VIII. THE EFFECT OF CARBON DIOXIDE AND ACETIC ACID ON THE OSMOTIC PRESSURE OF HAEMOGLOBIN.

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THE work of Martin [1896], Reid [1905], Hufner and Gansser [1907], and Roaf [1910] has demonstrated that solutions of haemoglobin, as free as possible from electrolytes, have a measurable osmotic pressure. Measurements of osmotic pressure, therefore, offer a means of studying various changes in the physical and chemical state of haemoglobin in solution.

The purity of preparations studied in this way is, of course, of fundamental importance. Since haemoglobin is believed to exist in the blood as a sodium salt, there is the possibility that it may be isolated as the sodium salt rather than as the free acid. In order to remove any base which may be present, the solutions can be dialysed against water saturated with CO_2 , as used by Bayliss [1911] in connection with his experiments on Congo-red. On carrying out such a procedure in an osmometer, preparatory to certain experiments, the osmotic pressure was observed to rise to a height several times greater than its original value when distilled water alone constituted the outer fluid. When the CO_2 solution was replaced by water the pressure fell to its former value. The present paper records a study of these changes, and similar changes caused by acetic acid.

There are, as far as I know, no observations recorded in the literature on the effect of CO_2 on the osmotic pressure of haemoglobin. Roaf [1910] reports experiments using laked corpuscles or a solution of once-crystallised, dried haemoglobin (to which alkali had been added to get it into solution) on the inside of the osmometer and acetic or hydrochloric acid as the outer fluid. In most cases when the acid used was strong enough to cause a rise in the osmotic pressure, the haemoglobin was changed to acid haematin, and the resulting increase in osmotic pressure must have been due, at least in part, to the increased number of osmotically active particles caused by the breaking up of the haemoglobin molecule. In two experiments (the only ones at all comparable to those reported below) methaemoglobin resulted from the treatment, and slight changes in osmotic pressure were observed. 0.001 *N* HCl gave an increase from 17.5 mm. (the control value against water) to 24.9 mm.

Hg and 0.005 *N* acetic acid gave an increase from 7.5 mm. to 11.2 mm. Hg. In neither case was the increase greater than 50 %, while in the experiments to be reported, a much greater rise in osmotic pressure was observed. No attempt was made to see whether or not the effect due to acids could be reversed by the substitution of water. Roaf concludes, on slight evidence [1910, p. 85] "It is most likely that the correct explanation is that haemoglobin forms ionising salts with alkalies and acids, and that the observed pressures are due to the sum of the pressure caused by haemoglobin and the ions into which the salt can split, for instance, sodium and "haemoglobyl" ions."

Preparation of haemoglobin.

The haemoglobin¹ was prepared according to the method described by Dudley and Evans [1921]. Well-washed corpuscles were laked by dialysing against distilled water in a collodion sac connected with a mercury manometer (see Hartridge [1917] for an earlier use of this method of laking corpuscles, to get a very concentrated solution of haemoglobin). Oxygen was bubbled through the thick haemoglobin solution after the stroma was removed by centrifuging. The crystals of oxyhaemoglobin which were formed in large amount by this procedure, were centrifuged from the mother-liquor and washed into a flask with one or two volumes of distilled water. By evacuating the flask, the oxyhaemoglobin was changed to the more soluble, reduced haemoglobin and the crystals passed into solution. Oxygen bubbled through this concentrated solution caused a recrystallisation of oxyhaemoglobin. Violent agitation of the flask when the oxygen is being bubbled through often helps to start crystallisation. This process may be repeated several times, though it is only fair to say that it becomes increasingly difficult to bring about this crystallisation. Perhaps a certain small percentage of inorganic salt is necessary for crystallisation, and this percentage may become reduced to a minimum as the purification continues. In these experiments, the haemoglobin preparations were always crystallised three, and usually four, times.

