SECRETION OF GASES AGAINST HIGH PRESSURES IN THE SWIMBLADDER OF DEEP SEA FISHES.

I. OXYGEN DISSOCIATION IN BLOOD [1]

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By studying the composition of the swimbladder gases in relation to depth it was established that both oxygen and nitrogen are deposited into the swimbladder against a concentration gradient to make up the hydrostatic pressure. The nitrogen is deposited as a certain fraction of the oxygen varying from 2 to 15%, according to the species. Carbon dioxide and organic gases contribute only insignificantly to the pressure. The oxygen pressure commonly reaches values above 100 atmospheres, and it is likely that it can be two to three times as much, as species with swimbladders are known to occur down to a pressure of at least 266 atmospheres. The nitrogen pressure in deep sea species frequently reaches 10–15 atmospheres (Scholander and van Dam, 1953).[2] In whitefish (*Coregonus*) pure nitrogen may be deposited into the swimbladder against 6–8 atmospheres' pressure (Hüfner, 1892). Saunders (1953) has verified this finding, and has added four more species of physostome fresh water fishes which also seem to deposit nothing but nitrogen in the swimbladder.

CURRENT THEORIES OF GAS SECRETION

Haldane (1922) in his celebrated book "Respiration" gives his views as to a possible mechanism for the gas secretion, which in its basic concepts expresses the ideas offered by later authors. He saw in the *rete mirabile* a counter current exchange mechanism (p. 214) : "The arrangement reminds us of that in a regenerating furnace, where the heat carried away in the waste gases is utilized to heat the incoming air." As to the chemical release of oxygen he points out the importance of CO_2 in expelling oxygen from the hemoglobin and states (p. 215) : "It seems probable, therefore, that the function of the *rete mirabile* is to enable venous blood to communicate part of its CO_2 to the arterial blood. The effect of

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[2] In our 1953 paper data are given on gas samples from *Alepocephalus* and *Cottunculus*. From the excellent review of Jones and Marshall (1953) we learned that *Alepocephalus* does not have a swimbladder and this we verified on our recent material. We found also that *Cottunculus* has no swimbladder. This raises the question as to where the gas came from of which we obtained a few cubic centimeters by poking around in these fishes with our needle. The presence of the gas was obvious because the fishes were floating on the water. The composition of this gas checked well with that of the other fishes in the same catch, and hence it is believed that the fish must have swallowed some of the gas gushing out from other fishes when the trawlbag approached the surface. Obviously a fish distended by swimbladder gas cannot pollute this gas by swallowing.

this will be to raise the CO_2 pressure of the blood supplied to the gland, and so raise the oxygen pressure." Already Müller (1840) and Woodland (1911), who presented accurate anatomical drawings of the *rete,* had considered that they must be interpreted as a diffusion apparatus between the arterial and venous capillaries.

Hall (1924) found that, if the gas gland in yellow perch (*Perca flavescens*) was stimulated to activity by removing gas from the swimbladder or by increasing the hydrostatic pressure, a dialysate of the active gland showed a decrease in pH from 7.1 to 6.4, as compared with a non-active gland. He assumed secretion of an acid by the glandular epithelium. This would diffuse into the capillaries of the *rete* and by releasing bound oxygen would raise the oxygen tension, permitting the oxygen to diffuse into the swimbladder. If the oxygen capacity were 9 vol. % and the solubility coefficient 3 vol. % per atmosphere, then this mechanism could raise the oxygen tension no more than 3 atmospheres. This would be ample for shallow water fish like the perch, but could not explain the secretion of 100 atmospheres or more in deep sea fishes.

Jacobs (1930) found a way out of this difficulty by elaborating somewhat further on the possible function of the *rete mirabile.* When acid is secreted by the glandular cells and diffuses into the blood, CO_2 is released, increasing the CO_2 tension. The CO_2 effect itself, or possibly the secreted acid, dissociates oxygen from the oxyhemoglobin, so that both CO_2 and oxygen tension increase. This would explain the fact that both O_2 and CO_2 tension increase when gas is generated following a puncture. The blood with elevated O_2 and CO_2 tension leaves the gland and enters the efferent (venous) *rete,* where the gases diffuse over to the afferent (arterial) side, boosting the tensions there. When this high tension blood enters the gland, new acid is added, the tensions are again stepped up, and so on, until high enough tension has been built up in the swimbladder.

