A SEROLOGICAL STUDY OF THE COMMON KILLIFISH, *FUNDULUS HETEROCLITUS* L.

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Roy Alton Hyle, II. A SEROLOGICAL STUDY OF THE COMMON KILLIFISH, FUNDULUS HETEROCLITUS L. (Under the direction of Dr. Patricia Daugherty) Department of Biology, February 1966.

The purpose of this study was to investigate the possibility of individual variations in the erythrocyte antigens of Fundulus heteroclitus and to investigate the use of serological techniques in subpopulation studies of the species. Slide agglutination with normal serum of the species and with other selected sera (human anti-A, human anti-B, and normal human serum, and anti-Fundulus rabbit serum) was employed.

Individual differences in the erythrocyte antigens were shown by titer and absorption reactions with the selected sera. Reagents for racial studies are discussed.

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INTRODUCTION

Isoagglutination, the agglutination of human blood cells by serum of other individuals observed by Karl Landsteiner in 1900, initiated blood group research as a field of immunology and led to the discovery of the ABO blood group system in humans. In 1910, Von Dungen and Hirszfeld demonstrated the genetic control of the antigens of the ABO groups. In 1919, Hirszfeld showed differences in the frequencies of these blood groups in various populations (Ridgway, 1957).

Blood group antigens are detected by their reactions with specific antibodies. Sera containing these antibodies may be prepared by injecting the erythrocytes of the species being studied into another organism. Heteroimmune antiserum is prepared by injecting erythrocytes into an individual of another species. A more specific antiserum, isoimmune antiserum, may be prepared by injecting erythrocytes into an individual of the same species. Normal sera of some individuals contain isoantibodies which are reactive with cell antigens of individuals of the same species; also sera of some individuals contain normal antibodies which are reactive with cell antigens of other species. A serum capable of detecting the presence or absence of an antigen is termed a reagent, but some sera are not in this form and require alteration. The technique of absorbing a serum with selected erythrocytes yields a serum in reagent form containing desirable antibodies (Cushing, 1964).

In the late 1930's, Irwin, Cumley, and Cole demonstrated the genetic control of the erythrocyte antigens of several species of Columbidae. Their classical work involved basic theory and practical technique in blood group research in animals. Specific antisera were prepared by

injecting rabbits with erythrocytes of two species of doves. Cells of each species were agglutinated by the antiserum prepared against cells of the same species, and also by the antiserum prepared against cells of the other species. Absorption patterns showed that there were particular antigens characteristic of each species. The mode of inheritance of the antigens was revealed by the study of hybrids of the two species. The antisera to each parent species agglutinated the cells of the hybrids even after absorptions by the other parent, showing that specific antigens were inherited (Irwin, 1947).

The erythrocyte antigens of fish received little attention until the early 1950's. Cushing (1952) was able to differentiate between the blood of the yellowfin tuna (Thunnus albacares, (Bonnaterre)) and that of the skipjack tuna (Katsuwonus pelamis (L.)) on the basis of the ability of their sera to agglutinate cells of the human blood groups. Cushing and Sprague (1953) demonstrated the occurrence of antigens similar to human A and B antigens in the bloods of various fish. They suggested the use of serological differences in migrational and evolutionary studies.

As serological studies progressed, advantages could be seen in the use of erythrocyte antigens in fishery biology. They had been shown to be genetically determined and unaffected by environmental variations. Also antigen frequencies had been shown to differ significantly in races of man and cattle. Once appropriate antisera were prepared, the test for the antigen was very simple; that these reagents would be most valuable in studies of migration or evolution was suggested by Ridgway (1957).

The frequency of a genetically determined erythrocyte antigen in a population could reveal whether subpopulations were alike or different, whether they were freely interbreeding and exchanging genes, or whether they were isolated races within the species (Cushing, 1956).

It was this aspect of blood group serology of fish that gained the interest of the student of migration and evolution and of the fishery biologist concerned with conservation and management programs. Prior to serological studies, information in fishery programs was obtained from tagging and from studies of age and growth. This new serological tool presented a criterion which offered more accurate conclusions, and therefore stimulated additional investigation into the serology of fish (Sindermann, 1961).

In 1956, Ashurst tried unsuccessfully to show isoagglutination differences in whiting (Gadus merlangus L.), pouting (Gadus luscus L.), and plaice (Pleuronectes platessa L.) (Cushing, 1964). However, Hildemann (1956) was able to show differences in the erythrocyte antigens of the goldfish (Carassus auratus (L.)) using isoimmunization and absorption techniques. Moreover, typing of siblings resulting from goldfish crosses showed specificities which definitely indicated inherited individual differences.

Others were able to reveal individual variations in the erythrocyte antigens of fish. In 1956, Cushing presented observations of individual variations in the erythrocyte antigens of the oceanic skipjack tuna and species variations among other tuna using a number of antisera prepared in rabbits. Cushing reviewed Ashurst's and Hildemann's studies concluding that either isoagglutinins may not occur in some species, or they are more difficult to demonstrate in

some species than in others.

In 1959, Sindermann and Mairs made an intensive study of the serology of the Atlantic sea herring (Clupea harengus L.) using prepared antisera and the absorption technique. They were able to isolate an antigen designated as antigen "C". Subpopulation studies were conducted by typing the frequency of "C" positive individuals of various area populations. On the basis of frequencies, an eastern and western subpopulation, in a sample area along the Maine coast, were distinguished.

Sindermann (1961) showed individual differences in the red cell antigens of the Atlantic redfish (Sebustes marinus (L.)). Absorptions of prepared antisera revealed two antigens, A_1 and A_2. A limited amount of frequency typing was undertaken, and racial studies were proposed. In the same year, Sindermann and Mairs established a blood group system in the dogfish (Squalus acanthias L.) using isoagglutination and absorbed antisera. As a result of this study, the "S" blood group system was established containing two antigens, S_1 and S_2. Although most of the work in serological taxonomy had been based on red cell agglutination, Mairs and Sindermann (1962) employed four methods, photronreflectometry, agar diffusion, paper electrophoresis, and erythrocyte agglutination to show antigenic relationships between five species of Clupeiods, alewife (Alosa pseudoharengus (Wilson)), bluback herring (Alosa aestivalis (Mitchell)), American shad (Alosa sipidissima (Wilson)), Atlantic sea herring (Clupea harengus L.), and Atlantic menhaden (Bervoortia tyrannus (Latrobe)).