Great care was taken to prevent decomposition. The corpuscles were kept as cold as possible from the time of their shedding until the recrystallised haemoglobin was put into the osmometers (7–10 days). The NaCl used to wash the corpuscles was ice cold and the dialysis of the corpuscles, which took several days, was carried out in an ice box, at 4 to 7°. In the earlier experiments, the first crystallisation was brought about by the use of a minimal quantity of ice-cold alcohol added to corpuscles laked with an equal volume of water (the stroma removed, as in the other method, by centrifuging). The subsequent recrystallisation was carried out as given above. An experiment described on a subsequent page of this paper, shows that samples prepared

¹ The haemoglobin used in these experiments was prepared from the corpuscles of oxalated horse blood, obtained, in England, through the kindness of Dr R. A. O'Brien of the Wellcome Physiological Laboratories, and in the United States, through the kindness of Dr John A. Murphy of the Mulford Biological Laboratories.

in the two ways give the same osmotic pressure readings. On theoretical grounds, at any rate, the method of Dudley and Evans is certainly preferable, for alcohol, which may denature the protein, is not used.

Unfortunately, there is no very satisfactory way of determining the purity of a sample of haemoglobin. To test how far the recrystallisation removed the electrolytes from the haemoglobin solution, the conductivity of the mother-liquor (found to contain about 1 % haemoglobin) was tested after each crystallisation. For instance, in one preparation, the conductivity, determined roughly at room temperature (9.5°) was:

Mother-liquor above 1st crop of crystals	...	...	1.7×10^{-3}	mhos
" " 2nd " "	...	...	9.5×10^{-4}	"
" " 3rd " "	...	...	2.4×10^{-4}	"
" " 4th " "	...	...	6.0×10^{-5}	"
A 13.8 % solution of haemoglobin from the 4th crystals			2.5×10^{-5}	"

Another preparation (1.96 % solution) had a conductivity of 3.9×10^{-5} mhos at 18° . This figure is practically identical with that given by Gamgee [1902] for purified haemoglobin. He gives 3.25×10^{-5} for a 2.24 % solution at 18° . The conductivity of the laboratory distilled water, not free from CO_2 at 18° , was 4.25×10^{-6} .

If a saturated solution of haemoglobin is made up quickly, by adding distilled water to oxyhaemoglobin crystals, it is seldom stronger than 2 %. But if the packed haemoglobin crystals are allowed to stand, after removing the supernatant liquid, they will go into solution, sometimes not for several days, sometimes in the course of a few hours, even when kept on ice, giving a solution of 12 to 14 %. This solution gives a definite oxyhaemoglobin spectrum, but as it is difficult, spectroscopically, to distinguish reduced haemoglobin when a small amount of oxyhaemoglobin is present, it is not known how far reduction plays a part in this re-solution. Such a solution after several weeks' standing gives a definite spectrum of reduced haemoglobin. By dialysing corpuscles in collodion sacs against water, Hartridge [1917] was able to obtain solutions of haemoglobin as strong as 48 %. In speaking of the change in colour on dialysing, he says, p. 254: "This is due to the laking of the blood, and not to reduction of the haemoglobin, for if a thin film of the haemoglobin solution is removed from the dialysers it is a bright red in colour and analysis in Barcroft's apparatus shows that it is fully oxygenated." Further study is needed on this point. Both weak and strong solutions were used in these experiments.

Apparatus.

Osmometers of two types were used. Those which will be referred to as Type A were Moore and Roaf osmometers [1907], as modified by Bayliss [1909], with the exception that no precautions were taken to exclude the CO_2 of the air from the solutions in the osmometer. Type B osmometers were made of collodion sacs, fitted with U-tube mercury manometers of 1 mm. bore,

and suspended in bottles (about 150 cc. capacity) containing the outer fluid (Fig. 1). In order to use the minimum of solution (a large yield of haemoglobin does not result after so many crystallisations) and quickly to bring about an equilibrium between the inner contents of the osmometer and the outer fluid, use was made of a glass float which occupied almost the entire space within the collodion sac. Before sealing off the test-tube from which the float was made, mercury was admitted into the tube so that the float, when placed in the sac filled with haemoglobin solution, would slowly sink to the bottom of the sac. This prevented pressure on the bottom of the collodion sac and kept it from floating on the surface of the fluid and interfering with the opening into the manometer. Only 5 cc. of haemoglobin was then needed to fill the osmometer and there was only a thin film of solution between the glass float and the wall of the collodion sac, thus facilitating diffusion by doing away with the central core of liquid. Another advantage is that it is possible to see through such a thin film of solution, and note the appearance of any precipitate, change of colour, etc., in the solution in the osmometer. 10 % Anthony's negative cotton in equal parts of alcohol and ether was used to prepare the sacs.

