CCVII. BASE BINDING IN ERYTHROCYTES

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(*Received 24 July 1937*)

The inorganic cation content of erythrocytes is greater than the content of inorganic anion and most writers have assumed that the excess is combined with haemoglobin. This is the view of Henderson [1928], who, however, points out that cell base is sometimes in excess of $HCO_3^- + Cl^- + Hb^-$. He considers this excess of base to be negligible in normals and due to the presence of small quantities of acids "not accounted for in the balance sheet." These acids he calls HX, and $X^- = B^+ - Cl^- - HCO_3^- - Hb^-$. Henderson calculates Hb^- from Van Slyke's data for horse blood [Van Slyke *et al.* 1923]. Henderson, however, was working with human blood and as the buffering power of human haemoglobin is much less than that of horse haemoglobin it follows that Henderson's value for Hb^- were too high and his figures for X^- too low. In a recent paper, Maizels [1936] using Adair's data [1925] for the buffering power of oxyhaemoglobin came to the conclusion that in normal human cells the amount of base combined with HX, though small, was not negligible, and that in the erythrocytes of the microcytic anaemias the value of X^- might rival that of Hb^- itself. But the figures quoted were only approximate and suffered from the defect of being based on several assumptions: firstly, the value of cell bicarbonate was derived by calculation from plasma bicarbonate; secondly, the presence of other cell anions like phosphate was neglected; and thirdly, as cell *p*H was not known, the amount of base actually bound by haemoglobin could not be determined. It was therefore necessary to assign certain limiting values to cell *p*H and in this way the value of X^- was derived.

Lately, Dill *et al.* [1937] have published experiments supporting the view of Henderson that X^- is insignificant.

In the present communication an attempt has been made to define the value of X^- more exactly and it will be shown that the value of X^- is often nearly as great as that of Hb^- itself. It may be said at once that our values for $B - Cl - HCO_3$, that is for $Hb^- + X^-$, agree with those of Dill *et al.*, but it will be shown that Dill *et al.* have used values for the buffering power of Hb^- which are too great and which exalt the value of Hb^- at the expense of X^-.

Experimental conditions have been simplified in the following way: erythrocytes were washed five times in isotonic potassium chloride solution by alternate suspension and centrifuging after which the supernatant potassium chloride solution was completely removed. It was assumed that all anions of low molecular weight had been washed out of erythrocytes and replaced by Cl^-. This assumption was based on the observation that if erythrocytes were washed in potassium nitrate solution, chloride was completely removed; hence if nitrate displaces chloride, it seemed probable that chloride washing would remove all other simple anions like bicarbonate, phosphate etc. In later experiments it was necessary to consider this more fully and it was found that bicarbonate in washed cells amounted to about 1·5% of the total anion present, but for the present it will be assumed that the only significant diffusible anion in washed cells is chloride. Under these circumstances, if X^- is insignificant, it may be said

that Base (B) − Chloride (Cl) equals the amount of base combined with Hb and (B − Cl)/Hb is the amount of base bound per unit of Hb. On the other hand, if X^- is significant, then (B − Cl − Hb^-)/Hb is a function of X^-.

In the first series of experiments, total base, Cl and Hb were estimated and the averages of twelve normal and anaemic cases are shown in Table I.

Table I

Group	Hb g. per 1000 ml. blood	Hb g. per 1000 ml. cells	Base m.eq. per g. Hb	Cl m.eq. per g. Hb	(B − Cl)/Hb m.eq./g.
Normals	144	323	0·353	0·255	0·098
Anaemics	70	251	0·487	0·324	0·163

It will be seen that cell chloride and especially cell base have a much higher value in the cells of microcytic anaemia than in normals and, further, the amount of base not combined with chloride is much greater in anaemic cells. It might be wondered if looser packing of centrifuged anaemic cells could account for these findings. Obviously, loose packing could not account for the increased value of B − Cl, since any increase of B due to intercellular KCl must be accompanied by an equivalent increase of Cl. But if intercellular KCl in anaemia were as high as 4% instead of the 1% or less occurring in washed normal cells, then the apparent increase in the absolute value of cell base would still be less than 1%. The cell content of chloride is naturally lower than that of base, but even here, the effect of intercellular salt inclusion would still be less than 2%—an increase which may be safely neglected.

