The Secretion of Oxygen into the Swim-bladder of Fish

II. *The simultaneous transport of carbon monoxide and oxygen*

JONATHAN B. WITTENBERG and BEATRICE A. WITTENBERG

From the Department of Physiology, Albert Einstein College of Medicine, Yeshiva University, New York, and the Marine Biological Laboratory, Woods Hole, Massachusetts

ABSTRACT   Toadfish, *Opsanus tau*, L., were maintained in sea water equilibrated with gas mixtures containing a fixed proportion of oxygen and varying proportions of carbon monoxide. The swim-bladder was emptied by puncture, and, after an interval of 24 or 48 hours, the newly secreted gases were withdrawn and analyzed. Both carbon monoxide and oxygen are accumulated in the swim-bladder at tensions greater than ambient. The ratio of concentrations, carbon monoxide (secreted): carbon monoxide (administered) bears a constant relation to the ratio, oxygen (secreted): oxygen (administered). The value of the partition coefficient describing this relation is ($\alpha = 5.44$). The two gases are considered to compete for a common intracellular carrier mediating their active transport. The suggestion is advanced that the intracellular oxygen carrier is a hemoglobin. Comparison of the proportions of carboxy- and oxyhemoglobin in the blood with the composition of the secreted gas proves that the secreted gases are not evolved directly from combination with blood hemoglobin. The suggestion is advanced that cellular oxygen secretion occurs in the rete mirabile: the rete may build up large oxygen tensions in the gas gland capillaries. It is suggested that the gas gland acts as a valve impeding back diffusion of gases from the swim-bladder.

The swim-bladder of fish is inflated by the secretion of an oxygen-rich gas mixture, which often consists of 95 per cent oxygen. In deep sea fish in which the secretory mechanism must operate against pressure, partial pressures of oxygen greater than 100 atmospheres are commonly achieved. Two sources of the secreted oxygen have been considered. On the one hand oxygen could be split off from the oxyhemoglobin of the blood through the action of acid or of oxidizing agents. On the other hand the cells of the gas gland and the associated rete mirabile may themselves be capable of transporting oxygen against

a pressure gradient. (The cells of the rete may transport oxygen from the venous to the arterial capillaries of the rete, and the gas gland cells may transport oxygen from the gland capillaries into the lumen of the swim-bladder.) A growing body of evidence indicates that such a cellular transport of oxygen does take place.

In order to study the cellular transport mechanism, conditions must be sought such that circulating oxyhemoglobin can make no contribution to the oxygen brought into the swim-bladder. An animal without blood hemo-globin would be ideally suited for the experiment, and indeed, fish totally lacking blood hemoglobin, are known in Antarctic waters (Ruud, 1954), but unfortunately have no swim-bladder. However, their very existence makes one bold to try to produce an animal lacking oxyhemoglobin. As has been known for some time, many fish and amphibia are very resistant to carbon monoxide (Fox, 1954). The toadfish, *Opsanus tau*, L., otherwise ideally suited for experiments on swim-bladder function (Fänge and Wittenberg, 1958), is found to survive indefinitely in an atmosphere containing up to 60 per cent carbon monoxide. The blood of such animals is essentially free from oxyhemoglobin, since all the circulating hemoglobin is tied up as carboxy-hemoglobin. At lower carbon monoxide concentrations the blood does con-tain a substantial proportion of oxyhemoglobin (Table II). Nevertheless, the results of all the experiments presented here are interpreted best in terms of glandular secretion alone and do not reflect the state of the circulating blood hemoglobin.

Toadfish maintained in sea water equilibrated with carbon monoxide containing gas mixtures are found to accumulate carbon monoxide in the swim-bladder in concentrations roughly seven times as great as the ambient. This finding provides for the first time an opportunity to study the properties of the cellular oxygen-transporting system.

The present communication presents the results of a quantitative study of the simultaneous transport of administered carbon monoxide and oxygen into the swim-bladder. The two gases are found to compete for a common transport system. The fact that they compete suggests that at some critical stage of the transport process, each of the two gases combines with one and the same intracellular carrier. By analogy with the convention introduced by Douglas and Haldane (1912) to describe the reactions of hemoglobin, the ratio of the affinities of the putative carrier for carbon monoxide and for oxygen will be expressed by a constant, the partition coefficient, denoted α. On the basis of reasonable assumptions, it is possible to derive a numerical value, $\alpha = 5.44$, for the partition coefficient exhibited by the intracellular carrier. The ability of the transport system to combine both with carbon monoxide and oxygen suggests that the intracellular carrier may be an intra-

cellular hemoglobin. The numerical value of the partition coefficient is consistent with this hypothesis.

The ultimate critical test of the hypothesis of an intracellular hemoglobin carrier must be the characterization of an intracellular hemoglobin in the gas gland and rete mirabile having an appropriate partition coefficient.