Fig. 1.
Type B Osmometer

Thymol and toluene were both found unsatisfactory for use in the osmometers or perfusing fluids, because after a time they caused precipitation of the haemoglobin. Haemoglobin solutions, however, used without any preservative did not undergo bacterial decomposition to any noticeable extent, even though kept for several weeks in the osmometers. Experiments were not continued any longer than necessary (usually less than two weeks) and in two cases osmometers were kept in an ice box at 4° throughout an experiment.

Briefly, the experiments were carried out as follows. The manometer tube was filled with haemoglobin above the mercury on the side to be connected with the collodion sac. The manometer carried a cork stopper which fitted into the reagent bottle containing the outer fluid of the osmometer. A float was placed in the collodion sac which was then filled with haemoglobin solution. A rubber stopper (with a hole for the manometer) was inserted securely, a rubber band placed around the neck of the sac and the joint covered with collodion. The manometer was then carefully inserted so that no air space was left in the system and the sac suspended in the bottle containing the outer fluid. The initial pressure in the manometer was due to the insertion of the manometer tube but this pressure rose or fell in a short time to the true osmotic pressure of the solution of haemoglobin. The osmometer and manometer could easily be removed from the bottle containing the outer fluid and held while changing the water in the bottle. Sufficient water was used completely to cover the collodion sac (about 100 cc.).

A comparison was made of preparations of haemoglobin obtained by the alcohol method and the Dudley and Evans method. Solutions of approximately the same strength (9 %) were placed in Type A osmometers and treated in the same manner in each case. The alcohol preparation had been crystallised four times and the D. and E. preparation three times. The following table will show that there is no appreciable difference in the osmotic pressure of the two solutions, either against distilled water or after treatment with acids.

Time of reading osmometers		Outer fluid	Osmotic pressure in mm. Hg	
			Alcohol preparation	D. and E. preparation
18. xi. 21	5 p.m.	Distilled water	39	52
19. xi. 21	1.40 p.m.	"	40	44
21. xi. 21	3.15 p.m.	Water + CO ₂ ($p_H = 4.0$)	64	68
22. xi. 21	10.35 a.m.	"	76	81.5
23. xi. 21	12.40 p.m.	"	93	100
Later in same experiment:				
12. xii. 21	10.45 a.m.	Distilled water	52	54
13. xii. 21	11 a.m.	Acetic acid ($p_H = 4.0$)	68	72
14. xii. 21	4.20 p.m.	"	74	81
15. xii. 21	11.15 a.m.	Distilled water	70	76
16. xii. 21	10 a.m.	" "	68	66

A comparison of oxyhaemoglobin and reduced haemoglobin was made. In this case the osmometers of Type B were used. The reduced haemoglobin was prepared from the same 2 % stock solution as the oxyhaemoglobin, by exposing a small amount of liquid to a vacuum for three and a half hours. In each case the pressure rose to practically the same extent (51–53 mm. Hg) with the use of CO₂. This was to have been expected, as the use of a saturated solution of CO₂ as an outer fluid probably changed the oxyhaemoglobin to the reduced form.

EXPERIMENTAL.

Below is given a detailed description of a characteristic experiment showing the influence of CO₂ and acetic acid on haemoglobin. A weak solution of haemoglobin was prepared by adding water to crystals of oxyhaemoglobin (four times crystallised) and centrifuging to get a clear solution free from crystals. By determining the percentage of solid and ash in 10 cc., it was found to be a 1 % solution of haemoglobin. Without delay, six osmometers of Type B were filled with the same solution. Distilled water of p_H 6.8 was used in all the osmometers as the outer fluid. On the second day of the experiment, and thereafter, oxygen was bubbled through the distilled water before using the osmometers, to keep the oxyhaemoglobin saturated with oxygen and to remove the CO₂ dissolved in the water from the air. When equilibrium had been obtained (the outer water having been changed frequently for two days), all six osmometers gave practically the same osmotic pressure (7 to 11 mm. Hg). This agrees quite well with the calculated osmotic pressure (10.9 mm.) of a 1 % haemoglobin solution at 20°, using 16,669 as the minimum calculated molecular weight of haemoglobin [Gamgee, 1898; Hüfner and Ganssner, 1907;

Barcroft and Hill, 1910]. The experiments were divided into two groups. Three osmometers Nos. 22, 23 and 24 were used for group I, and the other three, Nos. 25, 26 and 27, for group II. One osmometer from each group (Nos. 22 and 25) was kept in an ice-box at 4° throughout almost the entire experiment. The data for these experiments are given graphically in Fig. 2 and Fig. 3.