Sprague and Vrooman (1962) reported an intensive study of the erythrocyte antigens of over two thousand Pacific sardines (Sardinops caerulea (Girard)). Two blood group systems, B and C, were distinguished. Frequency typing of the antigens of the C system demonstrated that there are northern and southern subpopulations along the California coast. In 1964, further frequency typing by Vrooman established a third subpopulation in the gulf of California. Also, in the Pacific, Sprague and Holloway (1962) obtained evidence of subpopulations in the skipjack tuna (Katsuwonus pelamis (L.)) by typing the frequency of erythrocyte antigens using various prepared reagents (plant extracts, normal cattle serum, and immune rabbit sera).

Sanders and Wright (1962) identified blood groups in the rainbow trout (Salmo gairdnerii (Gibbons)) and the brown trout (Salmo turtta L.) using prepared antisera and absorptions. A two-allele genetic system with three phenotypes was identified in the rainbow trout. It was also found that the frequencies of these phenotypes differed among rainbow trout populations. Four blood group phenotypes were shown in the brown trout; however, only two phenotypes were identified among the yearlings of the brown trout. Ridgway (1962), using isoimmunization, found individual variation in the antigenic content of the erythrocytes of the rainbow trout and red salmon (Oncorhynchus nerka Walbaum), and distinguished four antigenic types in the trout.

Utter (1964) used plant extracts in serological studies of various species of fish. Bark extracts of legumes revealed

individual variations in the red cell antigens and were recognized as potentially useful reagents in blood group research in fish.

One point should be noted. These agglutination patterns are referred to as blood group systems, however, this term should be regarded as a special one, since in most of these studies the necessary knowledge concerning the genetic control is not available (Sprague, 1962). It may be assumed, however, that these erythrocyte antigens are under genetic control as are the blood cell antigens of other animals.

The major objectives of this study were to investigate the possibility of individual variations in the erythrocyte antigens of Fundulus heteroclitus and to investigate the use of serological techniques in subpopulation studies of the species. Slide agglutination with normal serum of the species and with other selected sera was employed.

MATERIALS AND METHODS

Collection and Maintenance of Specimens

During the month of June, 1965, specimens for this study were collected in the James River on inshore tidal flats around Deep Creek Harbor, Newport News, Virginia (Figure 1). Collecting was done at night during low tide. Funneled traps, and a seine were utilized for collecting. Crab or menhaden served as bait.

Because facilities were not available for maintaining large numbers of fish in the lab, a float was constructed and anchored in the James River collecting area. This float was used for holding fish. The fish in the float were fed crab.

As fish were needed, they were brought from the float to the college in styrofoam ice chests. Upon arrival, all fish were dipped in a mild formalin solution (1 part formalin to 50 parts sea water), after which they were transferred to tanks of synthetic sea water (Seven Seas Marine Mix) maintained at a salinity of approximately 27 to 30 parts per thousand. The tanks were aerated and filtered. Fish in the lab were fed a commercially prepared fish food.

Blood Sample Collection, Preparation and Preservation

For blood sample collection, a fish was anesthetized with MS-222 Sandoz (Engle and Davis, 1964). The fish was then pinned to a dissecting pan. The gill opercula were clipped away and the ventral aorta exposed and cut at its narrowest constriction (Cushing, 1956). Immediately after cutting the aorta, the blood was drawn with

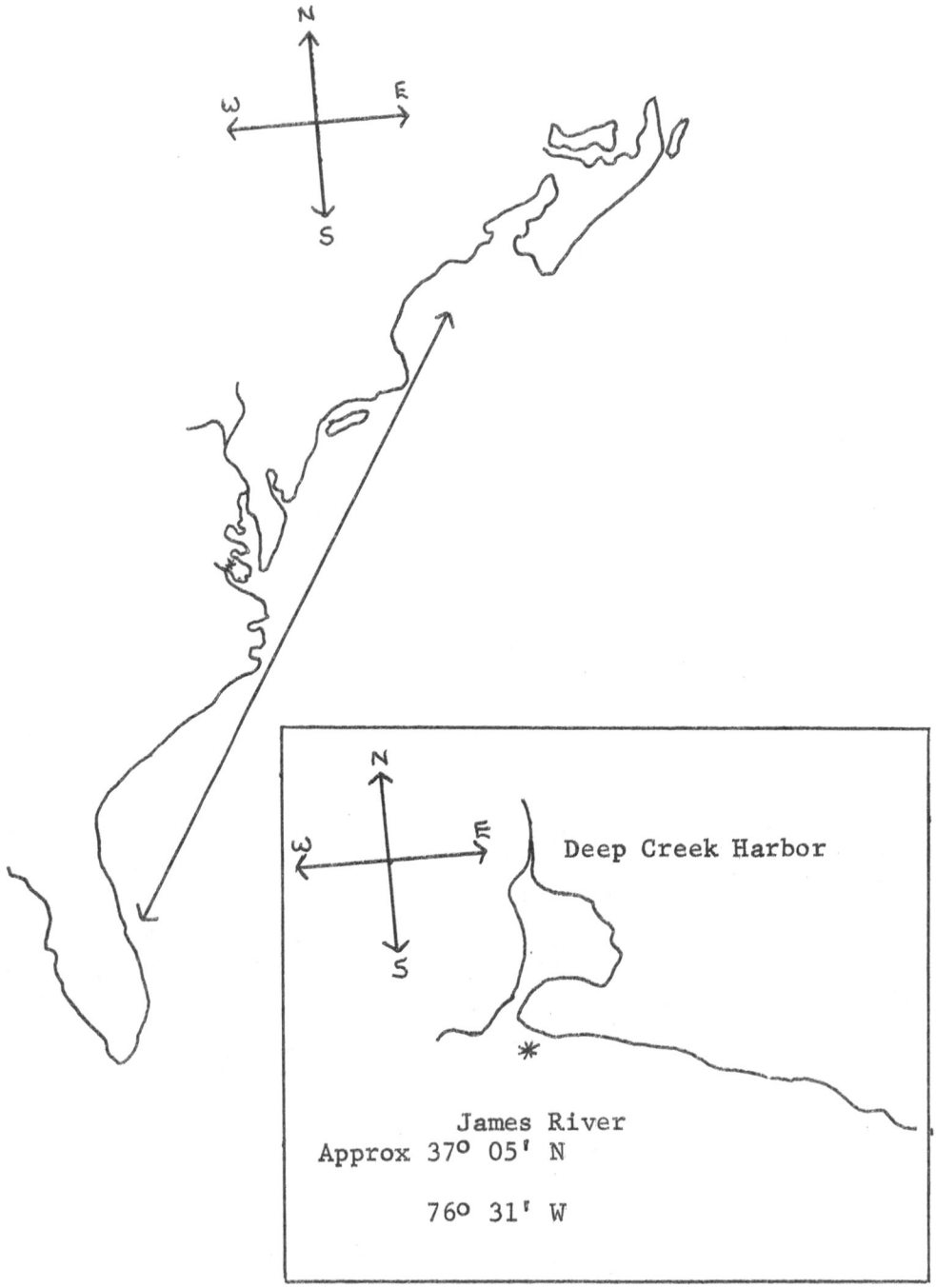

Figure 1. Range of Fundulus heteroclitus and collecting area.