EXPERIMENTAL

Choice of Experimental Animal The toadfish (*Opsanus tau*, L.) was chosen for its ability to resist anoxia (Hall, 1929), for its powerfully developed oxygen-secreting ability (Fänge and Wittenberg, 1958), and because it exhibits a relatively uncomplicated pattern of gas secretion (Wittenberg, 1961). The structure of the swim-bladder and gas gland in this species has been described (Fänge and Wittenberg, 1958).

The Gas Mixture All gas mixtures administered contained about 50 per cent oxygen. The carbon monoxide content was varied systematically from 0.2 to 35 per cent. Although toadfish will survive exposure to carbon monoxide in 20 per cent oxygen for long periods, gas secretion is not so well maintained as in higher oxygen concentration. Oxygen concentrations greater than 50 per cent are not advantageous.

Apparatus and Procedure The animals were exposed to gas mixtures in the apparatus described previously (Wittenberg, 1961) except that the gas mixtures were circulated at the rate of 1.5 liters per minute.

The animals were exposed to the carbon monoxide–containing gas mixtures for 24 hours prior to the experiment to allow them to acclimate to the stress imposed by the carbon monoxide and to allow time for the tissues to equilibrate with the gas mixture. The gases initially present in the swim-bladder were then removed by puncture and the volume noted. After an interval of 24 or 48 hours, the secreted gases were withdrawn into a syringe, the volume was noted, and the gases analyzed for their content of CO_2, O_2, and CO. In some instances it was possible to obtain several successive 48 hour samples from the same animal.

Oxygen and Carbon Dioxide Analysis Analysis for O_2 and CO_2 was performed by the method of Scholander *et al.* (1955).

Carbon Monoxide Analysis Carbon monoxide was determined volumetrically by absorption with Winkler's reagent, cuprous chloride in ammonium chloride (Scholander and Roughton, 1942, 1943), in a screw-driven capillary gas analyzer (Krogh, 1908; Scholander *et al.*, 1955).[1] The procedure described here is substantially more rapid and convenient than previous methods, and promises to be of general utility.

[1] Available from Otto K. Hebel Scientific Instruments, 80 Swarthmore Avenue, Rutledge, Pennsylvania, or from the Mark Company, 31 West Street, Randolph, Massachusetts.

Reagents

1. Acid citrate: 85 gm. sodium citrate dihydrate and 3 gm. citric acid monohydrate in 100 ml. water. The solution is boiled when made up to overcome the gas supersaturation that results from dissolving the salts.

2. Oxygen absorber: A solution of 20 gm. NaOH in 100 ml. of water is covered with a layer of paraffin oil, and 15 gm. pyrogallol added through the oil layer. The pyrogallol is dissolved under the oil layer by stirring with a glass rod.

3. Dilute acid citrate: 30 gm. sodium citrate and 1 gm. citric acid in 100 ml. water The solution is boiled when made up.

4. Winkler's solution: 20 gm. cuprous chloride, 25 gm. ammonium chloride, and 75 ml. water are placed in a bottle just large enough (*i.e.* 100 ml.) to contain them, and the solids dissolved by shaking. The precipitate which forms is allowed to settle.

The supernatant solution is withdrawn and stored in contact with a coil of fine copper wire. It is protected from air by a layer of paraffin oil. After the solution becomes colorless, one-half volume of boiled water is added with care to prevent excessive local dilution and consequent formation of a precipitate. The water may conveniently be drawn into a syringe while still boiling vigorously, be allowed to cool in the syringe, and be delivered into the solution below the oil layer through a length of plastic tubing.

The analyzer is charged with acid citrate and the gas sample delivered into the cup from a syringe. The analyzer is held cup down, the gas bubble is teased to the top of the cup with a fine wire, and drawn into the capillary. With the cup up, the bubble is drawn down into the barrel, and then brought back into the capillary. The volume is read. Oxygen and carbon dioxide are absorbed by drawing one cupful of pyrogallol solution down over the bubble with instrument held cup up. The remaining bubble of nitrogen and carbon monoxide is moved up into the capillary and the volume is read. The seal of pyrogallol is cautiously brought to the bottom of the cup. The cup is rinsed twice with portions of dilute acid citrate (conveniently delivered from a syringe), and is filled to the half-way mark with dilute acid citrate. The dilute acid citrate solution is drawn into the capillary. Carbon monoxide is absorbed by drawing one cupful of dilute Winkler's solution over the bubble, with the instrument held cup up. The dilute acid citrate forms a more or less distinct layer in the barrel of the analyzer preventing excessive reaction between Winkler's solution and the alkaline pyrogallol below. The analyzer is held strictly upright during the absorption of CO to prevent mixing of the layers of solution in the barrel. The remaining bubble of nitrogen (and other non-absorbable components) is returned to the capillary, and the volume read as soon as drifting of the meniscus subsides. If necessary, the meniscus is freed from precipitates by small forward and backward movements. Details of the manipulation of the analyzer will be found in the paper of Scholander *et al.* (1955).

