THE RELATIVE NUMBERS OF IMMATURE ERYTHROCYTES IN THE CIRCULATING BLOOD OF SEVERAL SPECIES OF MARINE FISHES

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During the course of a study of the reactions of the erythrocytes of fishes to vital dyes (Dawson, 1932) in which brilliant cresyl blue was used to produce patterns of reticulation, it became increasingly obvious that the numbers of immature erythrocytes present in the circulation varied widely in different species. In some fishes the numbers of immature cells were negligible while in others they attained considerable proportions.

These observations were made in the summer of 1931 at the Marine Biological Laboratory on seventeen different species. At that time blood was routinely drawn from the heart. Later it seemed possible that the source of the blood samples might in some measure be responsible for the variations encountered, since in many of the lower vertebrates the final differentiation of a considerable number of erythrocytes commonly takes place in the intertrabecular spaces of the ventricle. Variations in the depth of penetration of the needle might then be suspected as a possible cause of the variations in the number of immature cells, depending on whether the tip of the needle stopped in the intertrabecular spaces or passed into the main channel of the ventricle.

Consequently it was decided to repeat the study, drawing samples of blood from both the caudal vessels and the heart. In the summer of 1932 it was possible to re-study the blood of many forms which had been available in 1931 as well as to extend the study to include several additional species. In no case, however, was the blood from the heart and the caudal vessels found to vary appreciably in the number of immature erythrocytes present. Accordingly, it was concluded that the earlier observations were significant and that the blood of the different species of fishes may show specific differences in the proportions of immature cells present in the circulation.

Information on this subject appears to merit some attention. The erythrocytes of fishes are widely used by physiologists in studies on respiration and hemolysis, and the varying number of immature cells present should be taken into consideration when comparisons of the behavior of the blood of different fishes are made.

Material and Methods

As previously stated, these studies were carried on at Woods Hole in the summers of 1931 and 1932. The blood of the fishes studied in 1931 was drawn only from the heart, while in 1932 samples were taken from both the heart and caudal blood vessels. In many instances it was possible to secure in 1932 specimens of species studied the previous year, but in a few cases some species were not obtainable in the second summer. On the other hand, several additional species were available in 1932. In all, the proportion of immature cells in the circulating blood of twenty species was determined. Freshly-drawn blood was prepared by three different methods. Supravital preparations were made by adding a small drop of blood to a slide previously filmed with a saturated solution of brilliant cresyl blue in absolute alcohol. A circular coverslip was added and the preparation sealed with warm vaseline. In order to secure by this method slides on which accurate counts can be made, it is necessary to add the proper amount of blood, just enough so that when the coverslip is added the blood film will be one cell thick and barely reach the margin of the cover. Preparations which do not fulfill these requirements are unsatisfactory for quantitative studies.

More permanent preparations, also demonstrating the patterns of reticulation, were made by mixing the blood thoroughly with an oxalated solution of brilliant cresyl blue in cold-blooded Ringer's solution (Cook, Meyer and Tureen, 1931). A one per cent solution of brilliant cresyl blue and one per cent solution of potassium oxalate were used in the proportions of one drop of oxalate to five drops of stain. Equal parts of blood and oxalated stain were thoroughly mixed in a capillary pipette, spread thinly on a slide, and stained lightly by Wright's technique. In addition, ordinary smears stained with Wright's were employed to demonstrate the varying degrees of basophilia and polychromasia of the immature cells.

In general it was found that more accurate counts could be made when the patterns of reticulation were adequately developed with brilliant cresyl blue. Corresponding counts of immature cells in blood stained only by Wright's method ran uniformly lower, probably due to the fact that fatigue occurs much more rapidly when the observer has to evaluate continuously the varying degrees of basophilia and polychromasia present. Reticulation patterns, on the other hand, are definite and the amount of reticulation present is much more readily determined.

As shown in an earlier study (Dawson, 1932), remnants of the reticulation patterns persist in practically all the mature erythrocytes of fishes. The degree of persistence varies somewhat in different species

and also in the individual erythrocytes of the same species. When erythrocytes of varying degrees of differentiation are present, the distinction between maturity and immaturity must at times be a relatively arbitrary one. In this regard the slides doubly stained with brilliant cresyl blue and Wright's stain proved valuable, since the amount of reticulation and the degree of basophilia, polychromasia, or eosinophilia present in the individual cells could promptly be correlated.