a bright-line hemocytometer blood pipette into Alsever's solution (Sprague-modified, 1962, 16 grams of sodium citrate, 41 grams of dextrose, 14.5 grams of sodium chloride, 1.1 grams of citric acid with volume brought to 2 liters by addition of distilled water). Cells were washed three times with Alsever's solution by centifugation at approximately 2500 rpms with an International Clinical Centrifuge. Cell suspensions, approximately four percent, were made in Alsever's solution and held. Absorptions were performed immediately. Agglutination reactions were run within an eight hour period after collection. Cells held well in Alsever's solution at 4° C; however, after storage, the cells became fragile and less reactive in agglutination tests.

Antiserum Preparation and Preservation

Anti-Fundulus rabbit serum was prepared by injecting rabbits with a four percent cell suspension pooled from twelve fish (Figure 2). Injections were made intraveneously in the marginal vein of the ear (Campbell, 1963). The injection schedule was as follows: an initial injection of 1 ml, a booster of 1 ml after two weeks, a booster of 0.1 ml after three weeks, the titer checked, and subsequent boosters of o.1 ml every two weeks. Blood was collected from the ear of the rabbit by allowing the blood to drip into a test tube from a longitudinal slit in the marginal vein (Campbell, 1963). By this method approximately ten mls of blood could be collected at each bleeding. The serum was separated from the cells, inactivated for complement by heating for thirty minutes at 56° C, and stored by freezing at -20° C (Sindermann and Mairs, 1961). The titer of the antiserum was checked and standardized with human blood cells, type A. The titer was also checked periodically

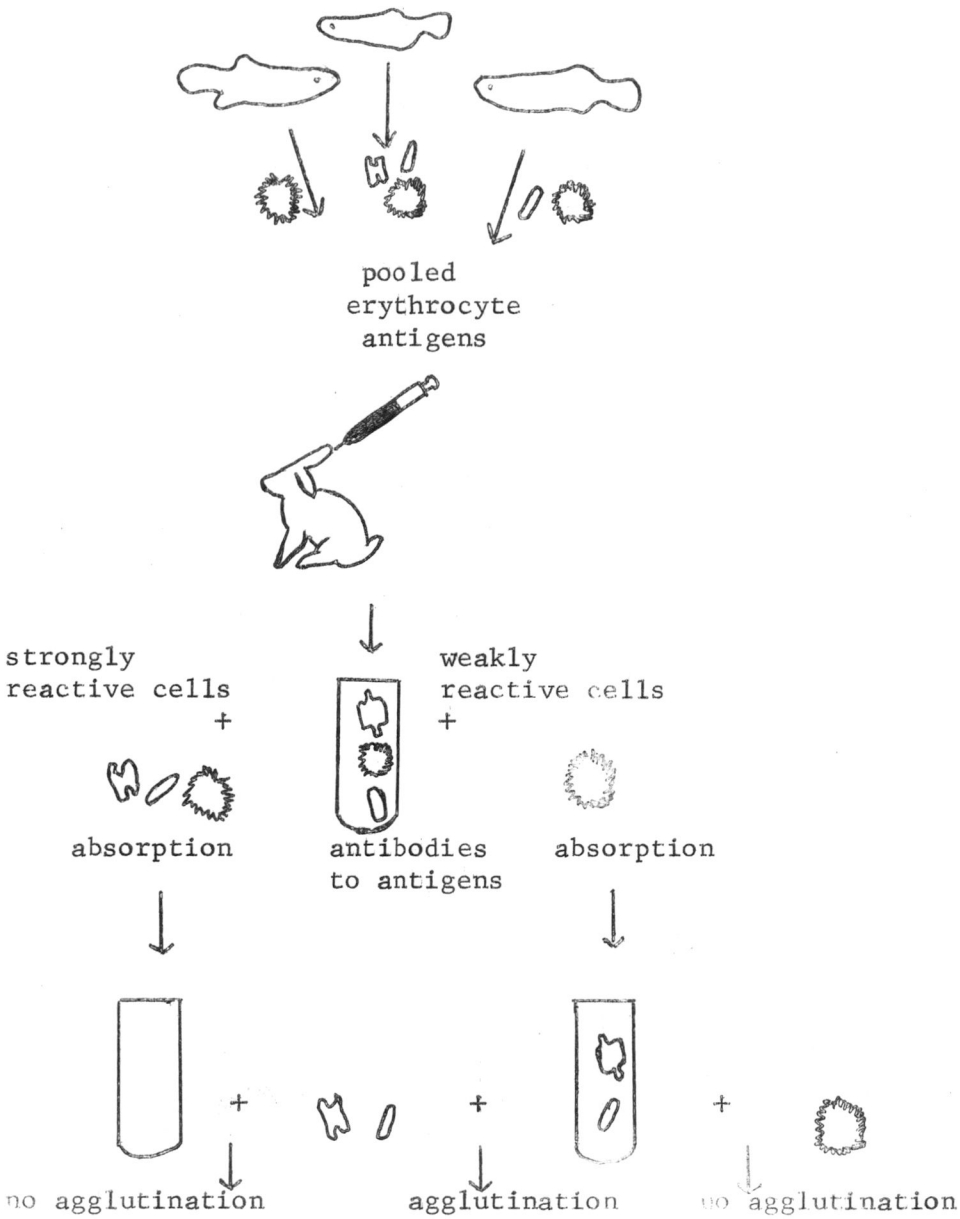

Figure 2. Diagramatic representation of the method employed to disclose individual differences in blood cell antigens.

and was found not to change throughout the experimental period.

Blood Typing Technique

Immediately after collecting the blood from the fish, four percent suspensions were prepared. The volume of the cell suspensions was divided; a portion was used for agglutination tests and a portion was used for absorptions.