Precipitates adhering to glassware may be removed with aqua regia.

The determinaton is accurate from zero to 100 per cent carbon monoxide. The precision obtained is 0.5 division in 100.

Gas samples high in oxygen and low in carbon monoxide were freed from oxygen

by treatment with alkaline pyrogallol drawn into the collecting syringe, and the oxygen-free residual gases analyzed for carbon monoxide.

The carbon monoxide content of the administered gas mixtures containing 0.19, and 1.32 per cent carbon monoxide was determined by the method of Allen and Root (1955).

Determination of the Proportion of Carboxyhemoglobin and Oxyhemoglobin in Red Blood Cells Blood was drawn from the gills into a heparinized syringe. Washed red blood cells were suspended in buffered isotonic saline (Green and Hoffman, 1953) (0.115 M NaCl; 0.05 M sodium phosphate buffer, pH 7.4) and equilibrated with gas mixtures containing oxygen at a partial pressure of 0.5 atmosphere and varying proportions of carbon monoxide. These gas concentrations are sufficient to completely saturate the hemoglobin and no reduced hemoglobin may be detected. The relative proportion of carboxyhemoglobin and oxyhemoglobin contained in the cells was determined by the method of Hartridge (1912, 1923) employing the reversion spectroscope.[2] Intact cells were studied because the equilibria between toadfish hemoglobin and oxygen are reported to be affected by hemolysis (Root, Irving, and Black, 1939). The absorption bands exhibited by cell suspensions containing less than 30 per cent carboxyhemoglobin are diffuse and the precision of measurement is low. The results at 0.002 atmosphere carbon monoxide are therefore presented as a range of values. The values given for 0.35 atmosphere carbon monoxide were calculated by extrapolation from measurements at this and somewhat lower carbon monoxide concentrations.

RESULTS

The Gas Secreted The compositions of the gas secreted into the swimbladder by animals maintained in sea water equilibrated with gas mixtures containing 0.2, 1, 5, 10, and 35 per cent carbon monoxide, 50 per cent oxygen, and the balance nitrogen are presented in Table I. It will be noted that the proportion both of oxygen and carbon monoxide in the gas secreted may be greater than in the gas administered, and that therefore both of these gases may be considered to be actively secreted.

The Partition Coefficient Values for the partition coefficient are calculated from the composition of the gas mixtures administered and secreted by Equation 1, given in the Discussion, and are presented in Table I. The average value found is 5.44. Although the values show scatter, no trend appears, and the partition coefficient may be considered to be a constant. The partition coefficient is seen to be independent of the concentration of carbon monoxide administered, and of course, of the relative proportions of oxygen and carbon monoxide which were changed 170-fold during the course of the experiment.

[2] Wavelength reversion spectroscope, R. and J. Beck, Ltd., 69/71 Mortimer Street, London, W.1., England.

TABLE I

COMPOSITIONS OF ADMINISTERED AND SECRETED GAS

Animal	Interval	Volume of gas secreted	Gas mixture					Partition coefficient
			Administered		Secreted			
			O_2	CO	CO_2	O_2	CO	α
	hrs.	per cent of initial gas volume	per cent	per cent	per cent	per cent	per cent	
122	24	16	49.0	0.19	4.1	85.5	1.33	4.00
123	24	16	49.0	0.19	7.2	82.5	1.32	4.13
124a*	24	65	49.0	0.19	6.2	89.4	1.09	3.15
124b	24	81	50.0	0.19	8.3	87.5	1.48	4.45
124c	24	53	48.5	0.19	5.3	90.3	1.23	3.48
124d	24	85	49.7	0.19	6.7	88.2	1.76	5.22
Average								4.07
86a	48	33	47.8	1.32	8.0	73.7	10.9	5.35
86b	48	44	49.9	1.32	6.4	75.5	9.16	4.59
86c	24	14	47.0	1.32	5.9	74.0	8.32	4.01
89a	48	42	49.9	1.32	5.4	76.2	11.5	5.70
89b	24	20	47.0	1.32	7.5	74.5	10.9	5.23
89c	24	15	47.2	1.32	9.1	74.8	9.25	4.44
90a	48	48	49.9	1.32	7.2	73.8	11.8	6.04
90b	24	19	47.0	1.32	5.3	74.5	11.8	5.61
90c	24	23	47.2	1.32	3.4	76.7	10.8	5.05
Average								5.11
14a	24	37	46.2	5.60	12.1	29.6	29.3	8.20
14b	24	7	45.5	5.84	11.8	32.3	30.0	7.25
15a	24	35	46.2	5.60	0.1	23.8	25.9	9.01
15b	24	20	45.5	5.84	10.6	32.1	33.5	8.12
15c	24	15	45.0	5.93	9.8	30.1	39.5	10.0
18	24	23	48.9	6.00	10.4	36.4	27.9	6.25
21	24	48	48.2	6.11	6.5	36.0	21.9	4.80
28	24	11	55.3	4.06	8.0	54.2	26.6	6.70
29	24	9	55.3	4.06	3.5	48.3	13.1	3.68
30	24	12	55.3	4.06	9.2	44.3	31.3	9.60
36	24	13	54.5	5.12	6.7	43.4	31.4	7.70
38	24	5	54.5	5.12	4.2	52.0	28.6	5.85
41	24	9	51.4	6.06	2.0	49.2	24.7	4.25
45	24	10	50.7	6.35	2.0	38.4	32.4	6.74
46	48	19	48.5	6.08	4.0	42.5	36.7	6.90
53	48	24	48.9	6.62	6.7	52.0	28.0	3.95
Average								6.81

* Samples marked a, b, c, are successive samples from the same animal.