For the purposes of this study the various types of erythrocytes encountered were arbitrarily divided into five classes as follows: (1) Mature erythrocytes, with the reticular material in scattered granules or isolated, irregular filaments and stained orthochromatically with eosin in Wright's; (2) less mature cells with the reticular material forming a loose, partially fragmented reticulum, staining orthochromatically with eosin or exhibiting a slight polychromasia; (3) younger cells with an open-meshed but practically complete reticulum and definitely polychromatic; (4) still younger cells with a close-meshed, complete reticulum exhibiting a decided basophilic tint when differentially stained; (5) young cells with the reticular substance massed in a dense, almost granular pattern, presenting little evidence of the true reticulation pattern. These cells give a definite basophilic reaction but are rarely seen except in the elasmobranchs.

Counts were made under oil immersion at a magnification of 900 diameters. In order to facilitate counting, a small coverslip was hand-ruled in India ink with a fine pen into areas 2.5 mm. square. This was used as an ocular micrometer at the level of the diaphragm. Four such areas could be used in the field of the microscope and their size was such that the number of erythrocytes seen in an individual square was small enough to be counted readily without any uncertainty. In 1931 only one sample was drawn from the heart but in 1932 an additional sample was drawn from the tail of each fish and two supravital preparations of each sample were counted. Twenty fields of four squares each were taken and the results averaged. In practice this meant that about one thousand cells were counted from each sample. These values were also checked by similar counts made on preparations doubly stained with brilliant cresyl blue and Wright's stain and on preparations stained with Wright's alone. The number of fishes of each species examined was not constant and data on this point will be included in the description of the findings.

Results

On the basis of the number of immature erythrocytes present in the circulation, the twenty species of fish fall naturally into four groups.

TABLE I

Counts of the several classes of erythrocytes present in the circulation of twenty species of fishes studied at Woods Hole during 1931 and 1932. Only the averages are given. Counts are recorded as "few" when no more than ten were encountered in two supravital preparations and "rare" when less than five were seen.

Groups	Species	Number of Individuals 1931	Number of Individuals 1932	I Isolated Granules and Filaments	II Fragmented Reticulum	III Open-meshed Reticulum	IV Close-meshed Reticulum	V Dense Granular Reticulum
				per cent	*per cent*	*per cent*	*per cent*	*per cent*
I	Mackerel, *Scomber scombrus* Linnaeus	4	2	83.8	3.3	4.3	8.6	0.0
	Menhaden, *Brevoortia tyrannus* (Latrobe)	4	2	83.5	3.0	2.4	11.1	0.0
	Alewife, *Pomolobus pseudoharengus* (Wilson)	4	0	83.3	3.6	2.2	10.9	0.0
	Summer Herring, *Pomolobus æstivalis* (Mitchill)	0	2	79.2	6.5	4.1	10.2	0.0
	Bluefish, *Pomatomus saltatrix* (Linnaeus)	0	2	84.7	5.9	3.2	6.2	0.0
	Common Eel, *Anguilla rostrata* (Le Sueur)	4	3	77.4	4.6	7.2	10.8	0.0
II	Variegated Minnow, *Cyprinodon variegatus* Lacépède	4	0	95.4	0.9	1.3	2.4	0.0
	Sea Robin, *Prionotus carolinus* (Linnaeus)	4	2	96.4	0.7	1.2	1.7	0.0
	Scup, *Stenotomus chrysops* (Linnaeus)	3	2	95.3	1.4	1.1	2.2	0.0
	Butterfish, *Poronotus triacanthus* (Peck)	3	3	96.2	0.8	1.0	2.0	0.0
III	Toadfish, *Opsanus tau* (Linnaeus)	4	0	99+	rare	rare	rare	0.0
	Tautog, *Tautoga onitis* (Linnaeus)	2	3	99+	few	rare	rare	0.0
	Cunner, *Tautogolabrus adspersus* (Walbaum)	3	1	99+	few	rare	rare	0.0
	Sea Bass, *Centropristes striatus* (Linnaeus)	4	0	99+	few	rare	rare	0.0
	Pipefish, *Syngnathus fuscus* Storer	0	4	99+	few	rare	rare	0.0
	Sand Dab, *Hippoglossoides platessoides* (Fabricius)	2	0	99+	rare	rare	rare	0.0
	Weakfish, *Cynoscion regalis* (Bloch and Schneider)	0	1	99+	rare	rare	rare	0.0
	Sharksucker, *Echeneis naucrates* Linnaeus	0	1	99+	few	rare	rare	0.0
IV	Smooth Dogfish, *Mustelus canis* (Mitchill)	4	4	80.6	5.4	3.4	7.6	3.0
	Spotted Skate, *Raja diaphanes* Mitchill	1	0	83.4	4.6	3.2	6.5	2.3

The first group comprises those in which the proportion of immature erythrocytes is strikingly high, approximately 20 per cent of the total erythrocytes. In the second group the number of immature red cells is much smaller, varying from 3 to 6 per cent. In the third group, the erythrocytes were practically all mature. The fourth group includes the elasmobranchs. These are grouped separately because their erythrocyte picture is quite different from that of the teleosts, resembling more closely conditions found in the cyclostomes, Dipnoi (Jordan and Speidel, 1930, 1931), and urodeles (Dawson, 1930). All stages of maturing erythrocytes, including erythroblasts, were seen and frequently erythrocytes in mitosis were encountered (Maximow, 1923). Summarized results are shown in Table I.