Fig. 2.

Fig. 3.

Figs. 2 and 3. Effect of CO_2 and acetic acid on the osmotic pressure of haemoglobin. Outer fluids:— a =distilled water ($p_{\text{H}}=6.8$); b^1 =water 1/10 saturated with CO_2 ($p_{\text{H}}=4.2$); b^2 =water half saturated with CO_2 ($p_{\text{H}}=4.0$); b^3 =water fully saturated with CO_2 ($p_{\text{H}}=3.8$); c^1 =acetic acid ($p_{\text{H}}=4.2$); c^2 =acetic acid ($p_{\text{H}}=4.0$); c^3 =acetic acid ($p_{\text{H}}=3.8$). The arrows indicate the first introduction of a particular outer fluid which was changed several times a day until a new outer fluid was used.

In the osmometers of group I, water one-tenth saturated with CO_2 ($p_{\text{H}}=4.2$) was used as outer fluid. This was made by adding nine parts of distilled water to one part of water saturated with CO_2 . Care was taken to minimise the loss of CO_2 but no special precautions were used. The osmotic pressure rose to 20 and 22 mm. Hg in the osmometers at room temperature and to 26 mm. Hg in the one in the ice chest. Half saturated CO_2 solutions ($p_{\text{H}}=4.0$) were used next, followed by fully saturated ($p_{\text{H}}=3.8$), each of which caused a further increase of pressure as may be seen in Fig. 2. This pressure remained constant until, two days later, distilled water was substituted, causing the pressure to fall to its former value. Next, acetic acid of the

same p_H (3.8) as the last carbonic acid solution was used. Although the molar strength of the acetic acid was only 0.0013, compared with 0.042 M of the CO_2 solution, the osmotic pressure rose to practically the same extent in each case. This seems to indicate that this rise in osmotic pressure is due not to the particular acid used, but to the hydrogen-ion concentration of the solution. One experiment with 0.00037 M lactic acid ($p_H = 3.8$) gave similar results.

The order of the experiments was reversed with the second group of osmometers (see Fig. 3). After determining the osmotic pressure of the solution of haemoglobin against water, the p_H of the outer fluid was decreased as in the experiments in group I, using acetic acid instead of CO_2 . The p_H of the solution was changed first to 4.2, then 4.0 and 3.8, with concentrations of acetic acid varying from about 0.0005 N to 0.0013 N . After maintaining this pressure for several days, distilled water was used to remove the acid and, finally, water saturated with CO_2 was put into the outer chamber of the osmometer. It can be seen from the figures that a rise in osmotic pressure occurs, and the rise is practically the same whether one acid or the other is used first as an outer fluid. In osmometer No. 25 the osmotic pressure, when CO_2 was used, was four times the value against water. The systems were considered to be in equilibrium when, after repeated changes of the outer fluid, the hydrogen-ion concentration became constant. The p_H of the outer fluid was determined colorimetrically, using bromophenol blue, bromocresol purple, and phenol red as indicators.

Results similar to those described above were obtained with a number of solutions of haemoglobin of different strengths using both types of osmometer. Haemoglobin prepared by both methods was used. With the stronger solutions the results are similar to those obtained with the 1 % solutions, but there was never proportionally as great a rise in the osmotic pressure produced by acids.

It is important to know whether or not the haemoglobin was decomposed or changed in any way during the experiments. There was no sign of decomposition other than the formation of a small amount of precipitated haemoglobin after acids were used. In the osmometers kept in the cold (Nos. 22 and 25) this amount was very slight. It was evidently too small appreciably to affect the osmotic pressure, for, whenever the solutions in these osmometers were dialysed against water, after the use of acids, the osmotic pressures returned to their former values. In the osmometers at room temperature, on the other hand, after repeated use of acids as the outer fluid, there was sufficient precipitation to account for a progressive reduction in the values against water, as, for example, in osmometer No. 24 from 9—8 to 5 mm. Hg.

