-labile, non-hemolytic in the presence of guinea pig complement, and inactivated after storage in the cold (20). A complete listing of the species in which humoral bactericidal and agglutinating substances occur has been compiled by Huff (10), and more recently Briggs (19) has reviewed the heat stable bactericidal factors and bactericidins. Further, current evidence derived from studies on the octopus has not demonstrated a homograft reaction (21). Neither the lobster (22) nor the crayfish (23) can exercise an immune response. At present, none of the experiments on invertebrate immunologic reactivity provides evidence for an adaptive immune response comparable to that observed in higher vertebrates.

Comparative studies on immune responses in vertebrates have also recently been

reviewed (12, 22, 23). These have indicated that teleost fishes and higher vertebrates are quite capable of giving immunologic reactions to a variety of antigenic stimulii including bacterial antigens, particulate antigens such as viruses and erythrocytes, proteins, homografts, and heterografts. Homograft second set reactions in amphibia and fish (24, 25) have clearly indicated that vigorous secondary responses can be achieved in these animals provided they are kept at appropriate temperatures (26). From this accumulated evidence, adaptive immunologic responsiveness would appear to have evolved early in vertebrate development in the most primitive fishes or protochordates.

The studies reviewed so far, however, do not exclude the possibility that some of the immune responses characteristic of adaptive immunity may be found in some invertebrates. Neither the present systematics nor the methods of immunologic analysis are so adequate that absolute differences may be established between invertebrates and vertebrates. The purpose of this paper is to explore the origins of the immunologic reactivity by studying responses to a variety of antigens among representatives of the lowest vertebrates. Investigations have been carried out on the responses of cyclostomes, elasmobranchs, holosteans, and teleost fish.

Materials and Methods

Experimental Animals.—

Hagfish: The California hagfish, *Eptatretus stoutii*, Fig. 1, is a free-living saprophytic form, part of the bathypelagic fauna inhabiting the deep waters below 100 fathoms off the Southern California coast (temperature approximately 4°C). The thermal death point of the hagfish is about 25–28°C (5). Approximately 120 hagfish were used in these experiments.[1] They were kept in a 10°C constant temperature cold room in well aerated sea water, 5 or 6 fish to a 2 gallon bucket, without feeding, for up to 7 months in good condition. They remain coiled unless stimulated by movement of the sea water, direct touch, or sudden approach of bright light. When aroused by handling, the hagfish secretes large amounts of slime; to extricate itself, it swims in a figure 8 or knotting motion, as described by Adam (27). The physicochemical characterization of hagfish slime was reported by Ferry (28).

Lamprey: The sea lamprey, *Petromyzon marinus* (4, 29, 30), an anadromous fish, has adapted completely to fresh water in the Great Lakes. All lampreys used in this study were mature adults trapped during upstream spawning migration in May and June, 1962.[2]

Holostean: Amia calva (31) is the common bowfin or dogfish of the northern lakes and rivers.[3] These fish were held in live tanks in the aquarium facilities of the Zoology Department, University of Minnesota.

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[1] Specimens of the California hagfish were obtained from Dr. David Jensen, Scripps Institution of Oceanography, University of California, La Jolla, California. The animals were shipped to the Heart Hospital Laboratories in cold sea water by air.

[2] Lampreys were obtained through the courtesy of the United States Government Fish and Wildlife Service, Bureau of Commercial Fisheries, Marquette, Michigan.

[3] Animals were obtained through the courtesy of Dr. Samuel Eddy, Department of Zoology, University of Minnesota, and from the Minnesota Conservation Department, Game and Fish Division.

Elasmobranchs:[4] Two species, common to the California coast (32), were studied: the guitarfish, *Rhinobatos productus*, and the horned shark, *Heterodontus franciscii.*

Teleost fishes:[5] The bullhead, *Ameiurus melas*, and the black bass, *Micropterus salmoides*, were studied.

Anesthesia.—The anesthetic used was a 0.1 per cent solution of MS-222 (Tricaine methane sulfonate, Sandoz Laboratories, New York) in sea water or dechlorinated tap water.

Immunization.—

Antigens: Bacteriophage T_2 (*Escherichia coli* B) (33), obtained from Dr. E. S. Lennox, prepared and purified as described by Brenner *et al.* (34); actinophage MSP-8 (35); bovine serum albumin (Armour Pharmaceutical Company, Kankakee, Illinois); hemocyanin (prepared from the giant keyhole limpet, obtained from Dr. Rimmon Fay, Pacific Biomarine Supply, Venice, California); *Brucella abortus* antigen obtained as in (36); typhoid-paratyphoid A and B vaccine, a phenol, heat-killed suspension of *Salmonella typhosa, Salmonella paratyphi A*, and *Salmonella paratyphi B* (Wyeth Laboratories, Marietta, Pennsylvania); *Ascaris lumbricoides* antigen (prepared as a crude extract of the whole worm by Dr. Raymond D. A. Peterson, Department of Pediatrics, University of Minnesota, by the method of Kailin, reference 37).

Adjuvant: Thirty mg/ml of dried *Mycobacterium butyricum* (Difco Laboratories Inc., Detroit) was added to Freund's complete adjuvant (Difco Laboratories, Inc.). Antigens were emulsified in adjuvant, 1 volume of antigen to 1 volume of adjuvant.

Antigenic stimulation: Hagfish were immunized by intracoelomic and intramuscular injections, or injections under the skin into the subcutaneous, lateral blood sinuses which approximates an intravenous injection. The circulatory system of the hagfish has been discussed by Jensen (5) and Johansen (38). Lampreys and other fishes were immunized by the intramuscular or intracoelomic routes.

Blood samples: Hagfish were bled by a median incision on the ventral surface of the anesthetized animal between the gill pores anterior to the heart, and insertion of a capillary tube in the ventral aorta. Tubes without anticoagulants, or with heparin, EDTA, or oxylate, were used to collect blood for serum or plasma. Collection was facilitated by grasping the hagfish between thumb and forefinger and drawing forward toward the heart. From 0.5 to 2.0 ml could be obtained from each fish.