The secretion of oxygen would accordingly involve two principal steps: (1) a chemical dissociation of oxygen by acid (and/or CO_2) from the oxyhemoglobin, and (2) a counter current exchange mechanism (the *rete mirabile*) which would retain the secreted gases within the swimbladder with a minimal loss to the outside, in spite of maintained circulation. These basic ideas have been accepted by almost all later workers as the likely mechanism for oxygen secretion in fishes (Powers *et al.,* 1932; Koch, 1934; Irving and Grinnell, 1939; Black, 1946; Copeland, 1952; Strittmatter, Ball and Cooper, 1952; Fänge, 1953). Von Ledebur (1937) expressed serious doubts as to the correctness of the chemical interpretation.

In the present account we shall deal with the oxygen dissociation at pressures from 0.2 to 140 atmospheres and its relation to pH and CO_2. The function of the *rete mirabile* will be dealt with in a later account.

Oxygen Dissociation in Blood from Deep Sea Fishes in Relation to pH and CO_2

The chemical or physical reaction which is in the end responsible for the oxygen deposition in deep sea fishes must be able to perform against a pressure which at least equals the partial pressure of oxygen known to occur in the fish in question. Many deep sea fishes secrete oxygen against 100 atmospheres or more, and by inference some secrete against considerably more than 200 atmospheres.

It has long been known that the blood of many fishes possesses a large Bohr effect (Krogh and Leitch, 1919, and many later authors). Root (1931) and Green and Root (1933) found that in several fishes the blood did not become completely saturated if acid or CO_2 was added at oxygen pressures up to near one atmosphere. The saturation curves were found to run virtually horizontal at pressures above 0.5 atmosphere. The *Bohr effect* is generally considered a change in the dissociation constant (K in the Hill equation) with 100% saturation obtainable at high enough pressure. The term *Root effect,* on the contrary, may be reserved for the situation where acidity completely blocks the oxygen from part of the hemoglobin.

Obviously, before we can know whether the Bohr or Root effect can be responsible for the oxygen secretion against 100–200 atmospheres' pressure, we must extend the dissociation curves to these pressures. This has been done for a series of ocean fishes inhabiting depths equivalent to pressures from 40 to as much as 260 atmospheres.

The investigation was commenced in the winter of 1952–1953 at the Lerner Marine Laboratory, Bimini, Bahamas, and was amplified by material caught on the dragger *Cap'n Bill II* during two cruises in the summer of 1953 off the New England coast.

Material

Fishes were caught at the bottom by hook and cable from 400–700 meters' depth in the Gulf Stream off Bimini between December, 1952 and February, 1953. For this purpose a gasoline-powered fishing rig was used, originally designed by Captain Eddie Wall at the Lerner Laboratory. The fishes were brought to the surface within 4–7 minutes after hooking, according to the depth. Blood was drawn immediately by hypodermic needle from the heart and transferred to a polyethylene bottle containing a little heparin. The sample, of about 20–50 cc., was stored on ice and brought to the laboratory within an hour. The blood was then put through all the necessary procedures at once.

Three species were obtained at Bimini:

			Depth of catch
3	*Epinephelus mystacinus*	Black grouper	320–400 m.
1	*Alphestes* sp.[3]	"Golden grouper"	410 m.
2	*Polyprion americanus*	Wreckfish	640 and 675 m.

The fishes weighed between 10 and 30 kg. each.

Deep sea material was likewise obtained on board the dragger *Cap'n Bill II*, operating from Woods Hole, on two cruises (June 16–23 and July 1–8, 1953). The fishes were caught by bottom trawl, mostly at depths of between 500 and 1300 meters, depending upon the species. It took up to three quarters of an hour to get the trawl to the surface from the time it left the bottom. The blood could in some cases be drawn from the heart (*Sebastes*). In other cases blood was obtained which dripped from the cut tail. It was usually necessary to pool blood from many specimens to get enough for the analyses, which were performed immediately.

[3] Called *Epinephelus,* Grouper, in Scholander and van Dam (1953).

The following species were caught and analyzed on the *Cap'n Bill:*

		Depth of catch
Antimora viola	Blue hake	1200 m.
Coryphaenoides rupestris	Round-nosed ratfish	1100 m.
Sebastes marinus[4]	Rosefish	550– 720 m.
Synaphobranchus pinnatus	Long-nosed eel	960–1100 m.
Urophycis chesteri	Long-finned hake	600–1300 m.