It may therefore be said that cell base, cell Cl and the value (B − Cl)/Hb all vary inversely with the content of cell Hb. The relative increase of base and chloride is doubtless associated with the greater water content of the anaemic cells. The increase in the value of (B − Cl)/Hb is less easy to explain, but that it in fact occurs is confirmed by Table III in which base, chloride and base-chloride per g. of cell dried weight are shown for a number of individual cases.

This apparent increase in the amount of base bound by Hb in anaemia may be due to three causes: either the anaemic washed cell solution is much more alkaline than the normal cell solution, or some other substance, X, is present in cells besides HCO_3, Cl and Hb, which binds base in considerable amounts, or else haemoglobin from anaemic cells binds more base than haemoglobin from normal cells. The last is unlikely. Further analysis of the figures, however, is hardly possible unless one knows the *p*H of the laked cell solution, since the amount of base bound by Hb increases with *p*H. The investigation of base, Cl, Hb and *p*H of laked cell solutions in normals and anaemics was undertaken in series 2.

Method. Blood was centrifuged in tubes shaped like inverted bottles open at the wide end. The narrower portion was about 5 cm. long and held a little more than 0·5 ml. Separated plasma and the excess of cells were removed down to the 0·5 ml. mark and the cells resuspended in fourteen volumes of isotonic potassium chloride solution. The wash fluid was warmed to between 30 and 38° to hasten the exchange of anions, but no attempt was made to control the temperature accurately. The process of centrifuging and washing was repeated four more times and finally, the supernatant solution was all removed and the last traces washed away from the upper face of the packed red cell column with several changes of distilled water. The cells were then haemolysed in a large volume of distilled water to give a solution containing about 0·5 % Hb. In this solution, total base was estimated by the method of Adair & Keys [1934], chloride by the

method of Claudius [1924] and haemoglobin with the Haldane haemoglobinometer. *p*H was determined with the hydrogen electrode [Harington & Neuberger, 1936] immersed in a constant temperature bath at 25°, this relatively low temperature being chosen so as to limit the *p*H changes which readily occur in dilute solutions of blood kept at 38°.

Base was estimated in duplicate or triplicate, the error for 0·004 m.eq. being less than ±3%, while for chloride, the experimental error was within ±2%. It is felt that some justification is needed for estimating haemoglobin with the Haldane haemoglobinometer. The method has speed, simplicity and above all economy of material to recommend it. For the more accurate oxygen capacity method it would have been necessary to have used as much blood as for all the other analyses combined—an important matter in the case of a patient whose haemoglobin is already half the normal. Further, estimation of oxygen capacity is not well suited to measuring haemoglobin in dilute solutions. There is admittedly a personal factor in colorimetric methods, but in experienced hands, a given sample of blood should give results differing by not more than 3%. Of course it is necessary to calibrate the apparatus, which in the present instance gave readings 6% too low. Calibration was effected by the refractometric and gasometric estimation of standard solutions: the former method being used for two solutions of dialysed haemoglobin and the latter for a fresh specimen of blood. Accordingly, all haemoglobin results have been corrected by 6% and, subject to this, it is felt that results obtained with the Haldane haemoglobinometer are accurate to within ±1·5%. Moreover, many of the results are referable to the dried weight (Dw.) of the cell solution and are quite independent of the haemoglobin values. Finally, in some of the experiments of Series IV, haemoglobin has been measured gasometrically. Calibration of the hydrogen electrode is described in greater detail in Appendix I.