In this study, four types of sera were employed, human anti-A, human anti-B, normal human, and prepared anti-Fundulus rabbit serum. Serum dilutions (1:2, 1:4, 1:8, 1:16, 1:16, etc.) were prepared in one percent saline (Boyd, 1957). Microscopic slide agglutination technique was used; a capillary tube (Kimax o.1-1.2 I.D. x 100mm) was used to transfer one drop of serum and one drop of cell suspension to a depression slide. The serum-cell suspensions were stirred with wooden applicator sticks and placed in a moist agglutination chamber (Boorman, 1961). The reactions were read after fifteen minutes using the conventional scale (4, 3, 2, 1, -), 4 to 1 representing declining degrees of agglutination and "minus" no agglutination (Figures 3-7). Saline controls were used in the titer agglutination reactions.

Absorptions were carried out by absorbing 0.3 ml of serum with approximately 0.01 to 0.05 ml of packed erythrocytes. The serum-cell suspensions were agitated by hand for several minutes and then placed in the refrigerator at 4^{o} C for a period of eight hours, usually over night. Absorbed sera were checked with the erythrocytes of the absorbing individuals, and if the absorptions were complete, the sera were held and utilized in the agglutination reactions. Because of the small cell

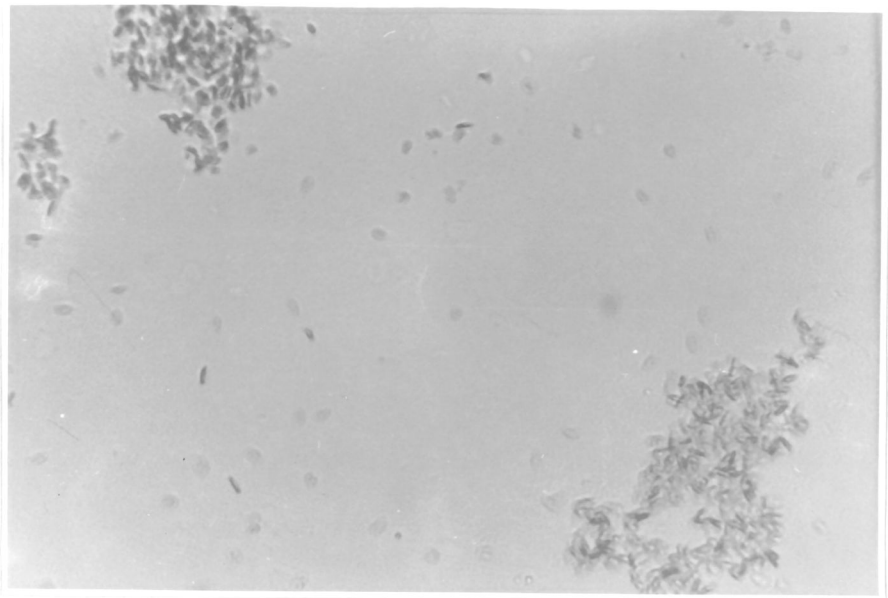

Figure 3. Photomicrograph of complete (++++) erythrocyte agglutination.

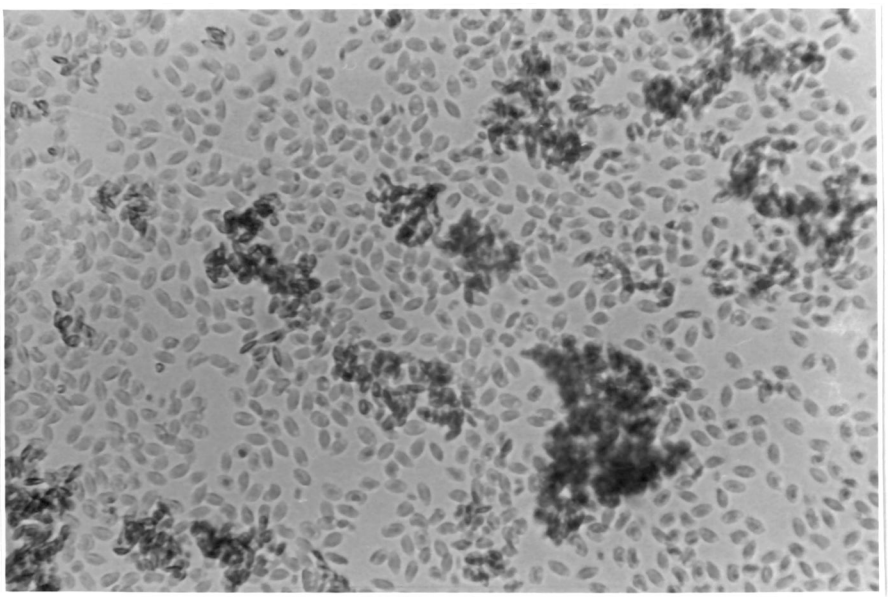

Figure 4. Photomicrograph of strong (+++) erythrocyte agglutination.

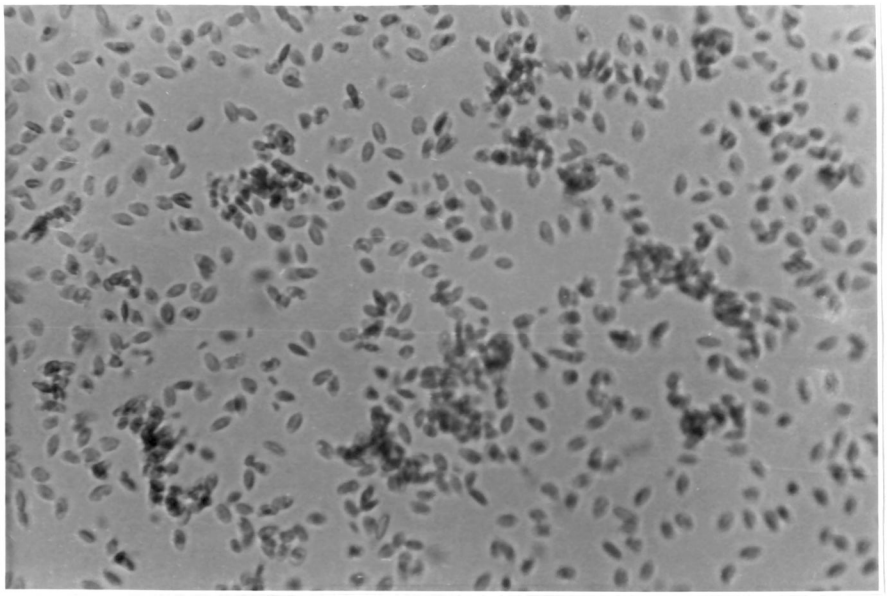

Figure 5. Photomicrograph of moderate (++) erythrocyte agglutination.

