

SEROLOGICAL STUDIES OF SCALLOP BLOOD

By Wayne Frair

1961 Summer Research done at

U.S. Department of the Interior, Fish and Wildlife Service, Bureau
of Commercial Fisheries Biological Laboratory, Woods Hole, Massa-
chusetts.

Woods Hole Laboratory

Report No. 61-10

SEROLOGICAL STUDIES OF SCALLOP BLOOD

1961 Summer Research of Wayne Frair
Fisheries Laboratory
Woods Hole, Massachusetts

This project was to determine if there were serological methods which could be used to distinguish among sea scallops (Placopecten magellanicus) taken at different regions along the Atlantic Coast. If differences other than environmentally-caused variations could be established, it would mean that separate gene pools existed and thus that distinct populations were present. The possibility exists that there is considerable mixing during larval stages; so we hoped that serological methods would be useful tools in determining similarities and differences so far not discovered with biometric tools.

Certain vertebrate populations have been distinguished by their blood groups and plasma proteins. For the most part these are hereditary factors. My plans were organized so that methods used in these other studies would be adapted to our present problem.

Where and how to obtain blood first was solved. In the scallop, blood passes through a network of vessels and sinuses. The heart lies in front of and to right of an observer looking down at an upper valve. A needle can be inserted into the heart and blood withdrawn without even separating the valves. Insertion is made just forward of the hinge at crotch of mantle. Blood (5 to 10 ml) can be taken periodically with no apparent serious injury to a large scallop (length of 12 cm plus). If a larger volume of blood is desired or if heart has stopped beating, blood may be withdrawn from the large sinus of adductor muscle just adjacent to pericardial cavity. Insertion of a needle is facilitated if valves are propped open. Fifty-five ml have been obtained from a 15 cm animal. Usually when more than 20 ml were removed, the scallop died shortly afterward or at least within a few days. As with heart-puncture method, periodic small bleedings from the sinus have been successful.

During course of this project, about 150 blood samples were taken usually from the sinus. Several comparisons were made between bloods from heart and sinus, and a higher concentration of cells and serum protein was found in sinus blood. This is to be expected because heavier components would tend to remain in sinuses where volume was greater and turnover less.

In order to carry on it was necessary to know osmotic pressure of blood. Earlier workers had found tonicity similar to surrounding sea water. I checked this by preparing two pools of blood and then measuring their density with a hydrometer. With simple calculations involving use of Knudsen's Tables, these values were converted to salinity. All measurements were done with solutions at 26 degrees. Several serial dilutions of sodium chloride solutions were checked along with sea water in our recirculation tank and sea water off government jetty.

Pool #1 came from 10 scallops (6 females and 4 males) ranging in size from about 13 to 16 cm, all having been taken from the channel of Georges Bank. These scallops had been in the tank of recirculating refrigerated water for several weeks. Pool #2 was composed of blood from 20 scallops (10 females and 10 males) from the northern edge of Georges Bank, and size was 11.8 to 14.0 cm. These were bled within 4 days from capture, but they had been in our tank for two days.

<u>Source</u>	<u>Salinity</u>
Jetty Sea Water	3.43%
Pool #2	3.29%
Pool #1	3.27%
Tank Sea Water	3.20%

Salinities as determined and shown above were equal to similar percentages by weight of sodium chloride dissolved in distilled water. On basis of data from these tests I decided to use 3.3% sodium chloride as suspension medium for scallop blood cells. These cells can tolerate a wide range of salinity, for in early tests I kept some cells in 1% sodium chloride for more than a week. pH of scallop blood checked by meter showed values of 7.1 to 7.4 with freshly-drawn blood (ph of sea water is near 8).

The next question was how should one treat blood cells to save them for testing. When blood is taken from a scallop, it is slightly cloudy because of suspended blood cells. Within several minutes these cells are forming aggregates which stick to sides or settle to bottom of blood container. Blood serum then appears clear as water. Some cells could be broken from aggregates and resuspended in isotonic saline, but I felt that it would be better to prevent aggregation and also to preserve from decay in one operation if this were possible.