Optical method for determination of dissociation curves at high pressures

The problem required a study of oxygen dissociation of fish blood at different pH or CO_2 tensions carried from an oxygen pressure of 0.2 atmosphere, if possible, up to values corresponding to the greatest depth at which the species was known to occur. As a routine the highest oxygen pressure employed was that of a fully charged commercial gas cylinder, *i.e.*, 140 atmospheres.

An earlier survey (van Dam and Scholander, 1953) showed that the blood of deep sea fishes had about the same oxygen combining capacity as that of shallow fishes, *i.e.*, about 6–10 vol. %. At 100 atmospheres' pressure the blood would physically dissolve about 300–400 vol. % oxygen, and hence it would be impossible by direct gasometric methods to determine the saturation of the hemoglobin. An optical method was therefore designed.

The method must be accurate to within a few per cent saturation, and it must be possible to operate it on a rolling ship. It was necessary to equilibrate the blood sample in the photometer cuvette, which must be accurately controlled to the same temperature as that at which the bottom fishes live. The cuvette must be able without significant distortion to stand a pressure of 140 atmospheres.

As a measuring instrument a Zeiss Pulfrich Stufen Photometer [5] was employed. The construction of the high pressure cuvette will be seen in Figure 1. The glass windows were ½-inch thick and the effective depth of the blood in the optical paths was 1.6 mm., increasing 1% at 140 atmospheres. Sealing of the cuvette chamber was obtained by a Neoprene "O" ring squeezed between the windows. Entrance to the cell was by means of a number 20 hypodermic needle which pierced the "O" ring. Through this needle blood could be introduced by a number 25 needle. The cell could be cleaned by rinsing it with 0.17 *M* saline solution, which was then removed by suction. A stainless steel ball was kept in the cuvette for stirring. The cuvette was dropped into an insulated water bath occupying the position where ordinarily the cuvette holder is placed on the instrument. Water of constant temperature (4° or 10° ± 0.1° C.) circulated through the water bath. Dewing of the water bath windows was prevented by keeping them wet with soap solution. To accomplish equilibration the cuvette was only partially filled with blood (see Fig. 1), and it was rotated back and forth through an arc of 45° by means of a motor. To permit rotation the high pressure line of small gauge copper tubing was wound as a loose helix around the cuvette water bath, seven to eight turns, before it was

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[4] In the paper of Scholander and van Dam (1953) the maximum depth of *Sebastes* is given as 1680 m., as recalculated from 917 fathoms given in Goode and Bean (1895, p. 261). This, however, represents a misquotation of the original figure of 179 fathoms (Townsend, 1901, p. 398).

[5] This was kindly lent to us by the Department of Biological Chemistry, Harvard Medical School.

connected to the oxygen cylinder. The oxygen pressure was regulated by means of a long handle on the main oxygen valve, and the pressure was read on a conventional high pressure gauge (10–140 atm.). Between one and 10 atmospheres the pressure was read on a special low pressure gauge, which could be shut off from the main line at pressures above 10 atmospheres. The "O" ring was always kept wet when under oxygen pressure and so far has not exploded.

The extinction of the blood at various oxygen tensions was read through a Wratten red filter, number 29F, and was compared with a constant gray filter held in a dry dummy-cuvette on the other side. This red filter gives the greatest separation between the oxy- and reduced hemoglobin. A gray filter (from a series of variously exposed photographic negatives) was chosen which gave a suitable reading for the oxygenated blood. As the light path on both sides was restricted to a diameter of one quarter of an inch, no wider diaphragm openings could be used
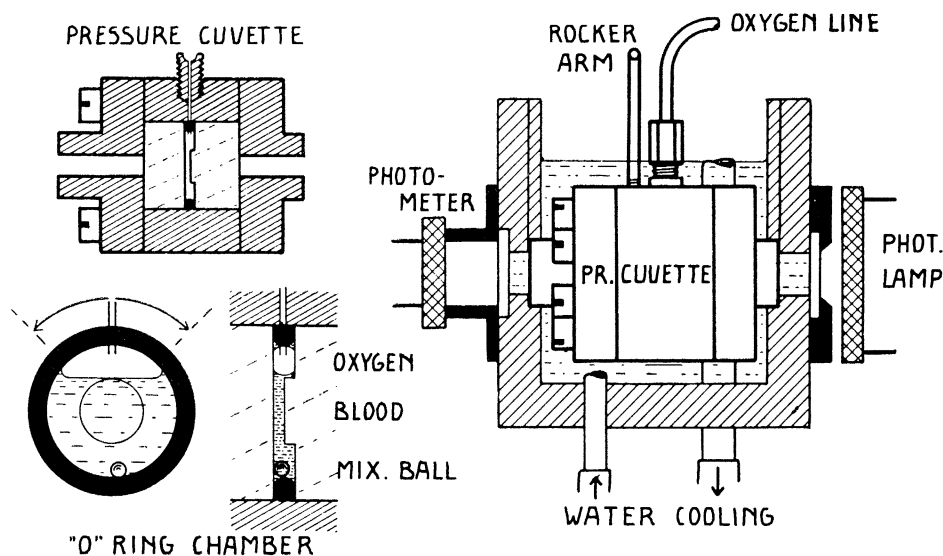