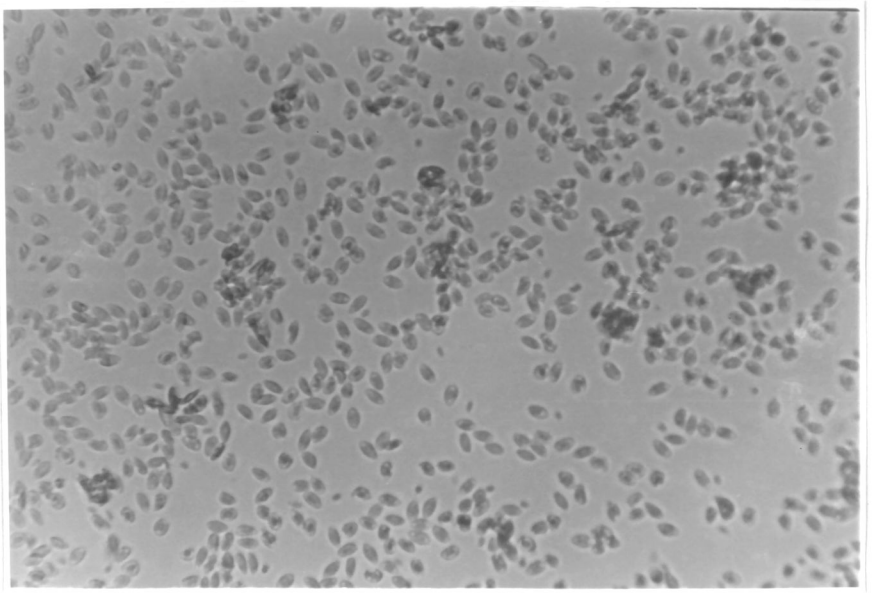

Figure 6. Photomicrograph of weak (+) erythrocyte agglutination.

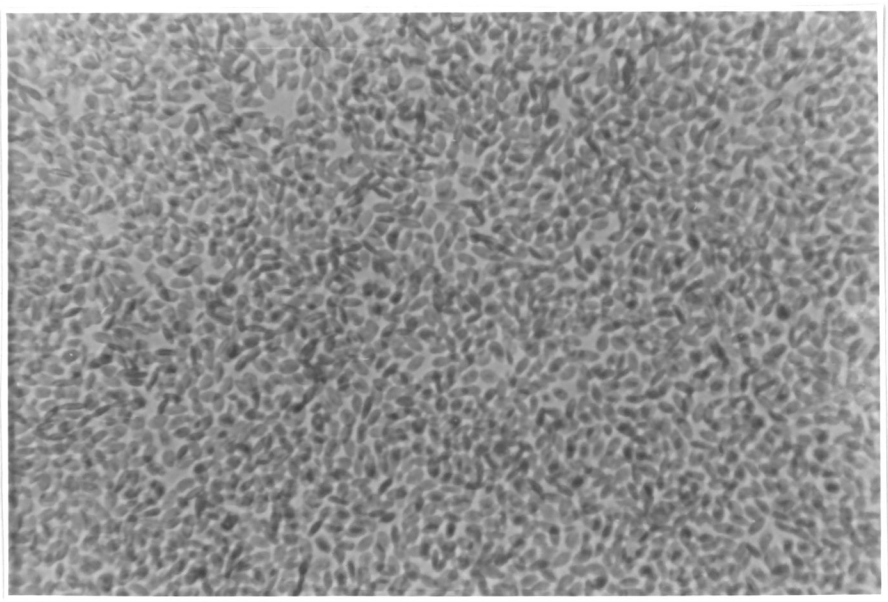

Figure 7. Photomicrograph of no (-) erythrocyte agglutination.

obtained from each fish, only one absorption could be performed. If it was not complete, the serum was rejected and not used in the agglutination tests.

Isoagglutination reactions were attempted. Blood was drawn from the ventral aorta by a capillary tube. One end of the capillary tube was sealed by heating. The tube was then centrifuged, after which the serum and cells were separated. The sera and cells were cross-matched by slide agglutination.

Anti-Fundulus rabbit serum in reagent form was prepared. Because of the time element, titer and absorption tests were run in sets. Cells of groups of ten individuals were used to check the titer of the serum. Serum was then absorbed with cells of the ten separate individuals. Agglutination patterns with the absorbed anitsera revealed weakly reactive individuals. The absorbed sera of these individuals were pooled and held as a reagent for future typing.

RESULTS

Individual differences in the erythrocyte antigens of Fundulus heteroclitus were first demonstrated by agglutination tests with undiluted human anti-A, anti-B, and normal sera. Fish nos. 4, 15, 17, and 21 (Tables 1a and 1b) showed distinct differences in the agglutination reactions as compared with the others observed. Dilution series reactions (Tables 2, 3, and 4) also revealed individual differences. Fish no. 38 exhibited a higher titer with all three sera than any of the others observed; fish no. 29 exhibited a lower titer than any of the other fish in the series of reactions.

Isoagglutination reactions (Tables 5a and 5b) were totally negative. No individual differences were disclosed through the use of normal serum of the species.

Agglutination reactions with anti-Fundulus rabbit serum disclosed individual differences in erythrocyte antigens. This difference was first indicated by titer differences (Table 6). Titer differences were observed in sets of dilution series reactions, and absorptions support - ed the observed differences in the titer. Certain cells are absent from the absorption results; these cells were lost through lysis, or they were rejected because of incomplete absorptions.

The following sets of reactions involved titer tests and absorptions. Some fish were disclosed which were less reactive than others of the set. The cells of these fish did not remove antibodies characteristic of other fish from the antiserum; these fish were designated "F" negative individuals.