Lampreys were bled without anesthesia. With an assistant to hold the animal firmly at the head and tail, a transverse cut on the ventral surface between the 5th and 6th gill slits was made to sever the ventral aorta only. A wide mouth test tube was held in front of the 6th gill slit while the heart pumped blood into the tube. Three to 10 ml were collected from each lamprey.

Other fish were bled by direct puncture of the heart or conus arteriosus with a 20 gauge needle.

Measurement of serum antibody: Sera were heated at 56°C for 30 minutes to inactivate heat-labile components of complement that might be present. The methods for measuring antibody to T_2 bacteriophage and actinophage have been described previously (33, 35). Phage was added directly to undiluted fish serum or serum diluted with 20 per cent normal rabbit serum (NRS) in 0.9 per cent NaCl, or 0.05 M veronal buffer (pH 7.5) containing 0.1

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[4] Horned sharks were collected by Dr. Rimmon C. Fay, Pacific Biomarine Supply, Venice, California. Guitarfish were collected by Dr. David Jensen. Holding facilities at Scripps Institute of Oceanography were obtained through the courtesy of Dr. David Jensen and Mr. Carr Tuthill, Scripps Aquarium. Holding facilities at Marineland of the Pacific, Palos Verdes, California were obtained through the courtesy of Mr. John Prescott, Curator at Marineland, and Dr. Rimmon Fay.

per cent bovine serum albumin. Diluent controls to measure rate of phage inactivation contained NRS or veronal buffer. Specificity of the antigen-antibody reaction was confirmed by a heterologous phage system as described previously (35).

Bacterial agglutinins were measured as described earlier (36). Antibody to bovine serum albumin was tested by capillary tube precipitation (39) and by the ammonium sulfate technique of Farr (40). Precipitating antibody to hemocyanin was tested by the capillary precipitin method using graded amounts of hemocyanin (0.01 to 1 mg) with undiluted fish serum (39). Antibody to hemocyanin was also measured by the bisdiazotized benzidine (BDB) hemagglutination technique (41). Hemocyanin was conjugated to sheep red blood cells with BDB. Serial twofold dilutions of serum samples were made, and the sensitized sheep red blood cells added. A rabbit hyperimmune serum standard was set up each time to serve both as a standard for reproducibility of the method and a check on the analytical procedure.

Persistence of circulating antigen: Persistence of antigen was studied with bacteriophage, hemocyanin, and bovine serum albumin. The assay for persistent phage involved use of the agar overlay technique. The whole or diluted serum being assayed for persisting phage was added to melted agar overlays inoculated with host bacterial cells and subsequently plated on nutrient medium. Persisting phage was indicated by plaque formation after incubation for 18 hours at 37°C. Persistent circulating hemocyanin was demonstrated using hyperimmune rabbit antihemocyanin antibody and serial dilution capillary tube precipitation. Circulating bovine serum albumin was similarly detected by serial dilution capillary tube technique using hyperimmune anti-BSA rabbit serum.

Delayed Hypersensitivity.—BCG (Bacillus Calmette-Guerin Research Foundation, Chicago) mixed with Freund's adjuvant, was injected intramuscularly and intracoelomically for primary sensitization. Challenge injections consisted of intradermal, intramuscular, and intracoelomic injections of old tuberculin (Eli Lilly and Company, Indianapolis), diluted 1:10 and 1:100. *Ascaris lumbricoides* antigen, 20 mg in complete adjuvant, was also used; challenge was provided with the particulate *Ascaris* antigen.

Homografts.—Grafts of skin 1 cm^2 from the caudal region of the hagfishes were removed from anesthetized animals and exchanged between pairs of fish. Autografts were exchanged between opposite sides in the tail region of the same animal. Skin sections were held on sea water-soaked sponges until ready to be sewed to adjoining skin at the graft bed with cutting edged needles and 5-0 silk suture. The skin of the hagfish is essentially avascular and does not adhere to the musculature over the sides of the animal because of the subdermal lateral sinuses. Autografts and homografts of muscle (1×2 mm) were implanted in graft beds prepared by incisions in the skin and myotomes. Following implantation the skin incisions were closed over the implants with 5-0 silk suture. Liver implants (2 mm^2) were autografted and homografted by implantation through ventral incisions posterior to the gill region over the liver. Autologous and homologous cell suspensions (prepared by repeated aspiration and expulsion from a syringe with a 20 gauge needle) were injected directly into the intact animal over the same area. Control animals were sham-operated by cutting into the myotomes, removing small sections of liver, or by incising the skin and closing the wound with silk.

Electrophoretic Analysis of Serum Proteins.—Samples of serum and oxylated and heparinized plasma were examined electrophoretically by filter paper, starch gel, and immunoelectrophoresis. Electrophoresis on paper was carried out at pH 8.6 in veronal buffer (ionic strength, 0.1) at 60 v and a current of 15 ma. Starch gel electrophoresis was performed according to the method of Smithies (42). For immunoelectrophoresis rabbits were hyperimmunized with fish serum by an initial intradermal injection in the foot-pad of 0.1 ml of an emulsion consisting of equal parts of fish serum and the complete Freund's adjuvant described above. An extended course of immunization consisted of 0.1 ml serum given intramuscularly every 2 to 3 weeks for approximately 3 months or as long as 1 year. Microimmunoelectrophoresis, as described by Scheidegger (43), was used to characterize serum samples further.

RESULTS

Formation of Circulating Antibody.—

Hagfish: Attempts to induce formation of circulating antibodies with diverse antigens were uniformly unsuccessful in the hagfish. Results of these experiments are presented in Table I. The antigens used included killed *Brucella*

TABLE I

Attempts to Stimulate Antibody Formation in the Hagfish

Antigenic stimulations	No. of fish	Time of bleeding	Method of measurement used	Response
10 mg BSA in 1 cc saline; 1, 2, 5, and 10 injections at weekly intervals	10	14 days after last injection	Capillary tube precipitation and antigen binding capacity*	0/10
100 mg BSA; 1, 2, and 3 injections at weekly intervals	9	14 days after last injection	Capillary tube precipitation and antigen binding capacity*	0/9
BSA, 10 and 1 mg in Freund's adjuvant; 1 injection	5	14 days after injection	Capillary tube precipitation and antigen binding capacity*	0/5
Typhoid-paratyphoid A and B vaccine; 1 and 2 weekly injections	6	10 and 14 days after last injection	Agglutination	0/6
Brucella abortus; 1 and 2 injections separated by 30 days	7	10 and 14 days after last injection	Agglutination	0/7
Hemocyanin; 1 and 2 injections separated by 30 days	4	14 days after last injection	Precipitation and hemagglutination	0/4
T_2 bacteriophage; 1, 2, 5, and 10 weekly injections	15	7 and 14 days after last injection	Neutralization	0/15
Actinophage; 1 and 2 weekly injections	7	7 and 14 days after last injection	Neutralization	0/7