FIGURE 1. Cuvette arrangement for a study of high pressure dissociation curves.

than those corresponding to 0.50 on the extinction scale. The reading from full oxygenation to full reduction usually went from around 0.50 to about 1.20–1.30, giving a resolution of this extinction interval of about 2%.

Procedure

The heparinized blood, kept on ice, was strained, if necessary, through a few layers of gauze. Blood was transferred into three 20-cc. Erlenmeyer flasks, A, B, and C, 3.0 cc. in each. To A was added 0.3 cc. 0.17 M NaCl. To C the same amount of 0.17 M lactic acid solution was added very slowly while agitating the blood. To B was added 0.3 cc. of an equal mixture of 0.17 M NaCl and 0.17 M lactic acid. Samples A, B, and C hence contained 0, 8, and 16 millimols added lactic acid. This would drop the pH from A to B by approximately 0.7 pH units and from A to C by approximately 1.5 units. The flasks were provided with

stoppers pierced with a glass tubing 6 inches long, open at both ends. They were then rotated in a water bath of the desired temperature (10° or 4° C.). A fine suction tube was passed frequently into the flask through the glass tubing to renew the air. After about 20 minutes the blood would be well aerated. Sample A was taken out and its pH taken on a Cambridge Electron Ray pH Meter. The cuvette was provided with a water jacket to keep the blood at the desired tempera- ture. If it was below about 7.4 a small known amount of NaOH would be added
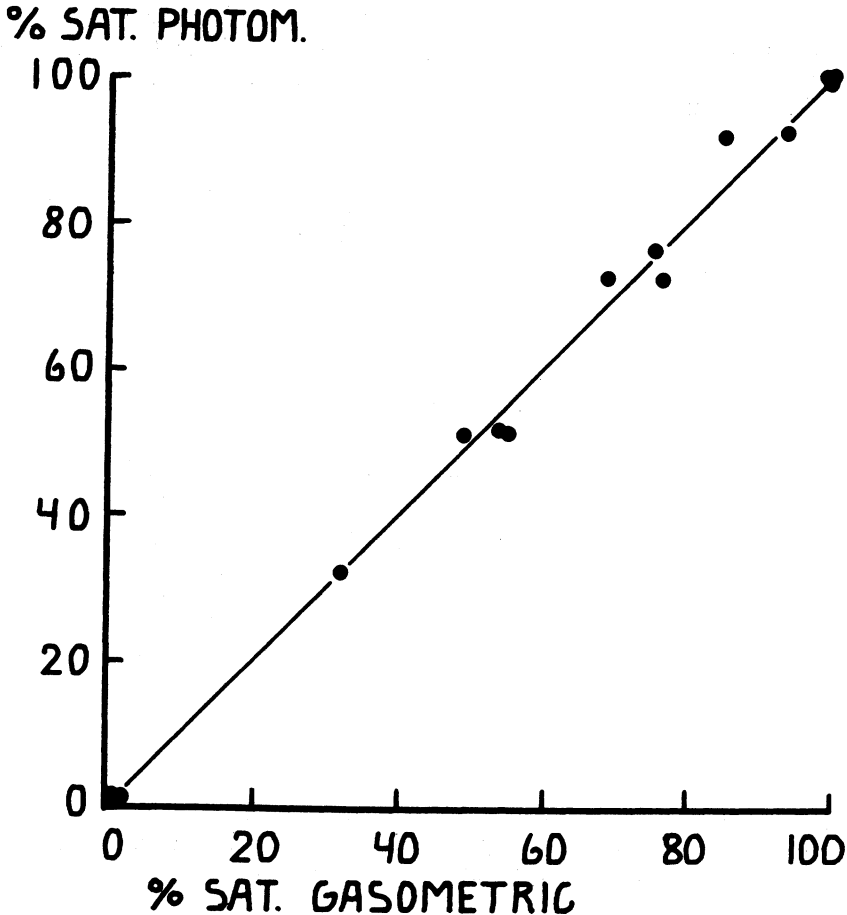

FIGURE 2. Correlation between optical and gasometric determinations of the oxygen saturation in the blood of tautog.

very carefully until a pH of about 7.6—7.8 was obtained. The flask was rotated again and the blood was then transferred to the pressure cuvette, which meanwhile had been temperature-equilibrated in its water bath on the photometer. Rocking of the cuvette was started, and the extinction was read as soon as a constant reading could be obtained. The oxygen line was flushed and connected with the cuvette and the pressure set to give one atmosphere O_2 in the cuvette ($= 1.8$ atm. total).