Clumping of scallop cells differs from blood coagulation of vertebrates where a fibrin network is formed. I found that usual anticoagulants, namely heparin, citrate and oxalate did not prevent aggregation. Answer came with a dilute solution of formaldehyde. When blood just after removal was squirted into about an equal volume of 3.3% saline containing 1/1,000 formaldehyde, aggregation was prevented probably because of some "fixing" (possibly a protein denaturation) of cells. If blood remained too long in a clean syringe, aggregation would begin there. Formaldehyde treatment not only prevented aggregation but also preserved cells for later agglutination tests.

In addition to preserving cells, formaldehyde prevented contamination of serum. When blood was taken under non-sterile conditions, serum became cloudy within hours (even under refrigeration) unless some preservative was added. Formaldehyde-treated serum remained clear. Other preservatives found effective were phenol at 0.3% and merthiolate at 1/5,000 to 1/10,000. Technics most satisfactory just for preserving serum involved centrifuging down aggregated cells and then pouring off serum. Serum then was frozen or had merthiolate added to it. As an additional precaution, preserved serum was refrigerated.

In serum phases of following studies I felt that it might be necessary to know how much protein was present. Literature revealed two studies in which protein had been determined on Pecten maximus off Europe (See Engle and Woods, '60). These values served as a guide to planning, but I found a lower concentration; in fact my results are as low as (and possibly lower than) protein values reported for circulating fluid of any animal.

Attempts first were made with turbidimetric procedures. Using a Beckman DU spectrophotometer I was able to get a good straight line with a bovine albumin standard dilution series in range of scallop blood using 0.75% potassium ferrocyanide in acid medium as protein precipitant. (Trichloroacetic acid at 10% also worked). Preliminary readings on scallop blood appeared valid and reproducible, but this method was abandoned because a slight turbidity of scallop serum could cause an increased reading.

Also I tried the new Bausch and Lomb refractometer and ultraviolet absorption (280 millimicrons) with a Beckman DU spectrophotometer to get protein concentration. The refractometer read zero with scallop blood, and values calculated from spectrophotometer readings were higher than expected possibly because of some turbidity of scallop blood.

Use of biuret reagent next was attempted. This reagent has been used directly with many vertebrate blood serums in a colorimetric method of protein determination, but with scallop blood (sea water also) a white precipitate is formed. Therefore, it was necessary first to precipitate protein from blood serum, 10% trichloroacetic acid being used. This was centrifuged at 10,000 RCF x G. Acid was added until no further precipitate formed. One half or one ml samples of scallop blood serum were used, and about 1 to 3 ml of trichloroacetic acid was used for each. Supernate was discarded, and precipitate was solubilized with one half ml of 9% (2.5 N) sodium hydroxide. Two ml of biuret reagent were added and the solution left for one-half hour at room temperature. Then it was checked for absorption in a Beckman DU spectrophotometer set at 550 millimicrons (slit 0.04 mm).

Standard was rehydrated sheep serum powder whose protein content had been determined by Kjeldahl analysis before lyophilization. After adding distilled water to this powder, I made a serial dilution of it and checked protein and amino acid contents by calculation from absorptions at 280 and 260 millimicrons. After the tubes of diluted standard were put through the process using biuret reagent, spectrophotometer readings at 550 millimicrons were plotted against serial protein dilutions (0.05 to 1.60 mg/ml). Points lay in a straight line.

8

Determinations on scallop blood were made using two pools of 20 serums each, one pool of 10, and 9 individual serums. Average value for the 59 was 0.23 mg/ml protein. My results indicate that normal range for protein is 0.14 to 0.29 mg/ml when blood is taken from a living animal. The Georges Bank pool #1 from 10 specimens in captivity for several weeks showed lowest value (0.16 mg/ml) whereas Georges Bank pool #2 from recently caught scallops was 0.27 mg/ml. A pool of 20 Cape Cod serums from freshly-caught scallops had an intermediate protein value of 0.23 mg/ml. Most determinations were repeated one or more times. Values for serum which had been untreated, thawed, and preserved with phenol and merthiolate did not differ significantly. One determination on a pool of 5 Aequipecten showed 0.50 mg/ml protein. Two comparisons of heart and muscle protein of different scallops showed 5 and 6 mg/ml higher for muscle than heart. This agreed with an earlier test by turbidimetric means with serum from another scallop. Addition of petroleum ether (a lipid solvent) to dissolved biuret-treated protein caused about 3 to 5 mg/ml drop in value after centrifuging. But centrifuging non-ether-treated controls also caused a drop in reading; so likely drop was caused by removal of a small amount of suspended matter. Even though a value of 0.14 to 0.29 mg/ml appears to be normal range for serum protein, one individual had a value of 0.35 for heart and 0.42 for muscle.