Knowing the amount of Hb and the *p*H of a solution it should be possible to determine the value of X^-. Under experimental conditions, however, the haemoglobin solution becomes reduced and unfortunately there are no figures for base binding of reduced human haemoglobin in dilute solutions. This was determined from a sample of pure human haemoglobin kindly prepared by Dr G. S. Adair.

Subsequent remarks therefore fall into the following sections.

(*a*) Titration curve of reduced human haemoglobin (0·552 g. per 100 ml. solution).

(*b*) Base bound by haemoglobin and other anions in washed anaemic and normal erythrocytes.

(*c*) Titration curves of washed and laked erythrocyte solution.

(*d*) Titration curves of washed, laked and dialysed erythrocyte solution.

The titration curve of reduced human haemoglobin

The observed osmotic pressure of the sample was in excess of what might have been anticipated from its haemoglobin content and indicated the presence of an amount of acid or base equal to $0{\cdot}238\,M$ per mol. haemoglobin and corresponding to a shift in the isoionic point of ±0·026 *p*H. As the preparation contained 1·2 volumes of carbon dioxide %, corresponding to a shift in *p*H of 0·028 it was assumed that the impurity present in the sample of haemoglobin was carbon dioxide and not base. Full details of this specimen are given in Appendix II.

10 ml. of a solution containing 0·552 g. Hb per 100 ml. were titrated with the hydrogen electrode using a saturated KCl-calomel half-cell. A sodium borate

buffer at *p*H 9·2 and 0·01 *N* HCl in 0·09 *N* NaCl were used as standardizing solutions and the E.M.F. recorded at 25° were 785 and 364·5 mV. corresponding to values of ϵ for the calomel cell of 242 and 241·5 mV. respectively, where $\epsilon = \text{E.M.F.} - (p\text{H} \times 59{\cdot}1)$ mV.

Table II. *Titration curve of haemoglobin solution (0·552 °/o) with N/50 NaOH at 25°*

Experiment I			Experiment II		
m.eq. base per g. Hb	E.M.F. mV.	*p*H	m.eq. base per g. Hb	E.M.F. mV.	*p*H
0	662	7·12	0	661·5	7·11
0·018	669	7·23			
0·038	677	7·37	0·035	676	7·35
0·054	686	7·53			
0·076	694	7·66	0·073	693	7·64
0·092	701	7·78			
0·110	710	7·93	0·112	709·5	7·92
0·127	718	8·06			
0·147	726	8·20	0·145	727	8·22
0·167	734	8·33			
0·185	747	8·55	0·182	747	8·55

Experiment III			Experiment IV		
m.eq. base per g. Hb	E.M.F. mV.	*p*H	m.eq. base per g. Hb	E.M.F. mV.	*p*H
0	662	7·12	0	663	7·13
0·038	676·5	7·36	0·035	676·5	7·36
			0·059	687	7·54
0·076	695	7·68	0·076	695	7·68
0·112	709	7·91			
0·148	728	8·23	0·147	728·5	8·25
0·182	747·5	8·56	0·183	748	8·56

It will be seen that the isoionic point of reduced haemoglobin is at *p*H 7·12 at 25°. To this must be added 0·03 owing to the presence of CO_2. It must be noted, however, that while the haemoglobin solution is in the electrode vessel, its average depth is only 3 mm., while it exposes a surface area of 30 sq. cm. to an atmosphere of pure hydrogen throughout the time during which equilibrium is being attained. Under these circumstances it is likely that much of the CO_2 will escape and that the isoionic point of haemoglobin lies between 7·12 and 7·15 at 25° and probably nearer the former figure. The effect of temperature may be taken as 0·018 *p*H per degree [Adair *et al.* 1929] so that the isoionic point at 38° ought to be at *p*H 6·88.