Table 1a. Reactions of cells of Fundulus heteroclitus with selected sera

Cells	Human Anti-A	Human Anti-B	Human Normal
1	++++	++++	++++
2	++++	++++	+++
3	++++	++++	++++
4	++	++	-
5	++++	++++	++++
6	++++	++++	++++
7	++++	++	++
8	+++	+++	++++
9	++++	++++	++++
10	++++	++++	++++
11	+++	+++	+++
12	++++	+++	+++

Table 1b. Reactions of cells of Fundulus heteroclitus with selected sera

Cells	Human Anti-A	Human Anti-B	Human Normal
13	++++	++++	++++
14	++++	++++	++++
15	-	-	-
16	++	++	-
17	-	-	-
18	++++	++++	++
19	++	+++	-
20	+++	++++	-
21	-	++	-
22	++	+++	++
23	++	++	++
24	++++	++++	++++

Table 2. Reactions of cells of Fundulus heteroclitus with human anti-A serum

Cells	Dilutions	1:2	1:4	1:8	1:16
29		++	-	-	-
30		+++	++	-	-
31		++++	++	+	-
32		++	++	-	-
33		+++	++	++	-
34		+++	+++	-	-
35		++++	+++	++	-
36		++++	+++	+	-
37		++++	++	-	-
38		++++	++++	++	++
39		++++	+	-	-
40		++++	+++	-	-
55		+++	+++	+	-
56		++++	+++	+	-
57		++++	+	-	-
58		++++	+	-	-

Table 3. Reactions of cells of Fundulus heteroclitus with human anti-B serum

Cells	Dilutions	1:2	1:4	1:8	1:16
29		+	-	-	-
30		++	++	-	-
31		++	-	-	-
32		++	-	-	-
33		+++	++	-	-
34		+++	++	-	-
35		+++	+++	+	-
36		+++	+++	-	-
37		+++	++	-	-
38		++++	++++	+++	+++
39		+++	+	-	-
40		+++	+	-	-
55		++	-	-	-
56		++++	++	+	-
57		+	-	-	-
58		++	+	-	-

Table 4. Reactions of cells of Fundulus heteroclitus with normal human serum

Cells	Dilutions	1:2	1:4	1:8	1:16
29		-	-	-	-
30		+	-	-	-
31		-	-	-	-
32		-	-	-	-
33		-	-	-	-
34		-	-	-	-
35		-	-	-	-
36		-	-	-	-
37		++	-	-	-
38		++++	+	-	-
39		-	-	-	-
40		-	-	-	-

Table 5a. Isoagglutination reactions (cross-matching of cells and sera of individual fish)

Cells	Sera	25	26	27	28
25		-	-	-	-
26		-	-	-	-
27		-	-	-	-
28		-	-	-	-

Table 5b. Isoagglutination reactions (cross-matching of cells and sera of individual fish)

Cells	Sera	48	49	50	51	52	53
48		-	-	-	-	-	-
49		-	-	-	-	-	-
50		-	-	-	-	-	-
51		-	-	-	-	-	-
52		-	-	-	-	-	-
53		-	-	-	-	-	-

Table 6. Reactions of cells of Fundulus heteroclitus with anti-Fundulus rabbit serum

Cells	Dilutions 1:2	1:4	1:8	1:16	1:32	1:64
54	++++	++++	++++	+++	+	+
55	++++	++++	++++	++++	+++	++
56	++++	++++	++++	+++	+	-
57	+++	++++	++++	++++	+++	+
58	++++	++++	++++	+++	-	-
59	++++	++++	++++	++++	++	-
60	++++	++++	++++	++++	+++	-
61	++++	++++	+++	++	+	-

In the first set of reactions (Tables 7a and 7b) one fish, no. 63, was shown to be less reactive than others of the set. Cells of this fish did not clear the antiserum of antibodies capable of agglutinating cells of fish nos. 64, 65, and 68. The cells of several other fish failed to remove the antibodies against antigens of fish no. 65.

The second set of reactions (tables 8a and 8b) disclosed one "F" negative individual; fish no. 75 failed to remove from the antiserum antibodies against antigens of any other fish in the set.

Fish no. 84 was distinguished as a least reactive individual in the third set (Tables 9a and 9b). After absorption with the cells of fish no. 84, antibodies remained in the antiserum which were reactive with the cells of fish nos. 83, 86, and 87.

In the fourth set of reactions (Tables 10a and 10b) two least reactive fish, nos. 101 and 103, were disclosed. Both revealed similar absorption patterns; neither removed antibodies against antigens of fish nos. 95, 96, or 98. In the fifth set of reactions (Tables 11a and 11b) only slight individual differences were indicated.

The sixth set of reactions (Tables 12a and 12b) distinguished two "F" negative individuals. Antiserum absorbed by fish nos. 116 and 123 gave positive reactions with cells of fish nos. 114, 115, 117, 119, 120, 121, and 122; antibodies against antigens characteristic of these fish were not removed.

Fish no. 126 was shown to be less reactive than the other fish of the seventh set (Tables 13a and 13b). Antibodies reacting with cells of other members of the set were left in the antiserum after absorption by fish no. 126.

The eighth set of reactions (Tables 14a and 14b) revealed one "F" negative fish, no. 139, whose cells did not remove antibodies for antigens of other fish in the set from the antiserum.

Pooled anti-Fundulus rabbit serum, pooled from abosrptions by "F" negative individuals, distinguished two antigenic types (Table 15). Again those individuals which reacted negatively to the pooled absorbed antiserum were designated "F" negative; those individuals which reacted positively to the pooled absorbed antiserum were designated "F" positive.

All saline controls were negative.

Table 7a. Reactions of cells of Fundulus heteroclitus with anti-Fundulus rabbit serum (set I)

Cells	Dilutions 1:2	1:4	1:8	1:16	1:32	1:64
62	++++	++++	++++	++	++	-
63	++++	++++	+++	+	-	-
64	++++	++++	+++	+	-	-
65	++++	++++	+++	+	+	-
66	+++	++	++	+	-	-
67	+++	++	++	+	-	-
68	++++	++++	+++	++	-	-
69	++++	++++	++++	+	-	-

Table 7b. Results of absorbing anti-Fundulus serum with cells of individual fish (set I)

Serum Absorbed by Cells	Cells 62	63	64	65	66	67	68	69
62	-	-	-	-	-	-	-	-
63	-	-	++	++	-	-	+	-
64	-	-	-	-	-	-	-	-
65	-	-	-	-	-	-	-	-
66	-	-	-	+	-	-	-	-
67	-	-	-	+	-	-	-	-
68	-	-	-	+	-	-	-	-
69	-	-	-	+	-	-	-	-

Table 8a. Reactions of cells of Fundulus heteroclitus with anti-Fundulus rabbit serum (set II)

Cells \ Dilutions	1:2	1:4	1:8	1:16	1:32	1:64
74	++++	++++	++++	+++	+	-
75	++++	++++	++++	++++	++	-
76	++++	++++	++++	++++	+++	+
77	++++	+++	+++	+++	++	+
78	++++	++++	++++	+++	+	-
79	++++	+++	++++	+++	++	+
80	++++	+++	+++	++	++	-
81	++++	+++	+++	+++	+++	+
82	++++	+++	+++	+++	+	-
83	++++	++++	++++	++++	+++	+