Much higher protein values (0.84 mg/ml) were obtained on a pool of 20 serums taken from scallops which had just arrived by plane from Canada. These animals were dead or just at point of death. Blood had to be obtained from base of muscle sinuses. Only a few ml could be taken from most.

For electrophoresis studies on serums from Canadian, Cape Cod and Georges Bank populations, I used the new cellulose acetate strip method of Kohn ('60; see also Scherr, '61). Serum was dialyzed against Karo or Carbowax (Polyethylene glycol 6000) to concentrate it about 10 times so that components could be observed more easily. (In one case I concentrated some serum 40 times.) On electrophoretic strip, the fastest-moving component could be observed in undialyzed serum and easily was seen when stained with nigrosin. Using Veronal buffer of pH 8.6 and ionic strength of 0.1 M in a run of 1 hour 45 minutes at constant voltage of 125 V with an 8 cm gap, after staining with Ponceau S, it was possible to distinguish 3 protein bands. Under above conditions, component moving fastest toward the anode was most abundant and had some mobility as human albumin. With TRIS buffer (Aronsson and Gronwall, '57) at 100 V for 2 hours, two components easily were separated and leading fraction moved faster than human albumin.

Two runs (16 samples) were made in agar, and some strips from cellulose acetate runs were used in attempts to get precipitation arcs after antiserum was added along edges of strips. These immunoelectrophoresis attempts with both agar and cellulose acetate oddly enough showed precipitin arcs only at position where sample was applied. It appears that possibly a highly antigenic substance did not migrate in the electric field.

Antiserums used in immunoelectrophoresis studies and following studies were produced in rabbits. Proteins were precipitated from the serums with about 1% tannic acid solution. Precipitate was centrifuged and supernatant discarded. Then precipitate was washed several times by shaking in physiological saline, and centrifuging. Supernatant was discarded each time. A suspension of this precipitate in saline was injected into ear vein of rabbit on 3 or 4 consecutive days. If no antibody was present one week after last injection, as shown by ring test, another series of injections was given. In these subsequent series, injections of first 1 or 2 days were given subcutaneously and following ones intravenously. Antibodies appeared to be produced quite easily in the 3 rabbits in response to precipitated protein.

11

Blood cells used as antigens were washed 3 times with physiological saline and injected in series as with serum. Three rabbits which were used responded well as antibody producers. One rabbit which produced antibodies against scallop serum and one which produced scallop-cell antiserum in addition to scallop material also were given injections of washed scup cells against which they produced antibodies as well.

Using antiserum to scallop serum, ring tests were made with undiluted serums. A titre as high as 1,000,000 was found. With Ouchterlony plates (See Ouchterlony, 61,) prepared with 0.5 M Veronal buffer pH 8.6 and 2% agar as many as 4 clear lines (and possibly another less distinct line) were observed. This was done using cellulose acetate medium (Kohn, '60) and several good precipitation lines developed. In agar and cellulose acetate, there was joining of major precipitation lines between serums of different populations. When antiserum to Placopecten was tested against Aequipecten, no precipitation lines developed in Ouchterlony plates.

12

Hemagglutination tests were made using rabbit erythrocytes coated with tannic acid (1/5,000 dilution). To individual aliquots of suspension of coated cells were added (1) serum from Georges Bank pool of 20 specimens, (2) serum from Canadian pool of 20 specimens, (3) serum from pool of 20 Cape Cod specimens, and (4) serum from an Aequipecten pool of 5 specimens. When these coated cells were tested against anti-Georges Bank serum, titre was 256 with Georges and Cape, 64 with Canadian and 32 with Aequipecten.