As the titration curve is almost straight over short ranges, the buffering power of haemoglobin per g., β_g, may be expressed by dividing the amount of base added to a known amount of Hb by the change in *p*H resulting. Thus the buffering power between *p*H 7·2 and 7·6 is 0·0545/0·4 or 0·136 m.eq. per *p*H. Between 7·6 and 8·0 it is 0·130 and between *p*H 8·0 and 8·4 the buffering power is 0·118. To convert these figures into eq. per mol. Hb (molecular weight = 67,000), it is necessary to multiply by 67 and this gives a buffering value of 9·1 between 7·2 and 7·6. This agrees well with Adair's [1925] figure of 9·2 for reduced human haemoglobin—although Adair's solutions were many times stronger than ours.

How far the above figures have an absolute value may be questioned. They provide no data as to the completeness of the reduction of haemoglobin in the electrode vessel, or of the CO_2 content after exposure of the solution to a current of hydrogen, nor has the considerable amount of KCl diffused out of the agar bridge been estimated. It may even be questioned if the hydrogen electrode

affords a suitable method for titrating haemoglobin solutions, although it has been used by Van Slyke *et al.* [1925] and by Cohn *et al.* [1937], while Sendroy *et al.* [1936] have shown that estimations of blood *p*H with the glass and hydrogen electrodes are in close agreement; the latter giving results which are about 0·01 greater. It is probable, then, that the figures we have given have in fact an absolute value; that the reduction of haemoglobin by saturation with hydrogen is practically complete and that the error due to the presence of CO_2 is less than 0·05 *p*H. (This probable error is calculated on the basis that a solution containing 0·5 % Hb will, after rocking in the electrode vessel, hold less than 0·08 volume % of CO_2—an assumption that is justified by the carbon dioxide analyses given with the bloods of series III.

In any case the absoluteness or otherwise of the preceding figures relating to the isoionic point and buffering power of haemoglobin is a matter with which we are not strictly concerned. The data state that under certain fixed conditions a solution of haemoglobin will bind a certain amount of base and contribute a certain E.M.F. to the current generated in a hydrogen ion concentration chain. These same data may be applied to estimating the base bound by haemoglobin present in a solution of laked cells contributing the same E.M.F. to a similar concentration chain. In short, if the haemoglobin data have no absolute value, they are at least strictly comparable and applicable to the haemoglobin present in laked cell solutions.

Base bound by haemoglobin and other anions in the erythrocytes of anaemic and normal bloods

In Table III the following are set out in vertical columns: (1) Case number. (2) Hb, g. per 1000 ml. blood; this is the conventional index of anaemia. (3) Hb, g. per 1000 ml. cells; this indicates the haemoglobin deficiency of the cells. (4) E.M.F. of the laked cell solution at 25°. The calomel cell was standardized against 0·01 *N* HCl in 0·09 *N* NaCl. The E.M.F. was 364 ± 0·5 mV. (5) *p*H of the solution at 25°; *p*H = (E.M.F. − 242)/59·1. (6) Base, m.eq. per g. dried cell material, that is B/Dw. (m.eq./g.). (7) Cl, m.eq. per g. dried cell material: Cl/Dw. (m.eq./g.). (8) Hb, g. per g. dried cell material: Hb/Dw. (g./g.). (9) Hb^- per g. dried cell material, m.eq./g., that is 0·136 × (*p*H − 7·12) × Hb/Dw. (10) X^-, m.eq. per g. Dw.

X^- is shown together with the extreme variations due to the sum of the experimental errors in the estimation of base and chloride.

