OBSERVATIONS ON THE TRANSPORT OF CARBON DIOXIDE IN THE BLOOD OF SOME MARINE INVERTEBRATES.

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While the respiratory properties of the blood of vertebrates, and more particularly of mammals, have formed the subject of numerous investigations, the study of the corresponding functions in invertebrates has received but little attention. Apart from the writings of those authors who have considered the question of the combination of oxygen with the blue copper-containing pigment, hemocyanine, the most important article dealing with the gas content of the circulatory fluids of invertebrates is that of Winterstein (1). This contains an account of the actual quantities of oxygen and of carbon dioxide contained in the bloods, drawn directly from the vessels of a number of marine animals, and serves to correct the erroneous figures previously given by Griffiths (2) who had claimed to have obtained values of the same order of magnitude as those which are normal for vertebrates. By means of the blood gas pump Winterstein showed that the circulating fluids of these invertebrates contained much smaller quantities of gas than Griffiths has supposed. The main result of this investigation was, therefore, to fix the physiological limits of gas content in the bloods of the forms examined. Since Winterstein's work the oxygen and carbon dioxide dissociation curves for mammalian, and more particularly for human, blood have been completely worked out (3). The dissociation of oxygen in invertebrate blood has also received some attention, more particularly in those forms in which either hemocyanine or hemoglobin is present; but the relationships of the carbon dioxide in these forms seems to have been almost entirely neglected. Recently, however, investigations bearing upon this subject have been carried out by Collip (4) whose paper became accessible to us on our return from Italy. Collip used the Van Slyke apparatus

(5), and with it determined the total content of combined carbon dioxide in the body fluids of a large number of marine forms after these fluids had been equilibrated with atmospheric air. In a few cases, also, the carbon dioxide content of the fluids was measured after equilibration with alveolar air. From Collip's results it is seen that, in these cases, the increased amount of carbon dioxide taken up at a pressure of CO_2 of about 40 mm. Hg over and above that taken up at the low tension of the gas existing in the atmospheric air is such as would be accounted for by the extra volume of the gas taken up in physical solution at the higher tension. This means that in the bloods of all the forms examined practically all the available alkali was combined with carbon dioxide at so low a tension of carbon dioxide as that existing in ordinary atmospheric air. It is evident, therefore, that the properties of these fluids are very different from those of human blood, in which, at body temperature, the amount of carbon dioxide taken up increases gradually as the tension of the gas is increased, so that it is not until comparatively high tensions are reached that the greater part of the available alkali has been converted to bicarbonate.

Thus the chief object of Winterstein's work was to measure the gas content of the body fluids of invertebrates, while Collip's investigations were directed towards the determination of the alkali reserve, or total concentration of available alkali in these fluids. Our object, however, has been to plot out the complete carbon dioxide dissociation curves of the body fluids of the commoner and larger invertebrates found in the Mediterranean at Naples, and to look for such correlation as might obtain between the form of curve given by any species and its particular respiratory conditions and habits.

Methods.

Naturally the methods used for withdrawing the blood or other body fluid varied according to the particular animal experimented upon. We shall therefore describe them separately along with the results obtained for each species. Suffice it to say that portions of the fluid obtained were saturated at a temperature of 15°C. with analysed mixtures of air and carbon dioxide by means of the apparatus already described for use with human blood (6). A measured volume of fluid was then run below the surface of 2 cc. of N/40 baryta solution

contained in one bottle of a Barcroft differential apparatus, in which the CO_2 was liberated by acidification in the usual way. The apparatus had previously been calibrated by the use of a standard solution of sodium carbonate. In each case we not only plotted the carbon dioxide dissociation curve of the blood but also attempted to fix the physiological limits by analysing the fluid collected without loss of gas directly from the body into a graduated pipette. In a number of cases we used the Van Slyke apparatus (5) for the determination of the carbon dioxide contents of the fluids, extracting the whole of the gas liberated on acidification and exposure to a vacuum, and afterwards determining the amount of carbon dioxide in this gas by exposure to potash solution contained in a tiny absorption pipette attached to the side tube of the apparatus. We shall now describe the results of the observations we made on the bloods of the various forms we examined.

I.

Maia squinado.