To see whether or not oxyhaemoglobin changes on merely standing, two osmometers were filled with the same 1 % solution used in the experiments described in detail above, and were left standing, occasionally changing the distilled water which was used as the outer fluid. The solution at the end of several weeks was apparently unchanged. There was no precipitate and the solution gave the spectrum of oxyhaemoglobin.

Haemoglobin solutions, saturated with CO_2 , are known to become reduced [Preyer, 1868]. But Heidelberger [1922] states that an oxyhaemoglobin solution will not become reduced when a mixture of 20 % oxygen and 80 % carbon dioxide is bubbled through the solution. In the experiments when water 1/10 saturated with CO_2 was used, there was probably enough oxygen present (18–19 %) to keep the haemoglobin completely oxygenated. When, however, water saturated with CO_2 was used, the oxyhaemoglobin was doubtless changed almost completely to reduced haemoglobin.

When air is again admitted to a solution of haemoglobin, through which CO_2 has been bubbled, methaemoglobin is formed [Preyer, 1868]. Oxyhaemoglobin treated with acetic acid [Menzies, 1895] is also changed to methaemoglobin. Hence it is not surprising that at the end of the experiments, after repeated use of CO_2 and acetic acid, the solution in the osmometers was found to give the spectrum of methaemoglobin.

Since a rise in osmotic pressure occurred in all the experiments when acids were used, whether or not oxyhaemoglobin, reduced haemoglobin, or methaemoglobin were present, evidently the effects of the acids are similar with each compound.

DISCUSSION.

The increase in the osmotic pressure of a haemoglobin solution, when treated with CO_2 or with traces of acetic acid must, obviously, be due to the presence of a greater number of osmotically active particles than before. This might possibly be brought about in one of a number of different ways.

1. As the membrane is permeable to the molecules and ions of H_2CO_3 and acetic acid¹, they cannot exist in greater concentration inside the osmometer than outside it. Hence the acid used cannot be responsible for the rise in osmotic pressure.

2. If the haemoglobin had been aggregated in water and the acid had caused de-aggregation, there would have been an increase in osmotically active particles. Both assumptions are, however, contradicted by certain evidence. The observed osmotic pressure against water was approximately the same as the osmotic pressure calculated on the assumption that the haemoglobin existed as single molecules. During the course of the osmotic pressure experiments with acids, the gradual formation of a small amount of precipitate was observed in the osmometers, showing that a certain amount of aggregation had gone far enough to cause gross precipitation, and this would, of course, tend to cause a fall in osmotic pressure.

The work of Barcroft gives additional evidence on both these points. Barcroft [1914, Appendix II, pp. 314–315], gives the values for n in Hill's [1913] formula,

$$y = \frac{100kx^n}{1 + kx^n}$$

for the dissociation curves of dialysed haemoglobin at 40°, equilibrated at

¹ For the sake of brevity, since the results were the same with CO_2 and acetic acid of the same p_{H} , the discussion will hereafter be confined to the experiments with CO_2 .

various CO_2 tensions. At 0 mm. CO_2 tension $n = 1$, at 8 mm. $n = 1.78$, at 33 mm. $n = 2.5$, and at 67 mm. $n = 2.7$. Barcroft [1913, p. 481] gives "the average number of molecules of haemoglobin in each aggregate" as the definition of " n ." According to these calculations, therefore, haemoglobin exists as single molecules in pure watery solution and is aggregated by CO_2 ¹.

Although, in the experiments described in detail in this paper, a weak solution of haemoglobin (1 %), dialysed against distilled water gave an osmotic pressure practically identical with the calculated value, stronger solutions (2–14 %) frequently gave lower pressures than those calculated for the particular concentration of solution used, indicating that some aggregation had taken place. Reid found values only one-third the calculated for 3–6 % haemoglobin solutions, and concludes [1905, p. 18] "that the molecular weight in solution in water would appear to be a multiple of the minimum calculated from analysis." Aggregation seems a more probable explanation.