* Antigen binding capacity was determined by the method of Farr.

abortus, typhoid-paratyphoid A and B vaccine, bovine serum albumin, hemocyanin, bacteriophage (T_2 coliphage), and actinophage MSP-8 (*Streptomyces griseus* host). Hagfish in groups of 4 to 15 were given 1, 2, and in some animals, up to 10 injections of the antigens, several given over periods up to 2 months. Groups of hagfish were injected with BSA, T_2 phage, actinophage, typhoid-paratyphoid, and *Brucella* antigens as well as BSA incorporated in Freund's adjuvant at the time of injection. Bleedings were taken at 7, 10, or 14 days after the last injection. In no instance were agglutinating, hemagglutinating, or neutralizing antibodies, or antigen-combining globulin found. In a separate experiment, 10 consecutive weekly injections of T_2 phage were administered intramuscularly, intracoelomically, or under the skin into the subcutaneous

lateral sinuses. Bleedings were taken 1 and 2 weeks following the last injection. Again, no evidence of neutralizing antibody was demonstrable. Immunized hagfish sera were tested for neutralizing antibody against T_2. Sera were heat-inactivated 30 minutes at 56°C and incubated with T_2 phage for as long as 72 hours at 37°C and 0°C. No specific neutralizing activity for T_2 was present in any of the sera.

The persistence of antigen in the hagfish circulation was demonstrated with 3 antigens: bacteriophage T_2, hemocyanin, and bovine serum albumin. Viable bacteriophage was regularly recovered from the circulation 14 days following intramuscular injection of 10^{10} T_2 bacteriophage. Similarly, both hemocyanin and BSA were detected in the serum at least 14 days after injection (Table II).

As a control on conditions of temperature and starvation, a fresh water

TABLE II

Persistence of Antigen in Circulation of Hagfish

Amount of antigen injected	Recovery of antigen 14 days after injection
Bacteriophage T_2 10^{10} particles intravenously*	4×10^3 particles per ml of serum
Hemocyanin 10 mg intravenously*	Positive qualitative precipitin test with rabbit antihemocyanin antibody
Bovine serum albumin 10 mg intravenously*	Positive qualitative precipitin test with rabbit anti-BSA antibody

* Injected into subcutaneous lateral venous sinuses.

teleost fish, ***Ameiurus melas*** (the bullhead) was immunized with 10^{10} T_2 phage at 10°C. Phage particles were cleared from the circulation within 4 days, and circulating antibody was detected in 14-day serum samples. Heterologous phage PLT_{22} (*Salmonella typhimurium*) was included in reaction tubes as a control on the specificity of the inactivation.

To evaluate the effect of temperature on ability to form circulating antibody, hagfish and bullheads were kept at 20°C for 7 days. Stimulation with 10^{10} T_2 phage particles failed to elicit antibody production in the hagfish, and large numbers of circulating phage particles were present in serum samples taken 4 and 7 days after injection. On the contrary, the bullhead cleared bacteriophage from the circulation within 4 days and heat-inactivated sera showed T_2-neutralizing antibody. Here also heterologous phage controls were used to establish specificity. Results are summarized in Table III.

Lamprey: The lampreys were maintained in their natural habitat in live boxes on the Cedar River, a tributary to Lake Michigan. Because of the rapid physical and physiologic degeneration that occurs in these animals at this phase in their life cycle, the mortality rate was as high as 70 per cent over the 2 month period

of these studies. Few animals survived for the long term studies of the secondary response. Two groups of 70 lampreys each were injected with 10^{10} T_2 phage or 10 mg hemocyanin per animal. Primary and secondary injections were made at the intervals indicated in Table IV.

The variations in phage-neutralizing activity for each of the animals sacrificed at 2 and 4 weeks after primary stimulation with 10^{10} phage per animal are compared with pooled non-immune sera and a buffer control in Table IV. Results are expressed in $t\frac{1}{2}$ values (time in minutes required to inactivate 50 per cent of the viable phage in the reaction tubes). From these data it can be seen that no primary antisera or pooled non-immune sera could inactivate 50 per cent of the bacteriophage in less than 360 minutes. Twenty animals

TABLE III

Effect of Temperature on Response to T_2 Bacteriophage in the Hagfish

Temperature	Fish studied	Day of serum sample	T_2 phage neutralized after 60 min. incubation
°C			*per cent*
10	Bullhead	14	40
	Hagfish	14	0*
20	Bullhead	4	30
	Hagfish	4	0*
		7	0*
25	Hagfish	Dead, 4 to 5 days	

* No deviation from diluent control.

were restimulated with 10^{10} phage per animal to study the secondary response. Only 5 lampreys were alive 2 weeks after secondary stimulation. The experiment was terminated by sacrificing the remaining lampreys for serum antibody assays. Two lampreys demonstrated some increased neutralizing activity when compared with primary groups; however, the results must be cautiously interpreted in view of the high mortality rate late in the season. The animals injected with hemocyanin each appeared to possess a feeble hemagglutinating antibody reaction to hemocyanin after primary injection of antigen; no precipitating antibody was demonstrated.