In reading this table it should be borne in mind that the mature erythrocytes of different species vary in the amount of persistent reticulation, the criterion for placing them in Class I being that the reticular material exists as isolated granules or scattered, irregular filaments which may even be branched. In general, the mature cells of the fishes of Group III contain relatively more remnants of the reticulation patterns than those of Groups I and II. Probably not too much significance should be attached to the relative numbers of cells in Classes II, III and IV; the end-points for separating them into these classes are not sharp, and different observers might vary appreciably in their determination of the classes into which such cells should be placed. The figures given are at least indicative of the conditions to be expected, but for quantitative work, where the degree of maturity of the erythrocytes is of importance, reticulation estimates should be made for each species studied.

Discussion

Many of the very active fishes are included in Group I. In general these fish are characterized by their inability to survive for any extended period in restricted quarters, such as the base of the aquarium stands in the Marine Biological Laboratory, even when a moderate inflow of sea water is provided. In the case of the common mackerel this sensitivity to confinement has been found by Hall (1930) to be due, in part at least, to the peculiar adaptation of its respiratory mechanism which permits the animal to secure sufficient oxygen only when it is moving rapidly forward, the ability to respire in a stationary position having been practically lost. The same explanation may hold for some others of this group, menhaden, alewife, summer herring and bluefish, but Hall's experiments apparently have not been extended to include them. In the case of the eel, however, there is no such sensitivity to

confinement; these animals may be kept in tanks for days at a time without exhibiting any signs of respiratory distress.

The fishes of Group II are less active than those of Group I and may successfully be kept in tanks, while most of those in Group III are relatively sluggish, frequently resting quietly in the water. Group IV, comprising the elasmobranchs, need not be discussed in this connection since their blood picture is quite unlike that of teleosts.

Several other sets of data compiled on the blood of teleosts parallel rather closely the present findings on the relative numbers of immature erythrocytes in the circulation. These data are based on determinations of hemoglobin concentration, size of the red corpuscles, numbers of corpuscles per cubic millimeter of blood, lowest oxygen tension at which fish are capable of removing dissolved oxygen from sea water, and the oxygen capacities of the blood.

A comparison of the hemoglobin concentration, measured in milligrams of iron per 100 cc. of blood, has been made of a group of marine fishes by Hall and Gray (1929). Highest concentrations, ranging from 41 to 35.5 mg., were found in the bonito, bull's-eye mackerel, common mackerel, and menhaden. A second group, with concentrations ranging from 23.7 to 27.7 mg., included the cunner, butterfish, scup and sea robin. The remaining fishes of this series vary more widely in this respect, showing concentrations ranging from 21.7 to 11.5 mg. Listed in a descending order these are the rudder fish, puffer, eel, silver hake, goosefish, toadfish and sand-dab. Reference to Table I will show a general agreement between my data and that of Hall and Gray, the most notable exceptions being the eel and cunner. The figures of Root (1931), covering several of the same species of fishes, also show that the high concentration of hemoglobin is associated with small cell-size, high corpuscle count, and high volume per cent of corpuscles.

It is quite evident that all those having high concentrations of hemoglobin are the more active fish. The conditions characterizing their blood presumably impose a greater burden on the erythropoietic centers in that a greater number of corpuscles must be maintained. Any considerable acceleration of the rate of erythrocyte production is rather generally accompanied by the appearance of less mature forms in the blood stream.

No satisfactory explanation has been given for this appearance of immature cells in the circulation. It is held by some that it is due to the limited volume of the erythropoietic organ. With a restricted storage capacity, partially mature cells are forced out to maintain room for the newly-forming cells (Barcroft, 1925). It is probable that some such factor as rate of erythrocyte production is operative in the fishes of Groups I and II.