TABLE I—(*concluded*)

Animal	Interval	Volume of gas secreted	Gas mixture Administered O_2	CO	Secreted CO_2	O_2	CO	Partition coefficient α
	hrs.	per cent of initial gas volume	per cent	per cent	per cent	per cent	per cent	
42	24	5	46.9	8.70	8.2	30.9	31.0	5.40
44	24	7	46.7	9.73	1.7	37.0	40.5	5.26
47	48	6	44.6	8.60	2.1	33.4	44.1	6.84
54	48	13	50.5	10.7	3.2	41.8	24.6	2.79
57	48	8	48.9	10.7	0.3	33.8	41.8	5.65
58	24	7	48.9	10.7	6.8	30.2	47.5	7.20
67	48	13	48.4	10.3	5.4	42.8	39.8	4.38
Average								5.36
59	48	4	47.5	35.4	5.1	13.3	70.1	7.10
60	48	6	47.5	35.4	6.8	24.0	58.9	3.29
64a	48	16	48.3	34.4	5.8	22.3	47.1	2.96
64b	48	6	49.1	33.4	6.4	21.7	52.7	3.58
66	48	2	48.3	34.4	7.8	23.0	54.4	3.31
76	48	5	49.0	34.0	1.5	14.3	34.8	3.50
77	48	17	49.0	34.0	7.5	14.2	38.4	3.90
Average								3.95
Over-all average								5.44

Carbon Dioxide The carbon dioxide content of the secreted gas is rather higher than that usually encountered in this species (Fänge and Wittenberg, 1958; Wittenberg, 1960). As can be seen in Table I, many of the samples contain between 5 and 12 per cent carbon dioxide. These concentrations are of the same order as those found by Scholander (1956) in gas samples collected directly from the gas gland surface in barracuda and cod. The source of this carbon dioxide is not known.

The Rate of Secretion The toadfish will normally replace the gas volume of the experimentally emptied swim-bladder in less than 24 hours. Inspection of Table I shows that secretion is slowed in the presence of carbon monoxide, and that the rate falls off with increasing carbon monoxide in the inspired gases. This may be a non-specific toxic effect of carbon monoxide, or a result of the limited supply of oxygen to the tissues.

State of the Circulating Hemoglobin The proportions of carboxyhemoglobin and oxyhemoglobin in toadfish red blood cells exposed to gas mixtures similar

to those administered to the experimental fishes are presented in Table II. The most striking finding is that blood hemoglobin is largely converted to carboxyhemoglobin only at high tensions of carbon monoxide.

The oxygen dissociation curve of toadfish hemoglobin is known to be of complex shape (Root, 1931; Green and Root, 1933; Hall and McCutcheon, 1938), and to be different in shape from the carbon monoxide dissociation curve (Root and Green, 1934). It is therefore not surprising to find that the partition between carboxyhemoglobin and oxyhemoglobin cannot be described by a simple relationship, and that the apparent partition coefficient is not constant. Although the value of the apparent partition coefficient of toadfish

TABLE II

PROPORTION OF CARBOXYHEMOGLOBIN IN TOADFISH RED
BLOOD CELLS EXPOSED TO GAS MIXTURES CONTAINING OXYGEN
AT A PARTIAL PRESSURE OF 0.5 ATMOSPHERE AND
VARYING PRESSURES OF CARBON MONOXIDE

Temperature, 20°C.

Carbon monoxide in gas phase	Carboxyhemoglobin		
	Animal		
	129	130	131
atmospheres	per cent	per cent	per cent
0.002	25 (±5)	15 (±5)	20 (±5)
0.01	60	64	63
0.05	83	83	80
0.10	91	92	87
0.35	97	97	96

blood hemoglobin has no theoretical meaning, one may note that the numerical values are totally different from, and roughly ten times greater than, the value of the partition coefficient of the intact transporting system. At carbon monoxide tensions of 0.002, 0.01, 0.05, 0.10, and 0.35 atmosphere respectively, the value of the apparent partition coefficient of toadfish blood hemoglobin, calculated from the data given in Table II, is 63, 82, 46, 45, and 46.