The equivocal nature of the response to these two antigens was further borne out in clearance studies. Fifty lampreys were injected intramuscularly with 10^{10} phage per animal to study the clearance of circulating antigen (Text-fig. 1). Ten animals were sacrificed at each interval for 28 days. Complete clearance of phage was never obtained in either 2-week or 4-week sera from the primary stimulation. A small amount of phage was still circulating 2 weeks after second-

TABLE IV

Inactivation of Bacteriophage T_2 by Lamprey Sera

Sample*	t½‡
	min.
Buffer control (veronal buffer, pH 7.5, with 0.1 per cent BSA)	1400
Normal lamprey serum (non-immune pool of 50 animals)	800
Immune sera, 10^{10} phage injected and serum sampled at:	
2 wks.	1400
	360
	2800
	1800
	1400
	1400
	1200
	2000
	480
	420
4 wks.	540
	1500
	1440
	1440
	2160
	1440
	420
	480
	2000
Secondary response, 10^{10} particles injected 4 wks. after primary stimulus, serum sampled at:	
2 wks.	1200
	60
	640
	560
	180

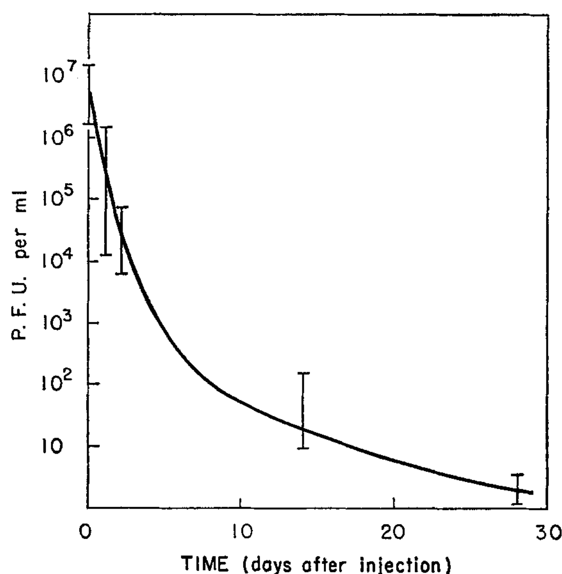
* Each sample represents serum from an individual lamprey.

‡ t½, time in minutes necessary for inactivation of 50 per cent of the viable phage in the reaction tubes containing undiluted sera.

ary stimulation with 10^{10} particles. Hemocyanin, on the other hand, was readily cleared from the circulation following injection of this antigen.

Bowfin or dogfish (Amia calva): Six animals were immunized with 10^{11} T_2 phage particles per animal intramuscularly. They were restimulated with 10^{10} particles for the secondary response 28 days after primary injection and for

the tertiary 4 months after primary injection (Table V). Serial bleedings were made by puncture of the conus arteriosus or the heart with a 20 gauge needle. In each instance phage particles were cleared from the circulation within 7 days of stimulation. Neutralization by primary response sera was not remarkably different from controls; however, secondary and tertiary response sera showed good neutralizing activity which was specific for T_2 phage, since



TEXT-FIG. 1. Clearance of T_2 phage in lamprey (*Petromyzon marinus*). Each animal was given 10^{10} phage particles intramuscularly; groups of 10 animals were sacrificed serially at the intervals shown (0 time = 15 minutes). The lines through the curve indicate the range. P. F. U., plaque-forming units.

no neutralization was observed with the heterologous PLT_{22} (*S. typhimurium*) system.

Both hemagglutinating and precipitating antibody to hemocyanin were measured in primary and secondary response serum from 2 animals (Table VI). The results indicate that the bowfin forms both precipitating and hemagglutinating antibody to hemocyanin.

Elasmobranch fishes: Elasmobranchs obtained for this study included guitarfish (*Rhinobatos productus*) and horned sharks (*Heterodontus franciscii*). All were held in live tanks with circulating sea water.

Guitarfish, 3 to 4 feet in length, were docile and could be bled from the heart without anesthetic. In 5 of 6 animals, injected with 10^{11} T_2 phage, 7-day sera contained circulating viable phage in concentrations up to 10,000 particles per ml (Table VII); 1 animal had cleared the phage in 7 days. Thirty days

TABLE V

Response to T_2 Phage in Amia calva

Sample*	Rate of phage inactivation, $t\frac{1}{2}$
	min.
Normal rabbit serum	1400
Veronal buffer with 0.1 per cent BSA	1080
Preimmune serum at 1:2 dilution	1200
	1020
	1560
Primary response to 10^{11} T_2 phage intramuscularly (serum 1:2 dilution) at:	
7 days	1080
	1440
	1500
	1020
12 days	560
	720
	420
14 days	540
	400
	120
Secondary response to 10^{10} T_2 phage intraperitoneally, 28 days after primary injection, at:	
7 days	80
	70
	40
14 days	80
	40
Tertiary response to 10^{10} T_2 phage intraperitoneally, 4 months after primary injection, at:	
7 days	240
	180
	70

* Phage particles were cleared within 7 days after each stimulation. Samples represent different groups of surviving animals and are not necessarily serial bleedings.

after the primary injection, the bacteriophage had been cleared from the serum in all animals studied (Table VII); the antibody response was present but was sometimes feeble. After a secondary injection of 10^{10} particles, 30 days after the primary, all animals cleared the phage within 7 days and all showed a vigorous antibody response at that time.

TABLE VI

Immune Response of Amia to Hemocyanin

Form of response	Time following injection	Antibody	
		Precipitin	Hemagglutinin titer
	days		
Primary response to hemocyanin	40	+	1/80
		−	0
Secondary response*	14	+	1/20
		+	1/20

* The animals were injected on the 40th day following initial injection and bled 14 days later.

TABLE VII

Response to Bacteriophage in Rhinobatos productus

Days after injection	No. phage injected	Animal No.	Viable phage per ml serum	Relative neutralization of added phage*
7 days after primary	10^{11} i.m.	1	0	Not measured
		3	1×10^4	
		5	5.8×10^3	
		6	1×10^4	
		7	3×10^3	
		8	3.1×10^2	
30 days after primary	—	1	0	++++‡
		4	0	±
		7	0	+
		8	0	++
7 days after secondary	10^{10} i.m.	1	0	++++
		4	0	++++
		7	0	++++
		8	0	++++

* Neutralization at 24 hours by undiluted serum.

‡ ++++ indicates complete neutralization of all phage added to reaction tubes to assay for circulating neutralizing antibody to T_2 bacteriophage.

The immunologic response of the guitarfish to hemocyanin is presented in Table VIII. The primary response to hemocyanin is feeble, and titers are only slightly increased following secondary stimulation. None demonstrated precipitating antibody by the capillary tube precipitin technique.