Other methods used in pilot runs have been Kohn's ('60) Oudin strip technic with scallop bloods and antiserum against 3 populations; and also a chromatographic technic with serums of Cyprina, Mercenaria, Mya, Spisula, and Placopecten (3 populations).

Above mentioned research with serums and antibodies have not, I feel, so far given evidence of population differences. There are species differences! It is possible that extension of some work here might prove fruitful along these lines, but it appears that studies with cells offer more hope of distinguishing populations.

Washed cells in about 2% suspension were put in equal volume (2 drops) with rabbit antiserum in Kahn tubes. These small tubes were left at room temperature, centrifuged periodically and checked for extent of agglutination during a 5-hour period.

Agglutinations were recorded as negative (0) or weak to strong positive (+ to ++++). Twenty cell suspensions were used from each population. All scallops were sexually mature and ranged in size from 109 to 165 mm. Record was kept of sexes (about half males). No correlation appeared to exist between sex and reactivity of cells.

Serum prepared against Canadian cells showed agglutinations with Canadian cells which equaled agglutinations with Cape cells, but Georges values were one third lower. Anti-Cape cells easily distinguished Cape cells from both Canadian and Georges, but Canadian values were closer to Cape than were Georges. Anti-serum to Georges cells gave powerful agglutination and did not distinguish among populations. See chart for summary.

From results of agglutinations, there is suggestion that separate populations do exist, and it appears that reactions of Canadian and Cape cells are more like each other than like Georges cells. These data lend support to a hypothesis that mixing of scallop larvae occurs between Canadian and Cape populations. Georges Bank scallops may be more isolated.

SUMMARY OF SCALLOP CELL AGGLUTINATIONS

No.*	Anti-Canadian against cells of			Anti-Cape Cod against cells of			Anti-Georges Bank against cells of		
	C	CC	G	C	CC	G	C	CC	G
1.	++	0	0	++	+	++	+	+	+++
2.	++	+	+	++	++++	+	±	+++	+++
3.	+	+	0	0	++++	+	+	+	+
4.	++	0	0	0	+	++	+	+	+
5.	0	0	+	+	++++	++	+	+	+++
6.	++	+++	++	+	+	0	++	++++	++
7.	++	++	++	+	++	0	+++	+++	++
8.	+	++	++	+	+	0	++	++	++
9.	++	++	++	0	+++	0	+++	++	++
10.	++	++	0	++	+	+	+++	++	++
11.	++	++	++	+	++	0	+++	+++	++
12.	0	++	+	0	+	0	+++	+++	+
13.	++	++	+	+	0	0	++	++	+
14.	+++	++	+	0	0	0	++	+++	+
15.	+++	++	0	+	0	0	++	++	+++
16.	0	++	0	0	++	0	+++	+++	++
17.	++	++	+	0	++	0	++	+++	++
18.	0	++	+	0	++	0	++	+++	++
19.	++	0	+	0	+	0	0	+++	++
20.	++	+	+	0	+	0	+++	+++	+++

* No. - Cell suspension number. This applies to C, CC, or G specific suspension.

- C - Canadian cells
- CC - Cape Cod cells
- G - Georges Bank cells

Bibliography

- Aronsson, T., and A. Grönwall. 1957. Improved separation of serum proteins in paper electrophoresis--a new electrophoresis buffer. *Scandinav. J. Clin. Lab. Investigation.* 9: 338-341.
- Engle, R. L., Jr., and K. R. Woods. 1960. Comparative biochemistry and embryology, p. 183-265. In F. W. Putnam, [ed.], *The plasma proteins.* Academic Press, New York.
- Kohn, J. 1960. Cellulose acetate electrophoresis and immunodiffusion techniques, p. 56-60. In I. Smith, [ed.], *Chromatographic and electrophoretic techniques.* Interscience, New York.
- Ouchterlony, O. 1961. Interpretation of comparative immune precipitation patterns obtained by diffusion-in-gel techniques, p. 5-19. In M. Heidelberger and O. J. Plescia, [ed.], *Immunochemical approaches to problems in microbiology.* Rutgers Univ. Press, New Jersey.
- Scherr, G. H. 1961. Cellulose acetate electrophoresis in microbiology and immunology. *Trans. N. Y. Acad. Science* 23: 519-530.