The underlying stimulus for rapid erythrocyte production is not readily determined. In general the blood picture of active fishes resembles the high-altitude effect obtained in mammals, namely, an increase in total number of red cells and hemoglobin as well as an increase in the number of young cells, reticulocytes. Barcroft has pointed out that the basal principle of the effects of high altitudes is that the blood cell count is a function of the degree of anoxemia. The findings of Hall (1930) on the lowest oxygen tensions at which teleost fishes are capable of removing dissolved oxygen from sea water appear to bear directly on the problem.

Of the fishes studied by Hall the mackerel is unique, being unable to remove dissolved oxygen below a partial pressure of 70 mm. Hg. Other fishes cease respiration at the following tensions: rudderfish, 15.1 mm.; cunner, 14.8 mm.; scup, 13.1 mm.; butterfish, 11.8 mm.; sea robin, 8.5 mm.; fundulus, 8.1 mm.; puffer, 4.7 mm.; sea bass, 3.2 mm.; tautog, 1.6 mm.; and toadfish, 0.0 mm. The eleven species listed may be roughly divided into three groups. One group, represented by the mackerel, removes oxygen only at a relatively high tension, 70 mm. Hg. The second group removes dissolved oxygen between 8 and 15 mm. Hg. pressure, while the third group survives at tensions varying from 0.0 to 4.7 mm. Hg. There is thus a striking correspondence between this grouping of teleosts based on ability to remove dissolved oxygen from sea water and the grouping of the author according to the number of the immature erythrocytes present in the circulation. The cunner, however, is a notable exception.

In an earlier study by Hall (1929) of the scup and toadfish it was found that the scup showed very little change in its oxygen consumption with the oxygen tension between 120 and 40 mm. Hg. pressure but below 30 mm. Hg. pressure a very sharp decline in oxygen consumption occurred and the fishes quickly died whenever the pressure fell below 16 mm. Hg. In the case of the toadfish the oxygen consumption was found to be directly proportional to variations in the oxygen tension between 0 and 118 mm. Hg. Also the toadfish lived for 24 hours with the oxygen tension between 0 and 1 mm. Hg. pressure.

The studies of Hall (1931), Hall and Gray (1929), and Root (1931) indicate that there is a definite correlation between the transportation of oxygen and the environment and habits of the fishes. Adaptations of the respiratory mechanisms are important as shown by Hall (1930, 1931) in his studies of the puffer and mackerel, permitting one form to secure an adequate oxygen supply in a stationary position while the other can do this only when swimming rapidly forward. As already pointed out, sluggish fishes have blood with larger and less

numerous corpuscles, low concentrations of hemoglobin and low oxygen capacities, while active fishes have smaller and more numerous corpuscles, higher concentrations of hemoglobin, and high oxygen capacities—definite adjustments between capacities and requirements. Further evidences of adjustment are also found in the behavior of the hemoglobin at low carbon dioxide tensions. In the presence of 1 mm. of carbon dioxide, a considerably greater tension of oxygen is required to saturate the hemoglobin of the mackerel than that of either the sea robin or toadfish. Root (1931) thinks this may account in part for the great susceptibility of the mackerel to asphyxiation (Hall, 1930).

These morphological and physiological features of the blood, representing adaptations to habits and environment, tend to make the active fish susceptible to variations in the oxygen content of sea water. The high numbers of immature erythrocytes, while probably largely the result of the normal, rapid rate of production of these elements, may also tend to be further increased by stimuli resulting from anoxemia. In other words the margin of safety of active fishes is reduced and the normal condition of their blood may readily pass into a state characteristic of the high-altitude effect found in mammals.

These observations immediately raise the question as to what extent the proportions of immature cells found in the teleosts studied are due to the immediate response of these fishes to varying degrees of asphyxiation caused by methods of transportation and retention in tanks. Studies on the effects of asphyxiation on the menhaden and puffer have been made by Hall, Gray and Lepkovsky (1926) and Hall (1928). These observations showed that asphyxiation produced a definite increase in the concentration of hemoglobin and iron and in the number of red-blood corpuscles, the increase being roughly proportional to the length of time of asphyxiation (19 to 50 minutes). The increased concentration of the blood constituents was explained by a release of water from the blood into the tissues, since the blood volume became diminished during asphyxiation. Iron, however, did not increase in proportion to the loss of water or to the increase in concentration of hemoglobin, but at a faster rate. The spleen showed a decrease in size during asphyxiation and it was suggested that the spleen is a store-house for iron which is released into the blood during asphyxiation.