After about 5–10 minutes' equilibration the reading would be constant, and the pressure was raised to two atmospheres' O_2 ($= 2.8$ atm. total) and so on. Our oxygen contained less than 0.01% CO_2 and 0.02% N_2.

Meanwhile the remainder of sample A (about 2 cc.) was put in a rotating 50-cc. syringe, which was repeatedly flushed with helium (containing less than 0.01% O_2) for reduction of the hemoglobin. The helium was ejected and replaced four or five times, during a total equilibration time of about three quarters of an hour.

The pressure-equilibrated blood was removed from the cuvette by suction, and the cuvette thoroughly rinsed with cold 0.17 M saline. It was then flushed with helium and the reduced blood was introduced, completely filling the cuvette, without any contact with air. The rocker was started and the extinction of the reduced blood read.

Sample B was now taken from the equilibrator, part of it transferred to the pressure cuvette, and part of it to the pH meter. The whole procedure was then repeated for B and finally for sample C. The oxygen saturation was calculated by taking the fully saturated reading as 100% and the reduced as 0%, interpolating the intermediary readings according to a linear relation which had been found to hold for tautog blood (Fig. 2).

In cases where the effect of various CO_2 tensions was tested (at Bimini only), CO_2 and air mixtures were made up in a small spirometer and accurately analyzed (Scholander, 1947). The sample was equilibrated in a 50-cc. rotating syringe with this gas mixture, which was changed four or five times. Concentrations of 5, 10, and 20% CO_2 were used, accurate to $\pm 0.1\%$. The cuvette was flushed with the proper gas mixture before it was filled with blood.

Although optical determinations of the saturation are satisfactory enough when dealing with the same blood sample, they may not be so satisfactory when comparing samples of different pH. It is not too easy to get an accurately fixed zero point for the reduced blood. Hydrosulfite as a reductant is unsuitable to use, as the blood gets darker and darker the more hydrosulfite is added. Vacuum technique involves much shaking and handling of the delicate fish blood, and easily gives evaporation errors. We have obtained most constant results by equilibrating with helium (99.99% pure). But even so, it seems that at least sometimes a change in pH by careful titration with lactic acid will change the light transmission somewhat, due to such things as change (increase) in cell size or slight degree of hemolysis and fibrin formation. We do not believe that this source of error can have seriously distorted our results, but it is likely to be the cause of much of the spread in our data. In most cases the extinction readings of the reduced blood have matched each other within a few per cent at the different pH, and nearly always the fully oxygenated values have checked each other at different pH. The latter is the most important in the present connection.