In this connection it is interesting to note that Lillie found a fourfold increase in the osmotic pressure of a gelatin solution, when sufficient HCl was added to the inner and outer chambers of the osmometer, to make $M/770$ HCl solutions. He says [1908, p. 140] "The increased pressure may safely be regarded as due to a simple change in aggregation state." He does not suggest ionisation as a possible cause. Either de-aggregation or ionisation would give an increased osmotic pressure. The osmotic pressure of his gelatin solutions returned to their former values if the acid was removed by dialysis against water. There was no increase, and sometimes even a decrease, when egg albumin was treated similarly.

3. Decomposition of the haemoglobin into smaller molecules, which were still too large to pass through the collodion sac, would give an increased number of osmotically active particles. The fact that the osmotic pressure can be brought back practically to its former value (see especially No. 25), by substituting water for the acid, is evidence that decomposition plays little or no part in the rise of osmotic pressure. Haemoglobin decomposed into haematin and globin, for instance, would not re-unite to form haemoglobin by merely substituting water for acid in the outer chamber of the osmometer.

4. Martin [1896] has shown that the osmotic pressure of haemoglobin varies with the absolute temperature. Roaf [1912] has shown that the osmotic pressure of haemolysed pig's corpuscles rises from 6.9 mm. (maximum pressure per 1 % of organic matter at 16°) to 10.5 mm. Hg at 37°. Thus, even with wide variations in temperature the variation in osmotic pressure is not great. Furthermore, in the experiments reported in this paper, the temperature seldom varied more than a few degrees, and in no case was there parallelism between a rise of temperature and a rise in osmotic pressure.

¹ *Note added Feb. 5th, 1923.* This aggregation should cause a lowering of osmotic pressure. The rise of osmotic pressure, which is observed in these experiments, must occur in spite of the aggregation caused by the CO_2 . The rise in osmotic pressure cannot, therefore, be as great as it would have been had there been no aggregation.

5. There still remains the possibility of the formation of ions by (a) ionisation of haemoglobin bicarbonate or (b) ionisation of the haemoglobin molecule into protein ions. These possibilities the author now proposes to discuss in some detail.

(a) Since haemoglobin is an amphoteric electrolyte it may be assumed that it can combine with acids to form salts. It has been shown that the osmotic pressure of haemoglobin against CO_2 may be nearly four times as high as against water. If haemoglobin bicarbonate is formed, the salt must have, under these circumstances, an average of three bicarbonate radicles to every haemoglobin radicle in order to furnish enough ions to bring about the increased pressure. A study of Donnan's theory of membrane equilibrium [1911] will show that there would be, under these circumstances, a counter osmotic pressure that must be taken into consideration. Donnan and Harris [1911] and Bayliss [1911] have shown that there is unequal distribution of an electrolyte, such as NaCl, between a solution of the sodium salt of Congo-red inside, and water outside, a membrane impermeable to the Congo-red. From thermodynamic considerations, Donnan [1911] showed that the product of the concentrations of the Na and Cl ions inside the membrane must equal the product of their concentrations outside the membrane. Since a part of the Na ions inside the membrane comes from the sodium salt of Congo-red, then the concentration of the ionised NaCl inside the membrane, will be less than that outside the membrane. This excess of NaCl outside gives rise to a counter osmotic pressure and therefore the osmotic pressure of the Congo-red salt will be less than the theoretical. The extent of the depression of the osmotic pressure depends on the relative concentrations of the colloid and electrolyte.

Applying this theory to our experiments, we have the following: where R = the cation of haemoglobin bicarbonate, (1) = outer fluid, (2) = inner fluid, and x , y and z are ion concentrations.

According to Donnan and Allmand [1914] the concentration of the undissociated electrolyte is the same on both sides of the membrane, and hence will not affect the osmotic pressure in any way. The ionic equilibria may be expressed:

$$y(y + z) = x^2.$$

Under these circumstances, instead of the osmotic pressure being dependent only upon the concentration $2z$, there is a counter osmotic pressure represented by $2x - 2y$. Furthermore, the H-ion concentration (y), inside the membrane, is less than the hydrogen ion concentration (x), outside the membrane. The following calculations, made on the basis of the foregoing, show

the extent of the counter osmotic pressure and the difference in p_H on the two sides of the membrane. The haemoglobin bicarbonate is this time written with three bicarbonate radicles for each haemoglobin radicle to account for the four-fold increase of osmotic pressure observed while using CO_2 .