Table III

No.	Hb g. per 1000 ml. blood	Hb g. per 1000 ml. cells	E.M.F. mV.	*p*H	B/Dw. m.eq./g.	Cl/Dw. m.eq./g.	(B − Cl)/ Dw. m.eq./g.	Hb/ Dw. m.eq./g.	Hb^-/ Dw. m.eq./g.	X^-/Dw. m.eq./g.
1	43	194	684·5	7·49	0·474	0·315	0·159	0·79	0·040	0·119 ± 0·014
2	48	220	691·5	7·61	0·476	0·297	0·179	0·80	0·053	0·126 ± 0·014
3	64	236	684·5	7·49	0·426	0·275	0·151	0·80	0·040	0·111 ± 0·016
4	81	240	686	7·51	0·412	0·257	0·155	0·81	0·043	0·112 ± 0·011
5	78	259	687	7·53	0·407	0·265	0·142	0·81	0·045	0·097 ± 0·008
6	68	261	685·5	7·50	0·377	0·232	0·145	0·84	0·043	0·102
7	97	270	688	7·55	0·364	0·225	0·139	0·85	0·050	0·089 ± 0·018
8	178	306	686	7·51	0·328	0·223	0·105	0·85	0·045	0·060 ± 0·002
9	143	311	694	7·65	0·293	0·197	0·096	0·87	0·063	0·033 ± 0·010
10	154	311	690	7·58	0·289	0·187	0·102	0·88	0·055	0·047 ± 0·008
11	128	318	680	7·41	0·267	0·187	0·080	0·87	0·034	0·046 ± 0·012
12	160	321	691·5	7·61	0·318	0·216	0·102	0·86	0·057	0·045
13	129	322	697	7·70	0·317	0·191	0·126	0·86	0·068	0·058 ± 0·008
14	154	333	680	7·41	0·276	0·199	0·077	0·86	0·034	0·043 ± 0·010
15	162	340	690	7·58	0·303	0·213	0·090	0·87	0·054	0·036 ± 0·007

Several observations in Table III call for comment. Column 5 shows that even after five washings cells remain comparatively alkaline. This is to be expected. If cells were freely permeable to anions and cations, a few washings would bring the cell to the *p*H at which Hb is isoionic. But as the erythrocyte is impermeable to cation, the free passage of anion is restricted, since such passage would involve a hydrolysis of KCl. Such a hydrolysis must in fact occur to some small extent but this could account for only a very small decrease in *p*H of the washed cell: a more important cause lies in the repeatedly washed cell losing its impermeability to cation so that a slow exchange of salts gradually occurs. Without this partial loss of cation impermeability, it would probably require many washings to bring about a significant change in cell *p*H.

The variations of *p*H in different experiments may be due to variations in the proportion of wash fluid to cells and also to differences in the temperatures at which wash fluid and cells are equilibrated. For if one system is equilibrated at a higher temperature than another, the increased acid dissociation of Hb will cause it to bind more base and the warmer system will contain less chloride. If now the hydrogen ion concentrations of all the systems are examined at the same temperature, the originally warmer system will appear to be more alkaline. It has been seen that no attempt was made to keep the temperature of the systems constant; indeed it would not have been possible to control cooling while centrifuging the cell suspensions. Nor is there any advantage in keeping the temperature of equilibration constant, provided that one estimates the final *p*H at constant temperature, since changes in the distribution of base and chloride due to variations in the temperature of equilibration are reflected in the *p*H recorded at the constant temperature of the hydrogen electrode and, knowing the values for base, Cl, Hb and *p*H at a given temperature in the laked cell solution, one can calculate Hb^- and X^- at that *p*H.

Columns 6 and 7 show that base and chloride increase inversely with the content of Hb per unit volume of original cells. As the increase in base is the more marked, the value B − Cl also increases inversely with cell Hb. Column 9 shows that the non-haemoglobin portion of the dried cell material is greater in anaemic cells.