Horned sharks were also stimulated with T_2 phage and hemocyanin. As shown in Tables IX and X, both primary and secondary responses to both antigens

TABLE VIII

Response of Guitarfish to Hemocyanin

Serum samples	Precipitating antibody*	Hemagglutinating antibody‡
Preimmune	—	0
	—	0
Primary bleeding 7 days after 10 mg hemocyanin i.m.	—	0
	—	10
	—	10
	—	10
	—	0
		0
Primary 30 days after 10 mg hemocyanin i.m.	—	10
	—	40
	—	20
	—	0
Secondary response 7 days after 10 mg hemocyanin i. m. on 30th day after first injection	—	10
	—	40
	—	20
	—	80
14 day secondary	—	20
	—	0
	—	10
	—	160

* By the capillary tube precipitin technique.

‡ Hemagglutinating antibody was determined by the bisdiazotized benzidine technique following absorption with sheep red blood cells. Since many lower vertebrate sera contain agglutinins to mammalian red blood cells in high titer, the unabsorbed sera, even in the preimmune state, regularly agglutinated sheep red blood cells. Following absorption with sheep cells, however, no agglutination was demonstrable in control sera. The figure given is the reciprocal of the highest dilution of antiserum producing pattern agglutination of the BDB-hemocyanin–carrying red blood cells.

were observed. However, in the assay for antibody against hemocyanin, none of the animals in the group produced sufficient quantities of antibody or antibody of the quality to be detected by the capillary tube precipitin technique.

Evolution of Antibody Production to Hemocyanin.—On Table XI are summarized data reflecting the immunologic responses of a variety of fish to stimulation with hemocyanin. It will be seen in this table that the hagfish does not respond with antibody production to this antigen by either the hemagglutination technique or capillary precipitin reaction. The lamprey, next in line on the phylogenetic scale, shows very weak hemagglutination responses

TABLE IX

Response to T_2 Phage in Horned Shark (Heterodontus francisci)

Sample	Shark No.*	Rate of phage inactivation, t½‡
		min.
Veronal buffer with 0.1 per cent BSA		>1440
Primary response to 10^9 T_2 phage i.m. (serum undiluted) at:		
11 days		§
21 days	1	>1440
	3	>1440
	4	510
	5	420
	6	>1440
Secondary response to 10^9 T_2 phage i.m. (serum undiluted) at:		
11 days	5‖	33
	6	21
21 days	1	165
	3	¶
	4	>1440
	5	84
	6	50

* Serial bleedings on same animals.

‡ t½, time in minutes necessary for inactivation of 50 per cent of the viable phage in the reaction tubes.

§ Phage not cleared from circulation at 11 days.

‖ Sharks 1, 3, and 4 had not cleared phage by 11 days after secondary stimulation.

¶ Phage not cleared from circulation at 21 days after secondary stimulation.

TABLE X

Antibody Synthesis in the Horned Shark (Heterodontus francisci)

Group	Day after injection of antigen	Circulating hemocyanin	Precipitating antihemocyanin	Phage persisting	Hemagglutinating antibody to hemocyanin	Neutralizing antibody to phage
Primary stimulation	11	6/6	—	5/5	—	0/5
	21	0/6	0/6	0/5	6/6	2/5
Secondary stimulation	11	0/6	0/6	3/5	5/5	2/5
	21	0/6	0/6	1/5	5/5	3/5

and no precipitating antibody as measured by the capillary tube technique. In both the guitarfish and the horned shark, elasmobranchs, a definite hemagglutination response is indicated but no demonstrable precipitin response is evident. *Amia calva* (the bowfin or dogfish), a representative of the primitive holostean fish, and the large mouth black bass (*Micropterus salmoides*), a representative of the higher teleosts, formed both precipitating and hemagglutinating antibody. These data, concerning the primary response to hemocyanin, suggest a progressive development of immunologic capacity as one ascends the phylogenetic scale among the lower vertebrates.

Delayed Sensitivity.—

Hagfish: Sensitization of the hagfish was attempted by intramuscular or

TABLE XI

Primary Antibody Response to Hemocyanin among Fishes

Fish	Precipitin response* (capillary tubes)	Hemagglutination
Large mouthed black bass (*Micropterus salmoides*)	+	+
Bowfin—dogfish (*Amia calva*)	+	+
Horned shark (*Heterodontus franciscii*)	−	+
Guitarfish (*Rhinobatos productus*)	−	+
Lamprey (*Petromyzon marinus*)	−	±
Hagfish (*Eptatretus stoutii*)	−	−

* Representatives of each species stimulated by injection of hemocyanin 10 mg intramuscularly.

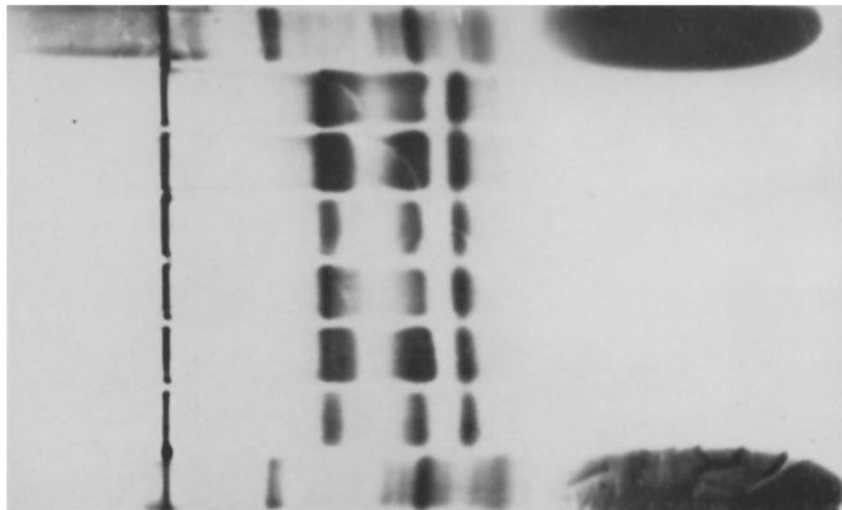
intracoelomic injection of 0.5 ml BCG in complete Freund's adjuvant. Several days after injection, no evidence of swelling or induration at the injection site could be noted by gross observation or palpation. There was no indication of diminished activity or change of normal position. Challenge intradermally, intracoelomically, and intramuscularly with BCG or old tuberculin 8, 20, 30, and 60 days following initial sensitization failed to produce any adverse systemic or local effects. No gross or microscopic lesions could be detected at the injection site.