DISCUSSION

The Secretory Product

Oxygen is brought into the swim-bladders of toadfish against a pressure gradient when the animals are maintained in air-equilibrated sea water (Fänge and Wittenberg, 1958; Wittenberg, 1961). In the present experiments, a

large fraction of the secreted gas volume was occupied by carbon monoxide, and the secretion of oxygen at tensions greater than that in the gases administered was demonstrated only by those animals exposed to low concentrations of carbon monoxide, 0.19 and 1.32 per cent.

The concentration of carbon monoxide in the secreted gas is in all cases greater than the concentration in the gas mixture administered. Animals exposed to gas mixtures containing 0.19 and 1.32 per cent carbon monoxide secreted gas mixtures containing 1.4 and 11.6 per cent carbon monoxide; seven- and nine-fold concentrations, respectively.

Evidence for a Single Carrier

Both oxygen and carbon monoxide are actively secreted into the swimbladder. It is necessary to inquire whether they are transported independently, in which case, the amounts of the two gases transported need bear no necessary relation one to another, or whether they are transported by a common transport system. In fact, the ratio of concentrations, carbon monoxide (secreted) to carbon monoxide (administered), bears a constant relation to the ratio of oxygen (secreted) to oxygen (administered) (Equation 1).

$$\alpha = \frac{pCO_{secreted}}{pCO_{administered}} \div \frac{pO_{2\,secreted}}{pO_{2\,administered}} \tag{1}$$

It should be emphasized that this relation, described by the partition coefficient, designated α, remained constant although the ratio carbon monoxide to oxygen was varied 170-fold in the course of the experiment.

The constancy of the partition coefficient constitutes decisive evidence that the transports of carbon monoxide and oxygen are mediated by one and the same carrier.

The Nature of the Intracellular Carrier

The ratio, α, as treated above, is essentially a ratio of rates of transport. On the assumption that carbon monoxide and oxygen combine in a reversible manner with one and the same intracellular carrier, the ratio, α, may be considered as the ratio of two equilibrium constants.
Let X indicate the intracellular carrier. Then:

$$O_{2\,administered} + X \rightleftharpoons X\text{-}O_2 \tag{2}$$

and
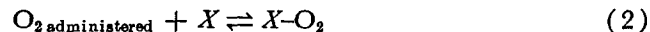
$$CO_{administered} + X \rightleftharpoons X\text{-}CO \tag{3}$$

The equilibrium constants for these reactions are:

$$K_{CO} = \frac{[X\text{-}CO]}{[X]\,pCO_{administered}}; \qquad K_{O_2} = \frac{[X\text{-}O_2]}{[X]\,pO_{2\,administered}} \qquad (4, 5)$$

The equilibrium constants describe the affinities of the carrier for carbon monoxide and oxygen respectively. As defined by Douglas and Haldane (1912) the partition coefficient is the ratio of the two equilibiium constants and describes the ratio of the affinities of the carrier for carbon monoxide and oxygen:

$$\alpha = \frac{K_{CO}}{K_{O_2}} = \frac{pO_{2\,administered}}{pCO_{administered}} \cdot \frac{[X\text{-}CO]}{[X\text{-}O_2]} \qquad (6)$$

To give meaning to Equation 6, both terms must be known.

The term $\frac{[X\text{-}CO]}{[X\text{-}O_2]}$, characterizing an intracellular happening, is not as yet accessible by direct experiment. However, if one assumes, as appears plausible in a system doing secretory work, that the complexes X–CO and X–O$_2$ are irreversibly and completely decomposed, the ratio of carbon monoxide to oxygen in the secreted gas will reflect the partition of the intracellular carrier between its carboxy- and oxy- forms.

The partition coefficient may then be approximated:

$$\alpha = \frac{pO_{2\,administered}}{pCO_{administered}} \cdot \frac{pCO_{secreted}}{pO_{2\,secreted}} \qquad (1\,a)$$

On rearrangement, this expression is seen to be identical with Equation 1. All the values it contains are accessible to experiment, and the values of the partition coefficient, α, for carbon monoxide concentrations ranging from 0.19 to 35 per cent are presented in Table I. As was noted before, the value is constant over the range of gas concentrations to which the fish were exposed. The average value is, $\alpha = 5.44$.

The partition coefficient for the entire transport process thus may be considered a chemical property of the intracellular carrier and provides a criterion by which this compound ultimately may be recognized in isolated glands or fragmented cells. The intracellular carrier must belong to one of the very few classes of compounds which combine both with carbon monoxide and oxygen. Of these, only the iron-heme proteins need be considered in formulating a working hypothesis. The value found for the partition coefficient of the oxygen-transporting system, average 5.4, is of the same order of magnitude as those known for myoglobin ($\alpha = 20$) (Roche, 1933; Theorell, 1934), and for other intracellular hemoglobins ($\alpha = 0.67$, $\alpha = 37$, $\alpha = 106$) (Keilin and Wang, 1946; Keilin and Wang, 1945; Rossi-Fanelli et al., 1958).