It is in column 10 that we are chiefly interested: this shows that in normal cells, Hb^- is combined with about half the non-chloride combined base and X^- with the rest. In anaemic cells, X^- binds about twice as much base as haemoglobin. These values for X^- relative to Hb^- are at variance with the work of Henderson and of Van Slyke and in view of this it was thought necessary to reinvestigate the value of X and to obtain more complete experimental data. Hitherto, the presence of CO_2 in the cell solutions has been neglected, it being assumed that most of the CO_2 in the cell solution would be removed by the current of hydrogen in the electrode vessel, but if this assumption were incorrect, then the high values obtained for X^- might be quite deceptive. In the experiments of series III, therefore, the following have been investigated: (1) Base, Cl, Hb and dried weight of a dilute solution of laked cells, together with the CO_2 content and *p*H at 25°. (2) Hb and dried weight of a concentrated solution (Hb, 3–5 g.) and also the *p*H and CO_2 content at 38°. From these figures the value of X^- has been derived, Hb^- per g. Dw. being calculated at 25° from the present writers' data, $Hb^- = 0{\cdot}136 \times (pH - 7{\cdot}12) \times Hb/Dw.$, and at 38° from the data of Adair [1925] who gives the buffering power of reduced haemoglobin as 9·2 eq. per mol. or 0·138 m.eq. per g. Reduced haemoglobin has been taken to be isoionic at *p*H 6·81 at 38°, so that Hb^- per g. of dried weight $= 0{\cdot}138 \times (pH - 6{\cdot}81) \times Hb/Dw.$

The results of series III are summarized in Table IV and two typical experiments are set out in detail in Appendix III. It will be seen that the findings confirm all the previous observations that X^- is nearly as great as Hb^- in normal bloods and is very much greater in anaemic bloods.

Table IV

No.	$T°$	Hb g. per 1000 ml. blood	Hb g. per 1000 ml. cells	pH	B/Dw. m.eq./g.	Cl/Dw. m.eq./g.	Hb^-/Dw. m.eq./g.	HCO_3/Dw. m.eq./g.	X^-/Dw. m.eq./g.
1	25	149	315	7·59	0·304	0·199	0·055	0·002	0·048 ±0·007
	38	—	—	7·29	—	—	0·057	0·002	0·046 ±0·007
2	25	147	318	7·56	0·291	0·195	0·054	0·004	0·038 ±0·006
	38	—	—	7·24	—	—	0·054	0·005	0·037 ±0·006
3	25	160	320	7·50	0·304	0·207	0·045	0·008	0·044 ±0·010
	38	—	—	7·26	—	—	0·055	0·002	0·040 ±0·010
4	25	81	240	7·41	0·415	0·261	0·033	0·005	0·116 ±0·016
	38	—	—	7·18	—	—	0·043	0·006	0·105 ±0·016
5	25	64	222	7·44	0·416	0·272	0·037	0·009	0·098 ±0·008
	38	—	—	7·23	—	—	0·046	0·005	0·093 ±0·008

(Solution at 25°—weak; at 38°—strong.)

Series IV

In this series X^- was estimated in fully oxygenated cell solution. pH was measured at 38° with the McInnes glass electrode. The strong cell solution was used and the time for equilibrium to be reached at 38° was noted. The CO_2 content of the strong cell solution kept for a similar time at 38° was also measured in cases 1*a* and *b* and 2*a* and *b*; this was not possible in cases 3 and 4 and an assumed average value for CO_2 has been applied to these. Hb and Dw. were also estimated in the strong solution and in cases 1*a*, 2*b* and 3, colorimetric values for Hb were confirmed by gasometric measurements. The strong solution was then diluted 7·5 times and the Hb, Dw., base and Cl of this standard dilution were measured. Results are summarized in Table V. *a* and *b* represent observations on the same case carried out at intervals of a fortnight. Adair [1925] gives the buffering power of HbO_2 as 9·88 m.eq. per mol. HbO_2 or 0·148 m.eq. per g. The isoionic point of HbO_2 has been taken to be at pH 6·65—a value midway between the usually accepted value of 6·6 and the value 6·7 given by Hastings *et al.* [1925] so that Hb^- per g. of dried weight $= 0{\cdot}148 \times (p\text{H} - 6{\cdot}65) \times \text{Hb/Dw.}$

Here again, the value of X^- is from one-half to three-quarters the value of Hb^- in normals and is very much greater in anaemic bloods.