Table 8b. Results of absorbing anti-Fundulus rabbit serum with cells of individual fish (set II)

Serum Absorbed by Cells \ Cells	74	75	76	77	78	79	80	81	82	83
74	-	+++	+++	+++	+++	+++	+++	+++	+++	+++
75	-	-	-	-	-	-	-	-	-	-
76	-	-	-	-	-	-	-	-	-	-
77	-	-	-	-	-	-	-	-	-	-
78	-	-	-	-	-	-	-	-	-	-
79	-	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-	-

Tables 9a. Reactions of cells of Fundulus heteroclitus with anti-Fundulus rabbit serum (set III)

Cells	Dilutions	1:2	1:4	1:8	1:16	1:32	1:64
84		++++	+++	++	-	-	-
85		++++	++++	++++	+++	++	-
86		++++	+++	+++	++	-	-
87		++++	++++	++++	+++	+	-
88		++++	++++	++++	++	-	-
89		++++	++++	++++	+++	++	-
90		++++	+++	+++	++	-	-
91		++++	++++	++++	+++	+	-
92		++++	++++	+++	+++	++	-
93		++++	++++	+++	+++	+	-

Table 9b. Results of absorbing anti-Fundulus rabbit serum with cells of individual fish (set III)

Serum Absorbed by Cells \ Cells	84	85	86	87	88	89	90	91	92	93
84	-	++	++	-	-	+++	-	-	-	-
85	-	-	-	-	-	-	-	-	-	-
86	-	-	-	-	-	-	-	-	-	-
87	-	-	-	-	-	-	-	-	-	-
88	-	-	-	-	-	-	-	-	-	-
89	-	-	-	-	-	-	-	-	-	-
90	-	-	-	-	-	-	-	-	-	-
91	-	-	-	-	-	-	-	-	-	-
92	-	-	-	-	-	-	-	-	-	-
93	-	-	-	-	-	-	-	-	-	-

Table 10a. Reactions of cells of Fundulus heteroclitus with anti-Fundulus rabbit serum (set IV)

Cells \ Dilutions	1:2	1:4	1:8	1:16	1:32	1:64
94	++++	++++	++++	++++	+++	-
95	++++	++++	++++	+++	++	-
96	++++	++++	++++	+++	++	+
97	++++	++++	++++	++	++	-
98	++++	++++	++++	++++	++	+
99	++++	++++	++++	+++	++	-
100	++++	++++	++++	++	++	-
101	++++	++++	+++	++	-	-
102	++++	++++	++++	+++	++	-
103	++++	++++	+++	++	-	-

Table 10b. Results of absorbing anti-Fundulus rabbit serum with cells of individual fish (set IV)

Serum Absorbed by cells \ Cells	95	96	97	98	99	100	101	102	103
95	-	-	-	-	-	-	-	-	-
96	-	-	-	-	-	-	-	-	-
98	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-
101	++	++	-	++	-	-	-	-	-
103	++	++	-	++	+	+	-	-	-

Table 11a. Reactions of cells of Fundulus heteroclitus with anti-Fundulus rabbit serum (set V)

Cells	Dilutions 1:2	1:4	1:8	1:16	1:32	1:64
104	++++	++++	+++	++	++	+
105	++++	++++	+++	++	+	-
106	++++	++++	++++	+++	+++	+
107	++++	++++	+++	++	++	+
108	++++	++++	+++	+++	++	+
109	++++	++++	++++	+++	++	+
110	++++	++++	+++	+++	++	+
111	++++	++++	++++	+++	++	+
112	++++	++++	+++	++	++	+
113	++++	++++	+++	++	++	+

Table 11b. Results of absorbing anti-Fundulus rabbit serum with cells of individual fish (set V)

Serum Absorbed by Cells	Cells 104	105	106	107	108	109	110	111	112	113
106	-	-	-	-	-	-	-	-	-	-
107	-	-	-	-	-	-	-	-	-	-
109	-	-	+	+	-	-	-	-	-	-
110	-	-	+	+	-	-	-	-	-	-
111	-	-	-	-	-	-	-	-	-	-
113	-	-	-	-	-	-	-	-	-	-

Table 12a. Reactions of cells of Fundulus heteroclitus with anti-Fundulus rabbit serum (set VI)

Cells	Dilutions	1:2	1:4	1:8	1:16	1:32	1:64
114		++++	++++	++++	++++	+++	++
115		++++	++++	++++	++++	+++	++
116		++++	++++	+++	++	+	-
117		++++	++++	++++	+++	+++	++
118		++++	++++	+++	++	++	-
119		++++	++++	++++	+++	+++	+
120		++++	++++	++++	+++	++	+
121		++++	++++	++++	++++	+++	++
122		++++	++++	++++	++++	+++	+
123		++++	++++	+++	+++	++	-

Table 12b. Results of absorbing anti-Fundulus rabbit serum with cells of individual fish (set VI)

Serum Absorbed by Cells	Cells	114	115	116	117	118	119	120	121	122	123
114		-	+	-	-	-	+	-	+	-	-
115		-	-	-	-	-	+	-	-	-	-
116		+	+	-	+	-	+	+	+	+	-
117		-	-	-	-	-	-	-	-	-	-
120		-	-	-	-	-	-	-	-	-	-
123		+	+	-	+	-	+	+	+	+	-

Table 13a. Reactions of cells of Fundulus heteroclitus with anti-Fundulus rabbit serum (set VII)

Cells	Dilutions 1:2	1:4	1:8	1:16	1:32	1:64
124	++++	++++	++++	++++	+++	+
125	++++	++++	++++	++++	+++	++
126	++++	++++	+++	++	++	-
127	++++	++++	++++	+++	+++	+
128	++++	++++	++++	++++	++	-
129	++++	++++	++++	++++	+++	++
130	++++	++++	++++	++++	+++	++
131	++++	++++	++++	+++	++	-
132	++++	++++	++++	+++	++	-
133	++++	++++	++++	++++	+++	+

Table 13b. Results of absorbing anti-Fundulus rabbit serum with cells of individual fish (set VII)

Serum Absorbed by Cells	Cells 124	125	126	127	128	129	130	131	132	133
124	-	-	-	-	-	-	-	-	-	-
125	-	-	-	-	-	-	-	-	-	-
126	++	++	-	++	++	+++	+++	++	++	++
127	-	-	-	-	-	-	-	-	-	-
128	-	-	-	-	-	-	-	-	-	-
133	-	-	-	-	-	-	-	-	-	-

Table 14a. Reactions of cells of Fundulus heteroclitus with anti-Fundulus rabbit serum (Set VIII)