Bowfin: Ascaris antigen was emulsified in complete Freund's adjuvant and injected intramuscularly and intraperitoneally into the bowfin, *Amia calva*. Thirty days after sensitization, subcutaneous challenge injections of *Ascaris* antigen produced an elevated indurated area of inflammation within 3 days (Fig. 2). Such a reaction did not occur in non-sensitized controls similarly injected with *Ascaris* antigen. An injection into the skin over the upper lip, 10 days following the initial test reaction in the sensitized group of two fish, produced another area of indurated inflammation. It is apparent from these

studies that *Amia calva* can develop a delayed allergic reaction whereas the hagfish cannot.

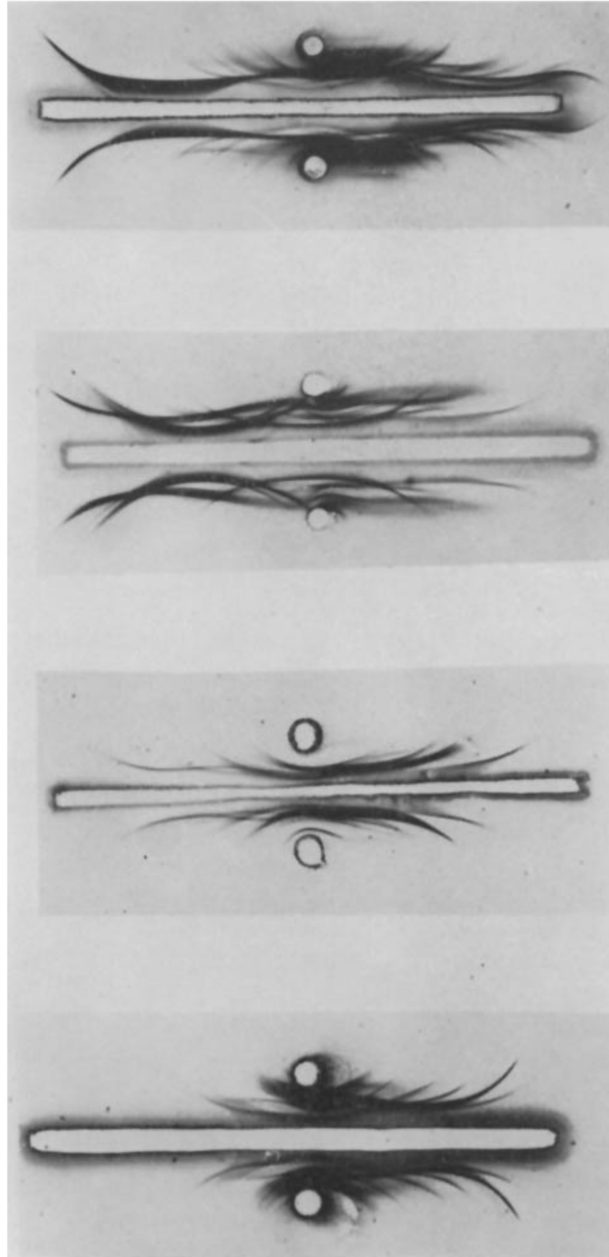
Studies on delayed sensitivity were not systematically attempted in other species. However, the bullhead (*Ameiurus melas*) regularly developed a large angry indurated lesion at the site of injection of Freund's adjuvant, whereas the hagfish showed no such reaction of inflammation to this stimulation.

Homograft Immunity and Wound Healing in Hagfish.—Autografts and homografts of skin from the caudal region of 1 animal to the caudal region of a



TEXT-FIG. 2. Starch gel electrophoresis of hagfish serum (six center strips) compared to normal human serum (top) and agammaglobulinemic human serum (bottom). The hagfish lacks proteins with mobilities of albumins and gamma globulins.

2nd did not firmly heal in the graft beds. Shortly after grafting, blood continued to ooze from the graft bed until small amounts of slime could be seen to coagulate around the sutures. Although two homografts of skin remained in place for as long as 30 days, gross and microscopic examination of the skin and suture areas revealed neither necrosis of the skin nor union of the homograft with the skin of the host. More usual was the situation in which both autografts and homografts merely pulled away from the graft bed and the containing sutures within 7 to 14 days because of the swimming movements of the animals. These results were comparable to those obtained by Triplett *et al.*, in skin homografts on a marine invertebrate worm, *Dendrostromum* (44). Homografts and autografts of myotomes and muscle implants also failed to heal in place. However, histologic studies of the graft beds failed to reveal significant differences in the cellular infiltrates of homografts and autografts.



TEXT-FIG. 3. Immunoelectrophoresis of serum from hagfish, lamprey, bowfin, and human being (from left to right). The complete lack of the gamma bands is evident in the hagfish (extreme left). A small gamma band is seen in the pattern of the lamprey, whereas a complexity of proteins in the gamma area is found in the bowfin. One of the bands in the bowfin is strikingly similar to the gamma$_2$ globulin component of man.

Grafts of liver tissue from autologous and homologous sources were inserted intrahepatically as liver cell suspensions. Eight to 20 days after such treatment, which included direct intrahepatic injection and implanting bits of tissue through a ventral skin incision, failed to reveal differences of the handling of homograft and autograft material.

A study of wound healing in the hagfish demonstrated the poor ability of this animal to heal wounds under the laboratory conditions. Gross and microscopic examinations of wound sites and skin incisions showed poor regrowth of the skin epithelium, non-joining of the tissue edges, and the formation of a rudimentary granulation tissue over the wound area. Similar observations on failure of wound healing had been mentioned previously by Jensen (5). From our studies it is clear that the technical difficulties of studying homotransplantation in the hagfish prohibit a definitive answer at this time. Our studies in the hagfish have not defined clear evidence of homotransplantation immunity.