The blood was collected by cutting through the appendages with stout scissors and collecting the fluid as it dripped from the cut surface. The animal showed a great tendency to throw off the injured limb by a process of autotomy at its base. If this happened before sufficient blood had been collected, further limbs were operated upon. The blood so collected has a reddish orange color owing to the presence of the pigment hemerythrin; hemocyanine is also present, but its blue color is masked. This blood does not clot, the animal relying upon its power of autotomy to prevent bleeding to death. The white corpuscles were caused to settle by gentle centrifuging, and the blood was then preserved in the ice chest until it was examined on the 2 days immediately succeeding that on which it was drawn. The points we obtained on the carbon dioxide dissociation curve for this blood were as follows:

CO_2 tension, *mm. Hg*	3.4	6.2	12.0	23.2	43.5	71.0	108.1
CO_2 content per 100 cc., *cc.*	11.9	15.3	17.5	18.6	23.4	26.9	29.6

These values are plotted in the figure, from which it will be seen that the blood of *Maia* possesses a carbon dioxide dissociation curve at sea

temperatures which, over the greater part of its range, ascends much less rapidly than does the corresponding curve for oxygenated human blood at body temperature which is reproduced from Christiansen, Douglas, and Haldane's paper (3) for the sake of comparison. In fact, the curve for *Maia* blood is practically parallel to that showing the amount of carbon dioxide dissolved in pure water at 15°C. But it will be seen that the blood of this animal contains a considerable amount of combined carbon dioxide and that the dissociation curve rises quite steeply at the lowest tensions of this gas. It was of interest, therefore, to determine the tension or content of the blood as it circulates in the animal in order to discover which portion of the curve —the initial steep portion or the later more gently ascending portion— falls within the physiological limits and so is used by the animal. In order to do this we collected a sample of the animal's blood directly into a 1 cc. measuring pipette attached to a hypodermic needle the point of which was inserted into a leg stump. The fluid flowed easily into the pipette from which a measured volume was run off and analysed at once. The carbon dioxide content of this sample was found to be 9.7 cc. per 100 cc. of blood. On another occasion we analysed the blood passing from the gills into the so called pericardium by inserting the needle into a small hole drilled in the dorsal carapace of the animal, in the distinctly marked area which marks the position of the heart below. During the withdrawal of the blood the animal was kept in a small tank of sea water with the back just projecting. The carbon dioxide content of this fluid was 4.9 cc. per 100 cc. of blood. After this experiment, which was carried out by means of the differential apparatus, a second sample of blood was withdrawn in a similar manner and analysed by means of the Van Slyke apparatus. A carbon dioxide content of 12.3 volumes per cent was now observed. From this result it seemed likely that carbon dioxide was accumulating in the animal's body in consequence of its confinement to a comparatively small volume of water. The specimen was therefore left untouched until the evening when further samples of its blood were withdrawn through the hole previously made in the carapace. One of these samples analysed in the differential apparatus was found to possess a carbon dioxide content of 20.4 cc. per 100 cc.; the second sample in the Van Slyke apparatus gave a corresponding value of 18.1.

By the time these last samples were taken the animal had become very "dull" and showed but little response when disturbed. Collip (4) mentions the need for keeping the respiratory conditions of an animal as normal as possible during determinations of its blood gases, and Winterstein (1) has followed the gradual accumulation of carbon dioxide in the pericardial blood of *Maia* while the animal was kept in moist air. But Winterstein's figures for the carbon dioxide content of the bloods of specimens of *Maia* kept under normal respiratory conditions are much higher than those which we observed.

II.

Palinurus vulgaris.

The blood of this lobster, obtained by cutting the appendages, presents a marked contrast to that of the crab *Maia*. In the first place the blue color of the hemocyanine is here not masked by the presence of other pigments. And secondly, the blood on standing clots to a firm jelly. This clotting can be prevented by the addition of potassium oxalate, but only in such amounts as would be likely to influence the carbon dioxide-combining power. These circumstances would seem at first sight to present an insuperable difficulty in the examination of this fluid, but we found it possible to "defibrinate" the blood by allowing complete clotting to occur and grinding the resulting jelly with a little coarsely powdered glass in a small mortar. In this way the threads of fibrin are broken and the original firm clot is converted almost entirely into liquid, the amount of solid remaining being surprisingly small. The glass was filtered off through a layer of clean linen at the pump, and the resulting dark blue defibrinated blood was used for the following experiments.