The observation on the reduction in the size of the spleen may be significant, since in the teleosts this constitutes the major erythropoietic center. In the menhaden, the red-blood cell count rose from 1,988,000 to 3,598,000 per cubic millimeter during 50 minutes confinement in a sealed jar containing 10 liters of water. Since differential counts for degree of persistent reticulation were not made, it is impossible to de-

termine whether the increased number of corpuscles per cubic millimeter was due solely to the withdrawal of water or whether additional and probably less mature erythrocytes had left the spleen to enter the circulation. If erythrocytes do leave the spleen during asphyxiation, as the decrease in its size suggests, it would seem to constitute a possible source of the immature erythrocytes encountered in the circulation.

In the present study the environment of the fishes, especially as regards oxygen tension, was not rigidly controlled. Fish were brought from the traps in the morning and delivered directly to the laboratory in pails of sea water, and many of the more active forms such as mackerel, menhaden, alewife, and summer herring frequently exhibited obvious signs of respiratory distress when received. However, counts made from alewife and mackerel taken directly from a floating "live car" did not differ appreciably from those made on fish delivered by the collecting staff to the laboratory. Since the oxygen tension of the sea water was not determined at the time blood was removed, no statement can be made as to the degree of asphyxiation, if any, to which the fish were subjected.

The prime purpose of this survey was to ascertain the relative homogeneity of the erythrocyte picture in fishes under ordinary laboratory procedures when no particular precautions are taken to insure an optimum environment—conditions such as may prevail when blood is obtained for studies on hemolysis and erythrocyte respiration. If the varying degrees of asphyxiation to which the fish were possibly subjected have modified the blood picture as described in this paper, then such errors are inherent in the data and no correction for them can be made at this time.

Accordingly the variations in the numbers of immature cells either may represent the varying ability of the fishes to respond to the adverse conditions encountered following removal from their natural environment or are evidences of the specific adjustments that have been made between the production of the oxygen-transporting elements, the red-blood cells, and the structural adaptations, the environment, and the habits of the fishes. In fact, these alternatives need not be mutually exclusive. The response to asphyxiation, if proven to occur, may be but an exaggerated picture of conditions normally present in a particular species.

In the elasmobranchs, as in the cyclostomes, Dipnoi, and urodeles, differentiation and multiplication of erythrocytes takes place to a variable extent in the peripheral circulation, and all stages, from their hemoblast progenitors to the completely differentiated cells, may be encountered. Mitosis may also occur in all stages of differentiation, short of

actual maturity. The counts include only cells which contain hemoglobin, the hemoblasts and proerythroblasts being omitted, and are based on large, freshly-caught specimens.

Young dogfish which had been kept in "live cars" for some time showed a higher number of immature cells as well as an increase in the number of mitoses. The blood appeared to be in an actively regenerative phase, probably brought about by the conditions of confinement and diminished food supply. The blood picture of elasmobranchs stands in striking contrast to that of teleosts where the erythrocytes enter the circulation in a much later stage of differentiation and no proliferation by mitosis is observed in the peripheral circulation.

Summary

The results of a study of the blood of the general circulation of twenty species of marine fishes are given. The number of immature erythrocytes present varies widely.

The differential erythrocyte counts were based largely on supravital preparations stained with brilliant cresyl blue. This material was supplemented by dry-fixed smears stained by Wright's method. The reticulation patterns produced by brilliant cresyl blue are discrete structures and more accurate counts can be made on these preparations than on stained smears where the varying degrees of basophilia and polychromasia are used as criteria of immaturity.

The twenty species of fish examined fall naturally into four groups. In Group I, including the mackerel, menhaden, alewife, summer herring, bluefish and common eel, the proportion of immature erythrocytes is high, approximately 20 per cent. In the second group, including the variegated minnow, sea robin, scup, and butterfish the percentage of immature cells is lower, varying between 3 and 6 per cent. In the third group, including toadfish, tautog, cunner, sea bass, pipefish, sand dab, weakfish and sharksucker, practically all the erythrocytes are mature. The fourth group, comprised of elasmobranchs, was treated separately since in these forms erythrocytes are continuously differentiated in the blood stream from primitive cells and may also proliferate mitotically in this location. The number of immature red cells in these fish is also great.

The varying blood pictures appear to represent the result of specific adaptations of fishes to such interrelated factors as their type of external respiratory mechanism, the efficiency of their oxygen-transporting system, their oxygen requirements and the oxygen tensions of their environment. In Group I the general blood picture is suggestive of the high-altitude effect observed in mammals.

Many of the fishes with high counts of immature erythrocytes quickly exhibit signs of asphyxiation when removed from their natural habitat and kept in tanks. Hall has noted that during asphyxiation the spleen of fishes decreases in volume. This may be correlated with the entrance of immature erythrocytes into the general circulation. Accordingly, it is possible that the figures presented in this paper are not exactly representative of the condition of the blood when such fish are undisturbed in their natural environment.

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