When the p_H of the outer fluid is 4, $x = 0.0001 N$. A 1 % haemoglobin solution (molecular weight 16,000) is $0.0006 M$. Assuming complete ionisation of the haemoglobin bicarbonate, $z = 0.0006 N$. Substituting in

$$\begin{aligned}
 y(y+3z) &= x^2, \\
 y(y+0.0018 N) &= 0.000,000,01 N, \\
 y^2 + 0.0018 N y &= 0.000,000,01 N,
 \end{aligned}$$

y^2 being infinitesimal as compared with the other values, may be disregarded. Hence

$$y = 0.000,006 N.$$

The counter osmotic pressure is dependent upon the excess of ionised H_2CO_3 outside the membrane over that inside the membrane

$$2x - 2y = 0.000,18 N.$$

The osmotic pressure of the haemoglobin bicarbonate, which is dependent upon the concentration $4z$ ($0.0024 N$), would, in this case, be reduced about 10 % due to the unequal distribution of the ionised H_2CO_3 . For example, if the osmotic pressure had been 10 mm. Hg against water, and a haemoglobin bicarbonate were formed (such as described above), the pressure instead of rising to 40 mm. Hg would rise to 36 mm. Hg. In these experiments, therefore, since the x concentrations are much less than the z concentrations, the counter osmotic pressure does not interfere much with the osmotic pressure determinations. With stronger solutions of haemoglobin the counter osmotic pressure would be proportionately less. A 10 % solution would show 99 % of the calculated osmotic pressure.

The effect of the Donnan equilibrium on the H-ion concentration of the inner fluid is more marked in the example given above than is the effect on the osmotic pressure. y , the H-ion concentration of the inner fluid, is $0.000,006 N$ ($p_H = 5.2$), while x , the H-ion concentration of the outer fluid is $0.0001 N$ ($p_H = 4.0$). With a 10 % solution of haemoglobin, in the form of haemoglobin bicarbonate, the inner fluid would have a p_H of 6.2. Since the isoelectric point of haemoglobin is about $p_H 7.0$ (Michaelis and Takahashi [1910] give 1.9×10^{-7} ($p_H = 6.75$) and Campbell and Poulton [1920] $p_H = 6.98$), undoubtedly a salt could be formed at a p_H of 5.2. A p_H of 6.2 might also be sufficiently acid for salt formation, although Bayliss [1921] has shown, for

dialysed blood serum, that there is a considerable lag in salt formation with acids, until the p_H is well on the acid side of the isoelectric point.

(b) Considerable evidence for the dissociation of proteins into two or more protein ions, due to the action of acids and alkalis, is given by Robertson. He says [1918, p. 20] "we may now regard it as an established fact that some elements in the protein molecule other than the terminal $-\text{NH}_2$ or $-\text{COOH}$ groups are responsible for the acid- and base-neutralising power which is possessed in such a marked degree by many proteins." Substituting H_2CO_3 for HCl in equations ii (p. 25) and vi (p. 26) we have:

In this case we would presumably have no Donnan effect, as the freely-diffusing dissociated carbonic acid has no ion in common with the haemoglobin, which has dissociated into protein ions. With the equal distribution of H and HCO_3 ions on both sides of the membrane there should be the same H -ion concentration on both sides. If the above theoretical considerations are sound, it should be possible to decide what kind of dissociation of haemoglobin has taken place by testing the p_H of the haemoglobin solutions in the osmometers, by means of the hydrogen electrode. If only protein ions are formed, the H -ion concentration of the inner fluid should be the same as the outer fluid. If haemoglobin bicarbonate is formed, and the Donnan equilibrium comes into play, the inner solution should be much less acid than the outer. The same relations should hold true for a haemoglobin acetate also. The author proposes to investigate these points shortly.

CONCLUSION.

It is shown that the osmotic pressure of solutions of purified haemoglobin is three or four times as great when dialysed against acetic acid, or water saturated with CO_2 (both about p_H 4), as against water alone. Reasons are given for believing that this is due to the formation of some sort of an ionising salt of haemoglobin, either one which ionises into several protein ions, or into protein and acetate or protein and bicarbonate ions.

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