CO_2 tension, *mm. Hg*.....	4.0	5.8	7.4	9.2	19.3	20.7	46.1	119.1
CO_2 content per 100 cc., *cc*.....	11.2	11.3	11.4	14.4	19.8	23.1	31.4	49.2

From the figure it will be seen that the carbon dioxide dissociation curve in this form rises more gently and to a greater maximum value for the combined carbon dioxide than does that obtained for the blood of *Maia*. The blood of *Palinurus* behaves as if its bicarbonate equilibrium is more profoundly modified by the presence of feebly acid

substances than is that of the blood of any other of the forms we examined.

Owing to the rapid clotting of this blood it was difficult to ascertain the carbon dioxide content of the blood of *Palinurus* drawn straight from the body. By rapid working it was found to be possible to collect a specimen from the cut surface of a leg stump and to measure it off and dilute it in the Van Slyke apparatus before it solidified. In this way we obtained a carbon dioxide content of 5.4 cc. per cent—a value which shows that in the case of this animal, as in *Maia*, it is the initial steep portion of the dissociation curve which is in use under physiological conditions.

III.

Octopus vulgaris and Octopus macropus.

The blood of the *Octopus* was collected by employing the technique devised by Fredericq (7). The animal was nailed by its tentacles to a board and then covered by a duster which left the body exposed. The respiration of the animal was maintained by inserting a glass tube conveying aerated sea water into the mantle cavity. An incision was then made into the body wall in the mid-dorsal line in order to expose the main dorsal artery. This was suitably ligatured to permit of the insertion of a long glass cannula, through which the blood was readily collected in abundance. An alternative method for dealing with this somewhat vigorous and troublesome, but interesting animal is to enclose its tentacles in a suitable bag and to support its body in a wire stand of appropriate shape during the operation. The blood so obtained possesses a deep blue color due to the presence of hemocyanine. It does not clot, the only change observed on standing being an agglutination of the leucocytes.

Our first experiments were carried out on the blood of *Octopus vulgaris* with the following results.

CO_2 tension, *mm. Hg*	2.4	4.5	7.8	14.6	26.2	52.8	102.0	148.7
CO_2 content per 100 cc., *cc.*	5.6	8.4	9.1	11.4	14.5	19.9	26.6	29.5

These figures show a carbon dioxide-combining power less than that observed in the case of *Maia*, but as Dr. Quagliariello, who kindly estimated for us refractometrically the percentage of hemocyanine

in the sample of blood, reported the abnormally small value of 2.9 per cent (about one-third of the amount usually found in this animal), it seemed possible that the specimen we had used was in some way abnormal or that the blood during its collection had become inadvertently mixed with sea water. The results were, therefore, not plotted in the figure. The curve labelled *Octopus* is plotted from the following results, obtained with the blood of a specimen of *Octopus macropus*.

CO_2 tension, *mm. Hg*	6.7	26.3	44.5	80.9	113.9
CO_2 content per 100 cc., *cc.*	12.2	20.3	28.4	34.3	41.0

It will be seen that the carbon dioxide-combining power of this blood is greater than that of *Maia*, and further that the curve has a more gentle slope. A determination of the carbon dioxide content of the arterial blood drawn directly into a pipette from the vessels of another specimen of *Octopus vulgaris* gave a value of 3 cc. CO_2 per 100 cc. of blood. Winterstein found values ranging from 3.94 to 7.09 cc. CO_2 per cent for the arterial blood and from 5.62 to 7.83 cc. CO_2 per cent for the venous blood of *Octopus vulgaris*, so that the physiological limits in this animal also include only the initial steep portion of the curve.

We carried out a few further observations on the respiratory phenomena of this animal. For example, we made a series of determinations of the difference in carbon dioxide content produced as the water circulated through a mantle cavity. In order to collect the outgoing water a short length of glass tube of appropriate diameter (about 1 cm.) was tied lightly into the funnel of the animal, and the collecting and measuring pipette was inserted into this. Each portion analysed consisted of a mixture of several separate fractions of 1 cc. collected from each of a number of "expirations." Successive analyses with the differential apparatus gave values of:

4.6, 5.1, 3.2, 3.1, 4.4, mean 4.1 cc. CO_2 per cent.