It is smaller than the partition coefficient of mammalian blood hemoglobins (α = 125 to 550) (*e.g.* Douglas and Haldane, 1912), and is larger than the partition coefficient found for cytochrome oxidase (α = 0.05 to 0.2) (Warburg, 1927; Keilin, 1929; Keilin and Hartree, 1938; Ball *et al.*, 1951).

The suggestion is advanced that the cellular oxygen carrier mediating the active transport of oxygen is a hemoglobin occurring within the cells of the gas gland and rete mirabile.

If intracellular hemoglobins are the carriers for the active transport of oxygen, it becomes reasonable to inquire whether tissue hemoglobins may not also be carriers for a passive transport of oxygen and facilitate the movement of oxygen under the influence of a pressure gradient. The predicted effect has been searched for and found, employing blood hemoglobin as a model system (Wittenberg, 1959; Scholander, 1960).

Possible Role of Blood Hemoglobin

Blood is supplied to the gas gland by way of a capillary countercurrent flow system, the rete mirabile. The afferent swim-bladder artery entering the rete breaks up into a large number of very long and straight capillaries which are interdigitated in a strictly parallel array with a similar development of capillaries in the efferent vein. The arterial and venous blood streams flow countercurrent one to another and a very large area is provided for exchange of materials (Woodland, 1911).[3]

Haldane (1922; amplified by Jacobs, 1930) suggested that acid generated in the gas gland might liberate oxygen from its combination with blood hemoglobin. The rete mirabile, acting as a countercurrent exchanger, was thought to conserve both the high oxygen tension so generated and the local concentration of acid. Haldane's theory has found general acceptance, although there is indeed no positive evidence implicating blood hemoglobin in oxygen secretion. Recently the theory has come under attack by Scholander and van Dam (1954) who have extended the oxygen dissociation curves of fish bloods to very high pressures, and argue (Scholander, 1954) that at least in some species of deep sea fishes, acidification of the blood cannot generate the large pressures of oxygen maintained in the swim-bladder.

The present experiments prove that the gases entering the swim-bladder are not evolved directly from combination with blood hemoglobin. In the extreme case, animals whose blood hemoglobin is almost entirely in the form of carboxyhemoglobin (animals exposed to 0.35 atmosphere carbon monoxide (Table II)) continue to secrete a gas mixture in which oxygen makes up

---

[3] The area of either the venous or arterial capillary surfaces in the rete of an eel comparable in size to the toadfish used in these experiments was 105 cm.[2] (Krogh, 1922). The surface area of the gas gland in an eel or toadfish of this size is about 5 cm^2.

about one-half of the total volume. This oxygen obviously comes from a source other than blood hemoglobin.

The situation obtaining in animals whose blood hemoglobin is only partially in the carboxy form can be assessed only approximately because the action of acid on mixtures of toadfish carboxy- and oxyhemoglobin is assuredly complex and the gas tensions that would be generated by acidification cannot be predicted. However, gas tensions so generated would at least reflect the proportions of carboxy- and oxyhemoglobin present in the blood (Table II). In fact, these proportions are grossly different from the proportions of carbon monoxide and oxygen brought into the swim-bladder (Table I). For instance, at a carbon monoxide tension of 0.002 atmosphere, the ratio of carboxy- to oxyhemoglobin in the blood is roughly 20/80, in marked contrast to the ratio of carbon monoxide to oxygen in the secreted gas which is about 1/50.

Blood hemoglobin may be ruled out as the primary source of the secreted gases. By exclusion, therefore, gas secretion must be a cellular process.

The Rete Mirabile as a Countercurrent Multiplier

The present experiments establish that oxygen is brought into the swim-bladder by a cellular transport process. The question arises, in which cells does oxygen transport take place?

The gas-secreting system in the swim-bladder consists of two major parts, the rete mirabile and the gas gland. Until recently, it appeared that some salmonid fishes, which lacked a rete mirabile, were capable of secreting oxygen by glandular action alone (Sundnes *et al.*, 1958). However, in every case which has been carefully studied (see Fänge, 1958; Fahlen, 1959) a rete has been found, and one must consider the rete to be an essential part of the oxygen-secreting mechanism.

In deep sea fish, the partial pressure of oxygen in the swim-bladder may be 100 atmospheres; the partial pressure in the blood is, of course, 0.2 atmosphere. It appears inherently unlikely that the gas gland, which in some deep sea fish consists only of a single layer of cells 10 micra thick, can support this large concentration difference. For this reason, the oxygen-concentrating mechanism may be referred to the rete mirabile. Denton (1960) considers in detail the possible functions of the rete mirabile and adduces a number of additional reasons for referring oxygen transport to the rete. If the oxygen-transporting function were displayed along the length of the rete capillaries, a relatively small concentration difference maintained across the capillary walls at any level of the rete could be cascaded by the flowing system into a very large concentration difference along the full length of the rete, with the result that the oxygen tension in the gas gland capillaries would be high and

roughly equal to the tension in the swim-bladder. Systems of this sort, termed countercurrent multipliers, have been brought to the attention of physiologists and their workings explained by Hargitay and Kuhn (1951).