As to the acidification of the blood we know in each case exactly the amount of acid added. The pH determinations were always made in aerated samples. A certain amount of drift and unsteadiness in the pH meter seemed unavoidable when it was used on our rolling moisture-laden ship, although the meter was kept in a dry-chamber. However, the fact that the drop in pH induced by the added known amounts of acid always came out with satisfactory regularity, as well as the fact that a colorimetric method used on our last cruise gave identical results,
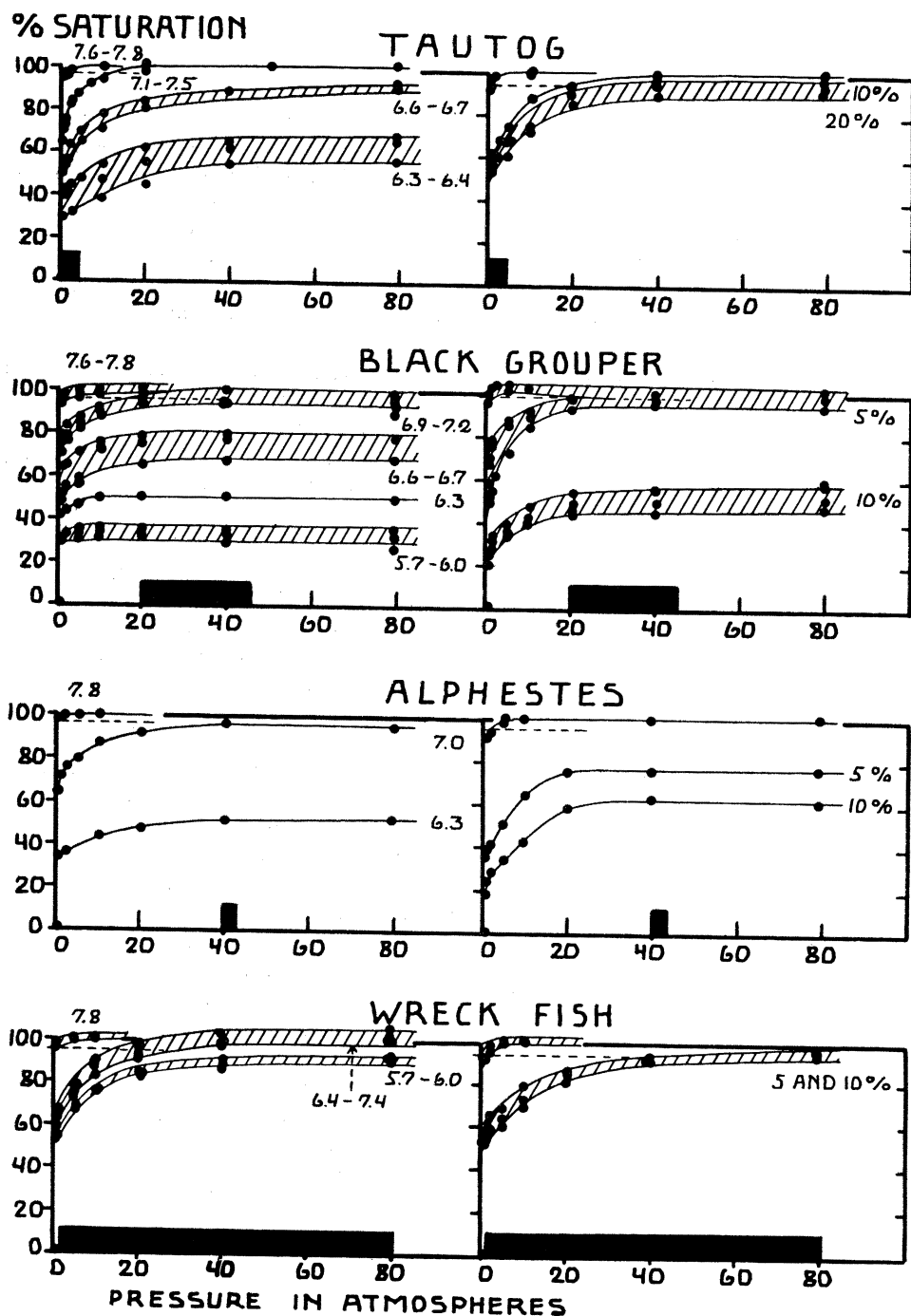
FIGURE 3. Oxygen saturation curves in various fishes at 10° C. Lactic acid was added to the blood in the curves on the left, CO_2 in those on the right. The pH values are given for curves of the lactic acid series. The equilibration pressure of CO_2 is given as per cent of one

lend assurance that our pH measurements must have been essentially correct. From very frequent checks back to our buffer standards, we consider our pH measurements to be reliable to within $\pm$ 0.1 pH unit.

RESULTS

In Figures 3 and 4 the oxygen dissociation curves are plotted for fishes living from shallow surface waters to the abysmal depths. The curves span a range of pH from 8.0 to 5.6, and the carbon dioxide tensions used were 0.03, 5, 10, and 20% of an atmosphere.

It will be seen that in half of the eight species of deep sea fishes investigated (grouper, *Alphestes,* long-finned hake, ratfish) lactic acid in a concentration of about 100–150 mg. % (pH 6.7—5.6) produces a clear Root effect, in that the hemoglobin does not become more than 30–80% saturated at tensions as high as 140 atmospheres.[6] The curves run horizontal from above 20 to 30 atmospheres. In these cases, therefore, the Root effect could be responsible for the oxygen secretion.