The "inspired" water gave readings of:

3.9, 4.4, 3.3, mean 3.8 cc. CO_2 per cent.

Even these few observations show how efficient is the washing out of the respiratory cavity by the sea water, and how large must

be the amount of water passing over the gills in comparison with the amount of carbon dioxide which has to be removed.

We also made an attempt to discover whether the carbon dioxide-combining power of the blood of *Octopus* is influenced by the oxygen content in any way similar to that well known to exist in mammals.

The following three points were obtained by saturating the blood with mixtures of nitrogen and carbon dioxide.

CO_2 tension, *mm. Hg*	45.5	104.1	122.1
CO_2 content per 100 cc., *cc.*	27.1	35.2	38.1

They are represented as black triangles in the figure, from which it will be seen that they fall somewhat below the curve for the oxygenated blood. These measurements are too few in number to enable us to draw certain conclusions, but it appears that oxygenation of hemocyanine produces no change in the carbon dioxide-carrying power of this blood comparable to the important change produced by the oxygenation of the hemoglobin in the blood of a mammal.

It should be mentioned that in these determinations the mixture of blood and baryta solution in the bottle of the differential apparatus was shaken in air before the acid was added to expel the carbon dioxide, in order to saturate the hemocyanine with oxygen and so to ensure that the readings were made under conditions similar to those which obtained during the determination of the "oxygenated" points.

IV.

Phallusia (ascidia) mammillata.

The blood of the ascidians is particularly interesting in some particulars. For Henze (8) has shown that while its plasma is faintly alkaline and possesses a salt content not very different from that of sea water the blood corpuscles contain a fluid which is strongly acid owing to the presence of about 3 per cent of free sulphuric acid. Furthermore, certain of the corpuscles carry a complex vanadium-containing chromogen which is thought to act as a catalyst during oxidative processes. The determinations by Quagliariello (9) of the reaction of the blood of these animals gave values decidedly less

alkaline than those given by other invertebrate bloods. We collected blood from these animals by making a careful incision through the basal part of the mantle on the side opposite to the expiratory orifice so as to expose the thin walled "heart." On puncturing this vessel by means of a hollow needle the greenish blood was quickly expelled. The color is due to the corpuscles. These were found to settle very rapidly on centrifuging, leaving a clear colorless plasma above a thin layer of cells. We have two determinations on the plasma obtained in this way from one animal and two on the whole blood of another specimen.

	Blood.		*Plasma.*	
CO_2 tension, *mm. Hg*	27.4	51.4	27.1	52.7
CO_2 content per 100 cc., *cc.*	0.3	2.9	2.9	5.3

From the figure it will be seen that the carbon dioxide-combining power of the plasma is appreciably less than that of sea water, while that of the whole blood—probably on account of the presence of the acid corpuscles—is still smaller. Winterstein similarly found a vanishingly small quantity of carbon dioxide in ascidian blood which had been equilibrated with air.

V.

Aplysia limacina.

The last body fluid for which we have data is the cœlomic fluid of this gastropod. The clear limpid liquid is obtained in abundance on making a cut in the foot of the animal. It is completely devoid of pigments and from the following results it will be seen that its carbon dioxide dissociation curve is practically identical with that of sea water.

CO_2 tension, *mm. Hg*	0.9	1.2	4.5	7.0	11.5	27.1	57.3	83.6	107.7
CO_2 content per 100 cc., *cc.*	5.0	5.4	5.5	5.8	7.5	8.3	11.4	10.7 (?)	14.5

A determination of the total CO_2 content of a sample of the fluid allowed to flow directly into a pipette attached to a hollow needle inserted into the foot of the mollusc gave a value of 4.4 cc. total CO_2 per 100 cc. of fluid—a value not appreciably different from the 3.7

cc. per cent which we found to be present in the sea water circulating in the aquarium tanks. It is to be concluded therefore that the body fluid in this form has no special function in connection with the carriage of carbon dioxide.

VI.

Sea Water.