Serum Protein Electrophoretic Studies.—Paper, starch gel, and immunoelectrophoretic studies were carried out on the sera from the cyclostomes and other fishes used in this study. A comparison of the starch gel pattern of normal and agammaglobulinemic human serum with that of hagfish is shown in Text-fig. 2. In the hagfish patterns, there is absence of protein migrating with mobilities comparable to gamma globulin and albumin of human serum.

A comparison of the immunoelectrophoretic patterns from the hagfish, lamprey, *Amia*, and human is shown in Text-fig. 3. While there are no proteins migrating with gamma mobility in the hagfish pattern, that from the lamprey indicates a low concentration of a slowly migrating component comparable to the mammalian gamma globulin. A number of bands in the gamma region are illustrated in the pattern from *Amia calva*, the holostean bowfin. One of these bands shows a striking similarity in mobility and form to that of human gamma_2 globulin components.

Here, as with antibody production, there appears to be clear evidence of development of complexity in the gamma migrating globulins with progression up the evolutionary scale among the lower vertebrates. It is apparent that a significant development in adaptive immune responses and complexity of protein chemistry occurred during the evolution from the level of the primitive cyclostome to the level of the lowest cartilaginous and bony fishes. In an effort to correlate the immune responses with appropriate lymphoid cellular elements, morphologic studies were performed in the several species studied, and are reported in the succeeding paper in this series (45).

DISCUSSION

The existing literature leaves great gaps in our knowledge of comparative immunology and does not provide information about immunologic responses in the large group of transitional animals important in the early evolution of the advanced fishes, the protochordates and cyclostomes.

The present experiments were begun with the intention of first testing the lowest vertebrates, the cyclostomes, for evidence of an immune response and then continuing the studies in animals representing simpler and more advanced stages of evolution. In this context experiments commenced with the California hagfish, *Eptatretus stoutii*. Our results with the hagfish led to studies of the lamprey, *Petromyzon marinus*, another cyclostome, and representatives of the bony and cartilaginous fishes.

The failure to elicit immune responsiveness to a battery of antigens in the hagfish, suggestive evidence of responsiveness in the lamprey, clear cut demonstration of anamnesis in elasmobranchs, and even precipitin formation in teleosts provide support for the gradual evolutionary ascendancy and emergence of the immunologic capacity which is associated with higher vertebrates.

In our studies the hagfish did not produce antibody when held at 10°C after a 3 month immunization schedule with T_2 phage and extensive immunization with other antigens including hemocyanin. However, the teleost species studied at this temperature (*Ameiurus melas*) developed neutralizing antibody to T_2 phage.

Hagfish held at 10°C also failed to show any gross or microscopic signs of an inflammatory reaction to complete Freund's adjuvant, while injections of the same material into *Ameiurus* produced an area of induration and inflammation at the site of injection. Hagfish survived so poorly at higher temperatures that reliable data could not be obtained under these conditions. Our evidence thus indicates that the hagfish reacts in an altogether different fashion from the higher vertebrates studied.

The temperature variable must be carefully considered for each species being studied. Hagfish acclimate only poorly to higher temperatures (5) and experiments at temperatures ordinarily compatible for other vertebrates are impossible in this species. The lamprey adapts more easily to warmer water, and many species of teleost fishes and elasmobranchs can tolerate temperatures between 4° and 30–35°C. That immune responses develop at cold temperatures has been reported both by Bisset (46) and Ridgeway (47) in certain teleost species studied at temperatures of 8°C or below. On the other hand, reptiles peculiar to desert habitats show reduced antibody forming capacity at temperatures even as high as 25°C when compared to the ability to synthesize antibody at 35°C (26).

Studies on skin grafting in the hagfish were inconclusive. The difficulty in maintaining both autografts and homografts in place was similar to the situation in the invertebrate marine worm, *Dendrostomum zostericulum* studied by Triplett *et al.* (44). Failure to demonstrate differences in the cellular infiltrate around autologous and homologous liver cell implants attested further to the lack of adaptive immunologic reaction to the homografted cells. These experiments can by no means be considered complete since very long term studies of homologous cell implants will be necessary before the homograft reaction in the

hagfish is described completely. More detailed studies of this response are in progress.

Homograft failure in some cases may not be based on immunologic reactivity. It may not be the limiting factor in establishing, for example, vascular channels to a homograft or other necessary cell associations leading to a graft "take" when histocompatibility differences are great (48). Cell contact reactions and mutual recognition phenomena appear to be important in organization and maintenance of the aggregated state in the Metazoa (49). Recognition of one cell by another has been studied by Abercrombie *et al.* by cinematographic analysis (50). Surface interactions characteristic of these primary organizing reactions in associated cell systems may be a primitive form of homograft reactivity, but are different from the highly specific reactions of sensitized lymphoid cells (51). Establishing the evolutionary point for the emergence of cell-mediated, adaptive immunologic reactions may be more difficult than for antibody synthesis, since the parameters of measurement are at present not clearly defined.

It is not possible to ascertain at present the evolutionary origin of the humoral components of adaptive immunity in vertebrates, the serum globulins. Mucoprotein-like substances are associated with the pharyngeal structures of the protochordates and the larval lamprey (29). Further studies on the components of the hemolymph, the coelomic fluid, and the blood of the invertebrates, along with protein studies on the blood of the cyclostomes, are needed to clarify the evolutionary origin of gamma globulin molecules. Preliminary studies with immunoelectrophoretic methods presented herein reveal an absence of proteins migrating as gamma globulins in the hagfish but provide suggestive evidence of such proteins in the lamprey. Evidence of globulins, or slowly moving proteins migrating toward the cathode, in the elasmobranchs, has been provided by Engle (52), and immunoelectrophoretic studies reported here reveal components with the mobility of gamma globulins to be present in the serum of *Amia calva.* Recent studies by Dr. Howard Grey (53) on the guitarfish used in our experiments at the Scripps Clinic and Research Foundation at La Jolla, indicate a 19S globulin antibody component in the serum, as obtained by ultracentrifugal analysis in a density gradient of sucrose. This antibody activity is destroyed by 2-mercaptoethanol and can be localized as specific antibody to hemocyanin in lymphoid cells with the fluorescent antibody technique.