The Function of the Gas Gland

The gas gland consists of a cellular layer interposed between the gas gland capillaries emanating from the rete and the lumen of the swim-bladder. It is a constant feature of the oxygen-secreting system and has been described in all groups of fishes capable of secreting oxygen.[4]

It would appear that the gas gland serves to transfer gases dissolved in the capillary blood into the gaseous phase. It has been argued (Wittenberg, 1958) that it does so through the formation of minute bubbles, assumed to be intracellular, which ultimately enter the swim-bladder. At least in some species of fish, all the oxygen entering the swim-bladder has been shown to enter by way of these minute bubbles. A corollary of this finding is that the gas gland cells must be impermeable to gases, or else part of the oxygen could have entered the swim-bladder by diffusion from the capillaries. The finding that substantial concentrations of nitrogen (up to 10 atmospheres) may be built up in the swim-bladder through the action of the proposed bubbles (Wittenberg, 1958) is in accord with the suggested impermeability of the gas gland to gases. A large difference in nitrogen concentration must be supported across the gas gland cells and the cells must therefore be relatively impermeable to gases. The barrier to gas diffusion must lie within the cells because the liquid phase within which the bubbles are formed has been shown to be in equilibrium with gas tensions in the capillary blood. Electron micrographs place the locus of bubble formation at the base of the gas gland cells, adjacent to the capillary wall (Copeland, personal communication).

The suggestion is advanced that the gas gland through its impermeability to gases serves as a barrier to the back diffusion of gases from the swim-bladder. The formation of minute intracellular bubbles may be thought of as a one way gate or valve allowing gases to move through an otherwise impermeable structure.

In summary, it would appear that the cells of the rete mirabile, by transporting oxygen from the venous to the arterial capillaries, are capable of originating an increased oxygen tension in the arterial capillaries. Countercurrent multiplication of this primary oxygen tension in the rete mirabile may bring about very high oxygen tensions in the gas gland capillaries. The gas gland serves to transmit the high oxygen tension to the lumen of the swim-bladder, while preventing back diffusion. The system may be likened to a piston pump and check valve.

[4] A gas gland has recently been figured in a salmonid (Sundnes *et al.*, 1958), a group in which its existence had been doubted.

REFERENCES

ALLEN, T. H., and ROOT, W. S., 1955, Colorimetric determination of carbon monoxide in air by an improved palladium chloride method, *J. Biol. Chem.*, **216**, 309.

BALL, E. G., STRITTMATTER, C. F., and COOPER, O., 1951, The reaction of cytochrome oxidase with carbon monoxide, *J. Biol. Chem.*, **193**, 635.

DENTON, E. J., 1960, The buoyancy of fish and cephalopods, *in* Progress in Biophysics, J. A. V. Butler and B. Katz, editors, New York, Pergamon Press, in press.

DOUGLAS, C. G., HALDANE, J. S., and HALDANE, J. B. S., 1912, The laws of combination of hemoglobin with carbon monoxide and oxygen, *J. Physiol.*, **44**, 275.

FÄNGE, R., 1958, The structure and function of the gas bladder in *Argentina silus*, *Quart. J. Micr. Sci.*, **99**, 95.

FÄNGE, R., and WITTENBERG, J. B., 1958, The swimbladder of the toadfish, *Opsanus tau*, L., *Biol. Bull.*, **115**, 172.

FAHLEN, G., 1959, Rete mirabile in the gas bladder of *Coregonus lavaretus*, *Nature*, **184**, 1001.

FOX, H. M., 1954, Addendum to paper of Ruud, *Nature*, **173**, 850.

GREEN, J. W., AND HOFFMAN, J. F., 1953, A study of isotonic solutions for the erythrocytes of some marine teleosts and elasmobranchs, *Biol. Bull.*, **105**, 289.

GREEN, A. A., and ROOT, R. W., 1933, The equilibrium between hemoglobin and oxygen in the blood of certain fishes, *Biol. Bull.*, **64**, 383.

HALDANE, J. S., 1922, Respiration, New Haven, Yale University Press.

HALL, F. G., 1929, The influence of varying oxygen tensions upon the rate of oxygen consumption in marine fishes, *Am. J. Physiol.*, **88**, 212.

HALL, F. G., and McCUTCHEON, F. H., 1938, The affinity of hemoglobin for oxygen in marine fishes, *J. Cell. and Comp. Physiol.*, **11**, 205.

HARGITAY, B., and KUHN, W., 1951, Das Multiplikationsprinzip als Grundlage der Harnkonzentrierung in der Niere, *Z. Elektrochem. angew. physik. Chem.*, **55**, 539.

HARTRIDGE, H., 1912, A spectroscopic method of estimating carbon monoxide, *J. Physiol.*, **44**, 1.