We have three measurements on the sea water of the Gulf of Naples as follows:

CO_2 tension, *mm. Hg*	0.8	58.1	125.3
CO_2 content per 100 cc., *cc.*	4.7	10.9	18.3

These are included in the figure and indicate a distinct combining power for carbon dioxide. As just mentioned, the sea water circulating in the aquarium tanks had a total content of CO_2 of 3.7 cc. per 100.

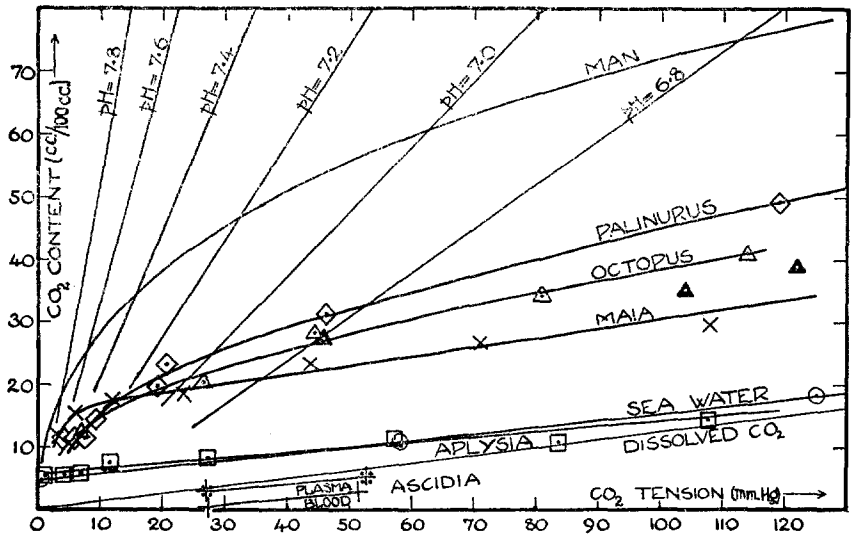

FIG. 1.

GENERAL CONCLUSIONS.

Although the results we have recorded merely serve to indicate the possibilities of this interesting field of investigation, we have sufficient data to enable us to draw certain general conclusions. In the first

place it is evident that the bloods of the more highly developed marine invertebrates, such as the active *Crustacia* and the *Cephalopods*, are specially adapted for the carriage of carbon dioxide. The quantity of carbon dioxide taken up by the blood of *Maia*, *Palinurus*, or *Octopus* at any given tension of the gas is, in general, about twice or three times as great as that which is taken up by sea water under the same conditions. On the other hand, the blood of a slow, creeping form, such as *Aplysia*, or of a sessile animal such as the ascidian *Phallusia* shows no more adaptation for the carriage of carbon dioxide than does sea water.

But our estimations of the CO_2 content of the blood as it circulates in the bodies of these more active invertebrates show that the conditions of transport of this gas differ considerably in some respects from those which obtain in mammals. For the invertebrate blood in the body contains only a relatively small quantity of carbon dioxide, averaging in the forms we examined from 3 to 10 cc. per 100 cc. of blood. This forms a marked contrast with the condition found in mammals where even the arterial blood contains about 50 cc. of CO_2 per 100 cc. of blood. The invertebrate, therefore, works at a very low CO_2 tension. There is a twofold significance in this circumstance. In the first place, it means that only the first portion of the carbon dioxide dissociation curve is in use in the respiratory mechanism. Now an inspection of our curves will show that at these low carbon dioxide tensions the dissociation curves tend to be steeper than at higher tensions. As we intend to show in a later paper it can be proved mathematically that, other things being equal, a blood with a carbon dissociation curve of moderate steepness, *i.e.* one in which the carbon dioxide content of the blood increases fairly rapidly with increase of carbon dioxide tension, is a more efficient carrier of the gas from the tissues to a respiratory surface than a blood in which the dissociation curve is either steeper or flatter. It would seem as if the active invertebrates avoid the use of too flat a part of their CO_2 dissociation curves by working over the initial steeper portion.