In the remaining four deep sea species (rosefish, wreckfish, blue hake, long-nosed eel), however, even at pH 6 the hemoglobin became 90% or more saturated when the pressure exceeded 50–100 atmospheres. The arterial saturation at 0.2 atmosphere is no more than 90–95% at pH 7.6—7.8. Since the oxygen tension in the swimbladder frequently exceeds 50–100 atmospheres it appears impossible that the Root effect can account for gas secretion in these cases, unless possibly the pH falls below 5.6—6 in the blood vessels of the gland.

It seems dubious that the pH in the blood could ever reach such a low value as 6.0. If we consider pH 6.5 as the lowest physiological limit, then only the black grouper and possibly *Alphestes* would be able to maintain part of the hemoglobin in a reduced state in the presence of the high oxygen tension. Of course we do not know what the pH might be in the capillary blood passing through the gland. The lowest pH found in the blood of dead asphyxiated fishes was 6.9. At the highest acidity used *in vitro* it was impossible to avoid the precipitation of some fibrin, and hemolysis occurred with the addition of very little more acid.

If a high CO_2 tension were to be considered responsible for the oxygen secretion via the Root effect, then the CO_2 must be present at high concentrations in the blood vessels of the gland in which case it would diffuse with the oxygen into the bladder where its concentration can be measured. In shallow fishes swimbladder CO_2 is usually below 1–2% (Table I). In the wreckfish the oxygen secretion could not be explained by the Root effect unless the CO_2 were more than 10% of an atmosphere, and the tautog would require more than 20% CO_2 to secrete in deep water. Accurate determinations were made of the CO_2 tension in the swim-

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[6] The Root effect was described from blood of shallow water fishes, dealing with oxygen pressures of no more than 700 mm. Hg (Root, 1931).

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atmosphere. The broken line (90–95% saturation) denotes the arterial saturation at 0.2 atmosphere O_2 pressure. The solid bar on the base line is the range of depth in atmospheres where these species live. For each range of pH or CO_2, 3 or 4 separate runs with different bloods were usually made to get an impression of the individual variability. All curves were continued to 140 atmospheres' oxygen pressure, but showed no change from the values at 80 atmospheres.
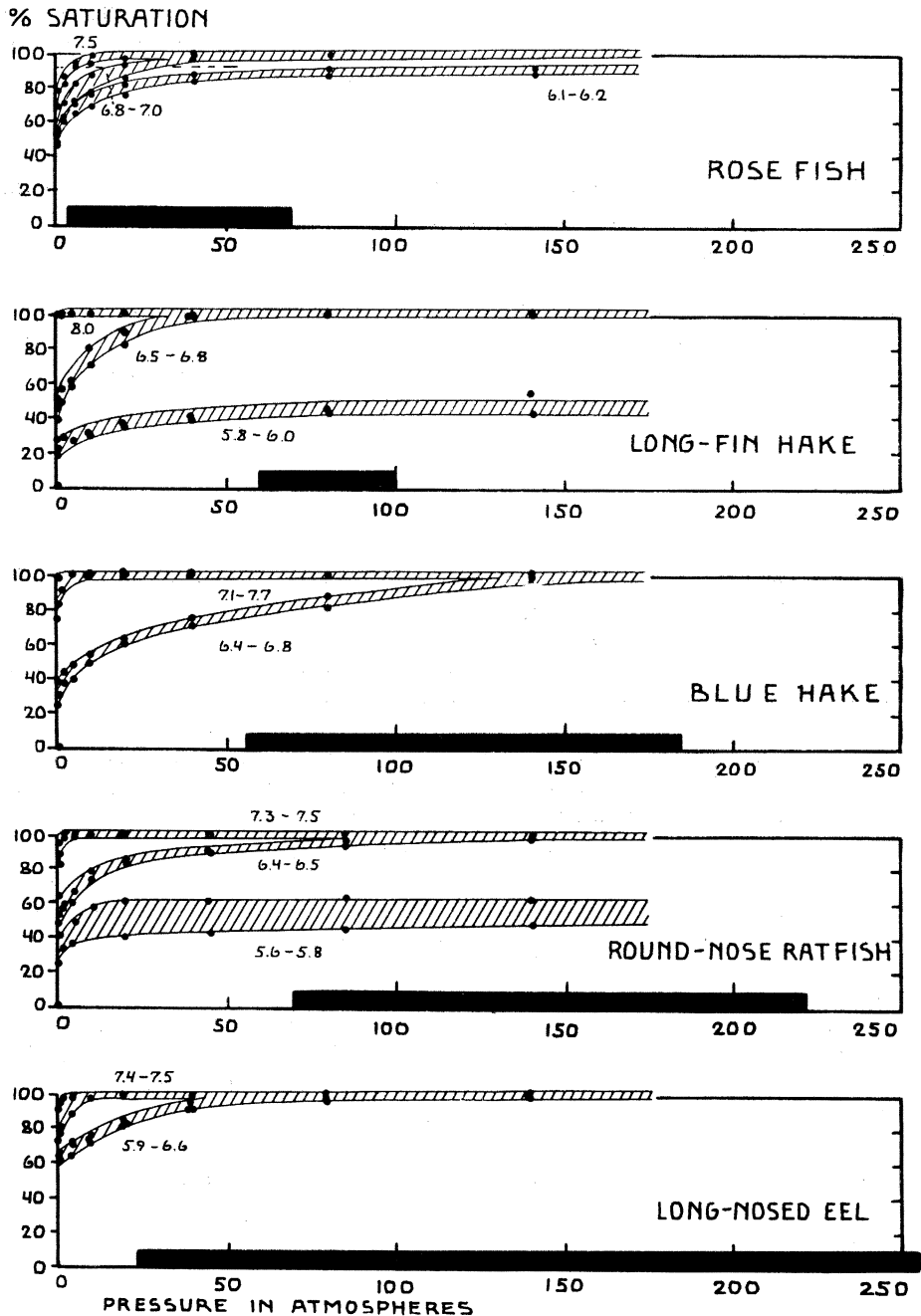
FIGURE 4. Oxygen saturation curves in blood of deep sea fishes at 4° C. The upper curve in each diagram represents the blood untreated, directly from the fish. In the other curves lactic acid has been added to give, after aeration, the pH stated for each curve. The solid bar on the base line is the depth range at which the fish lives. Each pH range was generally covered by two or three separate runs, using blood from different specimens. Arterial saturation would be no more than 90–95 vol. %.