The failure to find proteins in the hagfish with mobilities comparable to the gamma migrating group of the higher vertebrates is not surprising. The cells ordinarily associated with gamma globulin synthesis in higher vertebrates (plasma cells) appear to be entirely lacking in the hagfish (45). A difference in the globin proteins in erythrocytes of the hagfish and lamprey has been noted by Manwell and his associates (7). The hagfish hemoglobin resembles that of

the invertebrate sea-cucumber, while the lamprey globin is similar to that of the higher fishes (7).

From the early and more recent studies, it is apparent that the invertebrates are capable of protecting themselves from invasive saprophytic and pathogenic bacteria and other harmful foreign organisms by phagocytosis and cellular proliferation. The reaction to injury in these species usually consists of an encystment of the foreign material by the phagocytic cells lining the body cavities (14–17). Host resistance in the lower forms appears to be, in most cases, a natural, non-induced capacity. The outcome of host-parasite interactions in invertebrates is usually determined by the ability of the host to phagocytose the invasive organisms and of the organism to reproduce and metabolize within the body of the host. The result of this struggle is based primarily on metabolic and physiologic functions *other than* the immune response characteristic of higher vertebrates.

Humoral bactericidal substances provide another means of protection in invertebrates and may also be of importance in the hagfish, although these responses have not yet been studied in cyclostomes. The substances occur naturally and can be increased in concentration by active immunization with killed or live bacteria or other materials (19). As was stated previously, invertebrate humoral bactericidins, usually non-specific both in action and in their induction, do not resemble the globulin associated with antibody in the vertebrates (19). Complement components may be present in the invertebrates, and evidence for one or another factors of complement was discussed by Cantacuzène (13). No recent studies have been done on complement components in the invertebrates which are conclusive, and studies of complement activity has only just begun in our laboratories.

The studies reviewed here provide no evidence which would suggest that the protective capacity or immunity *per se* is lacking in the invertebrates or even in the cyclostomes. What appears to be lacking is adaptive synthesis of immunoglobulins and the regular recognition of such fine antigenic differences as the polymorphic antigens characteristic of the histocompatibility antigens within a species. This ability in the higher vertebrates seems to be associated with the lymphoid family of cells. It is apparent that the evolution of adaptive immunologic responses may not be related to protective functions alone, even though immune responses of higher vertebrates are closely associated with protection of these animals from invasive parasites, both bacteria and viruses. The ability to protect themselves from such invaders would presumably need be no more highly developed in the vertebrates than in the lower animals, since representatives of both groups are found together in most of the ecologic niches of the earth.

The evolution of the vertebrate immunologic system, if it is considered in a broader biologic perspective, represents development of a complex cell system.

Even a superficial examination of the reactive elements in an advanced vertebrate's immunologic armamentarium discloses that all or nearly all of the invertebrate mechanisms are retained in some fashion. With regard to the phagocytic capacity, the histiocytes and macrophages in the tissue and polymorphonuclear leukocytes of the peripheral blood actively phagocytose foreign particles. The extent and function of these cells was well described by Aschoff (54), who termed the functioning elements the reticuloendothelial system. Most authors have also included immunologically competent cells as part of the reticuloendothelial system in mammals. As Benacerraf, Biozzi, Halpern, and their associates (reviewed in reference 55) have shown, the phagocytic uptake of bacteria is enhanced in the immune host. The normally occurring bactericidal substances in mammalian serum have also been studied carefully, and a complete discussion of these is found in the review by Skarnes and Watson (1). Thus, with an evolutionary legacy from the invertebrates, consisting of both humoral and cellular components, we may usefully inquire into the reasons for the development of immunologically reactive lymphoid cells (45) in the more advanced vertebrates.

SUMMARY

1. The California hagfish, *Eptatretus stoutii*, seems to be completely lacking in adaptive immunity: it forms no detectable circulating antibody despite intensive stimulation with a range of antigens; it does not show reactivity to old tuberculin following sensitization with BCG; and gives no evidence of homograft immunity.

2. Studies on the sea lamprey, *Petromyzon marinus*, have been limited to the response to bacteriophage T_2 and hemocyanin in small groups of spawning animals. They suggest that the lamprey may have a low degree of immunologic reactivity.

3. One holostean, the bowfin (*Amia calva*) and the guitarfish (*Rhinobatos productus*), an elasmobranch, showed a low level of primary response to phage and hemocyanin. The response is slow and antibody levels low. Both the bowfin and the guitarfish showed a vigorous secondary response to phage, but neither showed much enhancement of reactivity to hemocyanin in the secondary response. The bowfin formed precipitating antibody to hemocyanin, but the guitarfish did not. Both hemagglutinating and precipitating antibody to hemocyanin were also observed in the primary response of the black bass.

4. The bowfin was successfully sensitized to *Ascaris* antigen, and lesions of the delayed type developed after challenge at varying intervals following sensitization.

5. The horned shark (*Heterodontus franciscii*) regularly cleared hemocyanin from the circulation after both primary and secondary antigenic stimulation, and regularly formed hemagglutinating antibody, but not precipitating anti-

body, after both primary and secondary stimulation with this antigen. These animals regularly cleared bacteriophage from the circulation after both the primary and secondary stimulation with bacteriophage T_2. Significant but small amounts of antibody were produced in a few animals in the primary response, and larger amounts in the responding animals after secondary antigenic stimulation.

6. Studies by starch gel and immunoelectrophoresis show that the hagfish has no bands with mobilities of mammalian gamma globulins; that the lamprey has a single, relatively faint band of this type; and that multiple gamma bands are characteristic of the holostean, elasmobranchs, and teleosts studied. By this method of study, the bowfin appeared to have substantial amounts of $gamma_2$ globulin.

7. We conclude that adaptive immunity and its cellular and humoral correlates developed in the lowest vertebrates, and that a rising level of immunologic reactivity and an increasingly differentiated and complex immunologic mechanism are observed going up the phylogenetic scale from the hagfish, to the lamprey, to the elasmobranchs, to the holosteans, and finally the teleosts.

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EXPLANATION OF PLATE 1

FIG. 1. The California hagfish, *Eptatretus stoutii*.

FIG. 2. Delayed reactivity to *Ascaris* antigen in *Amia calva*. The lesion developed after 12 hours, was red and indurated, typical of the delayed reaction in higher forms.

(Papermaster *et al.*: Evolution of immune response. I)