HARTRIDGE, H., 1923, The coincidence method for the wave-length measurement of absorption bands, *Proc. Roy. Soc. London, Series A*, **102**, 575.

JACOBS, W., 1930, Untersuchungen zur Physiologie der Schwimmblase der Fische. I. Über die "Gassekretion" in der Schwimmblase von Physoklisten, *Z. vergleich. Physiol.*, **11**, 565.

KEILIN, D., 1929, Cytochrome and respiratory enzymes, *Proc. Roy. Soc. London, Series B*, **114**, 206.

KEILIN, D., and HARTREE, E. F., 1938, Cytochrome oxidase, *Proc. Roy. Soc. London, Series B*, **125**, 171.

KEILIN, D., and WANG, Y. L., 1945, Haemoglobin in the root nodules of leguminous plants, *Nature*, **155**, 227.

KEILIN, D., and WANG, Y. L., 1946, Haemoglobin of *Gastrophilus* larvae. Purification and properties, *Biochem. J.*, **40**, 855.

KROGH, A., 1908, On microanalysis of gases, *Skand. Arch. Physiol.*, **20**, 279.

KROGH, A., 1922, The Anatomy and Physioloy of the Capillaries, New Haven, Yale University Press.

ROCHE, J., 1933, Recherches sur les globines. II. Sur le role des hematines et des globines dans la specificité des pigments hematiniques, *Bull. Soc. Chim. Biol.*, **15**, 110.

ROOT, R. W., 1931, The respiratory function of the blood of marine fishes, *Biol. Bull.*, **61**, 427.

ROOT, R. W., and GREEN, A. A., 1934, The effect of acidity on the carbon monoxide-combining power of hemoglobin in the blood of marine fishes, *J. Biol. Chem.*, **106**, 545.

ROOT, R. W., IRVING, L., and BLACK, E. C., 1939, The effect of hemolysis upon the combination of oxygen with the blood of some marine fishes, *J. Cell. and Comp. Physiol.*, **13**, 303.

ROSSI-FANELLI, A., ANTONINI, E., and POVOLEDO, D., 1958, Chemical and biochemical properties of a new type of myoglobin in molluscs, *in* Symposium on Protein Structure, (A. Neuberger, editor), London, Methuen and Co.

RUUD, J. T., 1954, Vertebrates without erythrocytes and blood pigment, *Nature*, **173**, 848.

SCHOLANDER, P. F., 1954, Secretion of gases against high pressures in the swimbladder of deep sea fishes. II. The rete mirabile, *Biol. Bull.*, **107**, 260.

SCHOLANDER, P. F., 1956, Observations on the gas gland in living fish, *J. Cell. and Comp. Physiol.*, **48**, 523.

SCHOLANDER, P. F., 1960, Oxygen transport through hemoglobin solutions, *Science*, **131**, 585.

SCHOLANDER, P. F., and ROUGHTON, F. J. W., 1942, A simple micro-gasometric method of estimating carbon monoxide in blood, *J. Ind. Hyg. and Toxicol.*, **24**, 218.

SCHOLANDER, P. F., and ROUGHTON, F. J. W., 1943, Micro gasometric estimation of the blood gases. II. Carbon monoxide, *J. Biol. Chem.*, **148**, 551.

SCHOLANDER, P. F., and VAN DAM, L., 1954, Secretion of gases against high pressures in the swimbladder of deep sea fishes. I. Oxygen dissociation in blood, *Biol. Bull.*, **107**, 247.

SCHOLANDER, P. F., VAN DAM, L., CLAFF, C. L., and KANWISHER, J. W., 1955, Micro gasometric determination of dissolved oxygen and nitrogen, *Biol. Bull.*, **109**, 328.

SUNDNES, G., ENNS, T., and SCHOLANDER, P. F., 1958, Gas secretion in fishes lacking rete mirabile, *J. Exp. Biol.*, **35**, 671.

THEORELL, H., 1934, Kristallinisches Myoglobin. IV. Myoglobin im Gleichgewicht mit Sauerstoff und Kohlenoxyd, *Biochem. Z.*, **268**, 64.

WARBURG, O., 1927, Über die Wirkung von Kohlenoxid und Stickoxyd auf Atmung und Gärung, *Biochem. Z.*, **189**, 354.

WITTENBERG, J. B., 1958, The secretion of inert gas into the swim-bladder of fish, *J. Gen. Physiol.*, **41**, 783.

WITTENBERG, J. B., 1959, Oxygen transport—a new function proposed for myoglobin, *Biol. Bull.*, **117**, 402.

WITTENBERG, J. B., 1961, The secretion of oxygen into the swim-bladder of fish. I. The transport of molecular oxygen, *J. Gen. Physiol.*, **44**, 521.

WOODLAND, W. N. F., 1911, On the structure and function of the gas glands and retia mirabilia associated with the gas bladder of some teleostean fishes, with notes on the teleost pancreas, *Proc. Zool. Soc. London*, 183.