Furthermore, it is seen that over the range of this initial steep portion of the curves the changes of reaction produced by the uptake of carbon dioxide are much smaller than at higher tensions of the gas; for these initial portions of the curves are more nearly parallel to the

lines of constant reaction calculated for a temperature of 15°C. according to Hasselbalch's method (10) on the assumption that the whole of the combined CO_2 is in the form of sodium bicarbonate. It is evident also that on this assumption the hydrogen ion concentration of the blood of invertebrates (with the exception of the tunicates) would appear to be practically the same as that of the warm-blooded vertebrates—a conclusion confirmed by the direct measurements of Quagliariello (9). On the other hand, our measurements do not lend support to the idea put forward by Collip (4) that in order to maintain an appropriate faintly alkaline reaction an invertebrate needs to retain carbon dioxide in its blood at a comparatively high tension. This idea was based on the observation that at comparatively high CO_2 tensions the blood of invertebrates contains considerably more sodium bicarbonate than does sea water. But our curves show that this is no longer true at the lower values of carbon dioxide tension, the amount of sodium bicarbonate falling off more rapidly in the blood than in the sea water with diminution of the carbon dioxide tension so that in order to maintain an appropriate reaction in the blood only a comparatively small tension of CO_2 is required. The largest amount of carbon dioxide that we found present in the circulating blood of any of the types examined was 9.7 cc. per 100 cc. of blood in the case of *Maia*, and in most cases the amount was considerably less. But even this lowest value corresponds to a tension of CO_2 of only about 3 mm., so that the tension gradient across the gill membrane must be even less than this. We would emphasize rather the circumstances that as the portion of the dissociation curve over which the reaction is approximately constant is of but small extent, it is necessary that in an active form like *Octopus* the carbon dioxide produced should be removed rapidly lest an accumulation of it should cause the limits of normal reaction to be exceeded; and this need is correlated with the extreme efficiency of the respiratory apparatus in this animal. It is interesting to notice that the mammal which, in order to obtain an appropriate reaction in the blood, has to work at relatively high carbon dioxide tensions where the dissociation curve is comparatively flat, secures a steeper physiological CO_2 dissociation curve in the body, and with it a more efficient carriage of carbon dioxide and a more constant reaction in the circulating fluid, in virtue of the effect of oxygenation on the carbon dioxide-combining power of its blood (3, 6).

Returning now to the consideration of the actual form of the dissociation curves we have obtained—it is a significant fact that it is in those forms such as *Maia*, *Palinurus*, and *Octopus* whose bloods are rich in proteins—particularly hemocyanine—that the initial steep portion of the curve is observed. This suggests that in these forms the blood proteins act as weak acids and expel carbon dioxide from the blood at the low tensions which include the physiological range, just as in vertebrates the hemoglobin similarly displaces carbonic acid from its combination with alkali metal. On the other hand the cœlomic fluid of *Aplysia* contains no pigment and only 0.00672 per cent of protein nitrogen (Bottazzi (11)) and shows no initial rapidly ascending portion of the CO_2 dissociation curve. This is supported by the observation of Quagliariello (9) that the acid-neutralising power of the blood of an invertebrate is roughly proportional to its protein content. It seems as if the proteins of invertebrate blood like the blood proteins of vertebrates, exist in the form of sodium salts which are capable of giving up sodium for the transport of carbon dioxide as sodium bicarbonate. That this is so in the case of hemocyanine follows from the fact that the isoelectric point of this pigment occurs at a hydrogen ion concentration of 2.12×10^{-5} N, *i.e.* at a pH of 4.67 (Quagliariello (12)) so that in the alkaline blood of the invertebrates possessing it, hemocyanine will act as a weak acid. It is probable that the initial steep portion of the carbon dioxide dissociation curves which we have found to be of such importance in the respiration physiology of *Octopus*, *Palinurus*, and *Maia* is produced by the competition of this acid with carbonic acid for the available sodium of the blood.

In conclusion we desire to express our thanks to the Master and Fellows of Sidney Sussex College, Cambridge, for a generous grant towards the expenses of these investigations; to the Director and Staff of the Zoological Station at Naples for the wholehearted manner in which they afforded us the facilities we required; and to the Ambassadors of France and Italy who, on the recommendation of the Royal Society, were good enough to supply us with permits to take our packages of glass apparatus unopened through the customs houses of their respective countries.

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