Cells \ Dilutions	1:2	1:4	1:8	1:16	1:32	1:64
134	++++	++++	++++	++++	+++	+
135	++++	++++	++++	++++	+++	+
136	++++	++++	++++	++++	+++	+
137	++++	++++	++++	++++	++++	+
138	++++	++++	++++	++++	++++	+
139	++++	++++	+++	++	+	-

Table 14b. Results of absorbing anti-Fundulus serum with cells of individual fish (set VIII)

Serum Absorbed by Cells \ Cells	134	135	136	137	138	139
134	-	-	-	-	-	-
135	-	-	+	-	-	-
136	-	-	-	-	-	-
137	-	-	-	-	-	-
138	-	-	+	-	-	-
139	+	+	+	+	+	-

Table 15. Reactions of cells of Fundulus heteroclitus with pooled absorbed anti-Fundulus rabbit serum

Cells	Pooled Absorbed Serum
144	+
145	+
147	+
148	+
149	-
150	-
151	-
152	-
153	-
154	+++
155	-
156	-
157	-
158	+++
159	-

DISCUSSION

Fundulus heteroclitus exhibits a wide north and south range (Perlmutter, 1963), and is found far up the estuaries along the Atlantic coast. It seems probable that this species occurs as genetically isolated subpopulations. Since, because of size, the species does not lend itself to tagging, and since its range is so broad, a logical approach to obtaining information about subpopulations within the species would be antigen frequency studies of various area populations.

Races may be distinguished by determining a variable character which is genetically controlled and by calculating its frequency in populations being studied. Racial studies have been the primary objective of blood group research on fish. A genetically determined character such as a blood cell antigen can be used to distinguish between races or subpopulations in species of fish. The investigations in the introduction indicate the extensiveness of studies with this view in mind.

The genetic control of erythrocyte antigens of vertebrates has been clearly demonstrated by blood group research in doves (Irwin, 1947) and in fish (Hildemann, 1956; Sanders and Wright, 1962). The most complete presentation of the inheritance of erythrocyte antigens of fish was reported by Sanders and Wright (1962). Their work involved two species of the genus Salmo. The assumption that the red cell antigens of Fundulus heteroclitus are under genetic control is based on the above works and other investigations.

Erythrocyte antigens are detected by their reactions with specific antibodies. A sensitive test for these antigen-antibody reactions is erythrocyte agglutination. Sera containing specific antibodies are obtained from various sources. Normal heterologous sera are normal sera of individuals of a species containing antibodies reactive with erythrocyte antigens of a different species under investigation. Normal homologous sera are normal sera of individuals of a species which may contain isoagglutinins or other antibodies reactive with erythrocyte antigens of the same species. Heteroimmune sera are sera prepared by injecting erythrocytes of a species into individuals of another species.

Individual differences in the erythrocyte antigens of Fundulus heteroclitus were shown in this study. Cell antigens were disclosed using normal heterologous sera (human anti-A, human anti-B, and undifferentiated normal human serum) and a heteroimmune serum (anti-Fundulus rabbit serum).

Cells of most of the fish tested were agglutinated by antibodies in human anti-A, human anti-B, and normal human serum. The cells of approximately 10% of the fish tested were not agglutinated by these undiluted heterologous sera. Similar variations in the erythrocytes were shown by titer differences in dilution series of all three normal heterologous sera. The relative affinity for cell antigens for antibodies of normal heterologous sera varied; some fish reacted strongly and others weakly. Absorptions of normal heterologous sera were unsuccessful.

Variations were also shown by titer differences with anti-Fundulus rabbit serum, and these differences were supported by absorption patterns. Tables 7-14 show titer and absorption reactions. Individual fish which exhibited a low titer were also observed not to remove antibodies from the immune serum. These individuals differed from the rest of a set in their erythrocyte antigens. Variations in titer and supporting absorption patterns indicate a qualitative difference in the erythrocyte antigens of Fundulus heteroclitus.

Isoagglutinins were not demonstrated in this series of experiments. This was not surprising in view of previous work; isoagglutinins were not shown in whiting, pouting, and plaice (Cushing, 1964); however, Cushing and Durall (1957) and Sindermann and Mairs (1961) have disclosed isoagglutinins in the brown bullhead and spiny dogfish, respectively. A small serum volume obtainable from each Fundulus heteroclitus limited the number of cross-matches of sera and cells. Another consideration is that the serum is diluted when mixed with the cell suspensions; low titers (1:4) were observed by Sindermann and Mairs (1961) in isoagglutination reactions of spiny dogfish. It is doubtful that isoagglutinins can be used to study the erythroycte antigen system of Fundulus heteroclitus.

The antigenic differences detected with heterologous and heteroimmune sera indicate differences in the genetic material controlling the antigens of Fundulus heteroclitus. The presence of an erythrocyte antigen is evidence of the presence of the controlling gene; the absence of this erythroycte antigen is evidence of the absence of the controlling gene.

The main technical problem in working with a species such as Fundulus heteroclitus is blood cell volume. The quantity obtained from each fish was exceptionally small (0.01 to 0.05 ml of packed erythrocytes). Attempts to prepare a large volume of specific antiserum or of human sera in reagent form were unsuccessful because it was not possible to clear the human sera by absorption, and only a small volume of specific immune serum could be prepared by absorption. It was necessary to pool absorbed immune sera of the "F" negative individuals to obtain a volume of immune serum for typing. Although this pooled serum was in reagent form, as indicated in Table 15, it was less specific than an immune serum absorbed by a single fish would have been. Because of the small immune serum volume and of inactivity and lysis of cells after storage, it was not possible to investigate differences or similarities among "F" negative individuals.

Since it is difficult to prepare a large volume of specific immune serum, normal heterologous sera containing specific antibodies appear to be the most promising reagents for distinguishing the presence or absence of the erythrocyte antigens of the species. Sera are needed which are in reagent form without alteration. Individual fish were found which reacted negatively to the unaltered human sera, indicating the absence of one or more antigens. These normal heterologous human sera appear to be potential reagents for studying the antigenic system of area populations of Fundulus heteroclitus.

SUMMARY

Individual differences in erythrocyte antigens have been demonstrated in the common killifish, Fundulus heteroclitus. Detection of such variations in erythrocyte antigens was possible with normal heterologous sera and with heteroimmune serum.

Specific immune serum can be prepared against the blood of the common killifish. However, it can be prepared in small volumes only. Normal heterologous sera appear to be potential reagents, and subpopulation studies are suggested.

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