bladder gas of the Bimini fishes (Table I, B), which were brought quickly to the surface. Assuming that only insignificant changes took place during the 4–7 minutes of getting the fish on deck, the tensions in the swimbladder gas were from 10–19% of one atmosphere CO_2 at the bottom. As the gas in the blown-up fish was dispersed all through its body cavity, it may well have picked up some CO_2 from the tissues.

If, as indicated by Root and Irving (1943), the CO_2 effect is simply a pH effect, then CO_2 cannot be the direct cause of the hypothetical oxygen dissociation from hemoglobin in our deep sea fishes. It was suggested by Black (1946) that HCl might be secreted rather than lactic acid. In tautog blood at pH 6.8 we found no greater Bohr effect with HCl than with lactic acid, and hence HCl does not seem to have any specific action beyond the pH effect.

In a discussion of the probable function of the *rete* it will be shown that in that connection, also, there are serious obstacles to regarding the Root effect as

TABLE I

Carbon dioxide content of swimbladder gas

Species	Depth of catch (m.)	Number of specimens	% CO_2 Low—High	Mean partial pressure of CO_2 $\left(\frac{atm.}{100}\right)$
A. Tautog (*Tautoga onitis*)	0–1	2	0.49–0.99	0.7
Queen triggerfish (*Balistes vetula*)	0–2	7	0.33–0.99	0.4
Blue parrotfish (*Scarus caeruleus*)	0–2	1	1.02	1.0
Black durgon (*Melichtys piceus*)	0–2	1	1.08	1.1
Sergeant major (*Abudefduf saxatilis*)	0–1	5	0.29–0.48	0.4
B. Black grouper	320–400	6	0.17–0.40	10
Alphestes	408	1	0.47	19
Wreckfish	640	1	0.22	14

Mean value for partial pressures of CO_2: Group A, 0.9% of 1 atm.; Group B, 14% of 1 atm.

the basic chemical reaction responsible for the oxygen secretion in deep sea fishes (Scholander, 1954).

SUMMARY

1. Oxygen dissociation curves at a P_{O_2} running from 0.2 to 140 atmospheres have been obtained from eight species of deep sea fishes, at a pH varying from 8.0 to 5.7 and at a CO_2 tension from 0.03 to 10–20% of one atmosphere.

2. The Root effect in acidified blood could be demonstrated in some of these species even up to 140 atmospheres, inasmuch as part of the hemoglobin remained unsaturated. In other species full arterial saturation occurred, however, at pH 6 or lower, at oxygen tensions much lower than those existing in the swimbladder. Hence the Root effect is not the mechanism for the oxygen secretion in deep sea fishes. If oxygen is derived from oxyhemoglobin it must be unloaded by some mechanism as yet unknown.

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