The values for X^- in this series are a little lower than in the preceding series. This may arise from the effects of oxygenation of Hb, which lowers the pH of

Table V

$T = 38°$

No.	Hb g. per 1000 ml. blood	Hb g. per 1000 ml. cells	pH	B/Dw. m.eq./g.	Cl/Dw. m.eq./g.	Hb^-/Dw. m.eq./g.	HCO_3/Dw. m.eq./g.	X^-/Dw. m.eq./g.
1 *a*	154	313	7·00	0·283	0·203	0·045	0·004	0·031 ±0·008
1 *b*	—	—	7·08	0·300	0·203	0·055	0·005	0·037 ±0·011
2 *a*	149	311	7·19	0·310	0·201	0·071	0·005	0·033 ±0·004
2 *b*	—	—	7·08	0·301	0·210	0·055	0·004	0·032 ±0·004
3	158	320	6·97	0·283	0·201	0·041	0·004*	0·037 ±0·010
4	61	252	6·90	0·423	0·277	0·031	0·004*	0·111 ±0·010

* Assumed figures.

the system. The isoionic point of Hb is also lowered; its buffering power is increased and base bound at lower *p*H is greater. But this effect will be absent in the case of X, which will accordingly bind less base at the lower *p*H.

The findings in series III and IV may now be contrasted with those of the writers mentioned previously. Van Slyke *et al.* [1925] give the following data for horse cells: per kg. cell water Hb^-, 0·050 eq.; X^-, 0·015 eq. Here X^- is by no means insignificant. In many of Henderson's experiments total base has not been determined. In some, however, base is known and X^- is calculated from the value $B-Cl-HCO_3-Hb^-$. Hb^- has been obtained from Van Slyke's data for horse haemoglobin, which has a much greater buffering power than human Hb, so that Hb^- is increased at the expense of X^- and the latter appears almost negligible. The most recent data are those of Dill *et al.* [1937] who give average values for twelve normals. Data calculated from the figures of these authors are shown in Table VI, together with our own. In order to make the data comparable, we have expressed our values not as m.eq. per g. Dw., but as m.eq. per 1000 ml. cells.

Table VI

Author	No. cases	Hb g. per 1000 ml. cells mean	*p*H	B m.eq. per 1000 ml. cells mean	$Cl+HCO_3$ m.eq. per 1000 ml. cells mean	Hb^- m.eq. per 1000 ml. cells mean	BP_c m.eq. per 1000 ml. cells mean	X^- m.eq. per 1000 ml. cells mean
D. E. C.	12	337 HbO_2	7·19	110·5	69·8	?	41·6	− 1·0
M. P.	3	318 Hb	7·26	109	74	20	—	+15
M. P.	5	315 HbO_2	7·06	107	74·5	20	—	+12·5

It will be clear from the table that when allowance is made for differences in oxygenation and *p*H, the values of $B-Cl-HCO_3$, that is of Hb^-+X^- are similar in all three series and differences in these values arise from the exact meaning that one chooses to give to X^-. For us, it is $B-Cl-HCO_3-Hb^-$; for Dill *et al.* it is $B-Cl-HCO_3-BP_c$. It is rather difficult to assign an exact meaning to BP_c. It is obtained by what is virtually an indirect titration of cell material by means of the Henderson-Hasselbalch equation and not of haemoglobin and it must of necessity include all cell substances which have buffering properties at *p*H 7. Such substances will include Hb and also a large proportion of X. It would appear that those substances designated by Dill *et al.* as X in their Tables consist merely of the unbuffered fraction of the unidentified cell anions. It must be realized that the precise values of X^- and Hb^- are not of importance to Henderson and his co-workers. They are concerned with a physico-chemical system comprising a cell and plasma phase and are interested in the interplay of the various constituents when one or other component is altered by physiological or pathological circumstances. For this purpose, the value BP_c suffices. Nevertheless, from a less general standpoint the conception that HCO_3 and Hb are the only significant cell buffers is undesirable, for it ignores other cell buffers which are present in quantity and which may well have a physiological and pathological significance.

It may perhaps be of interest to compare the values for X^- obtained in our own experiments and in those of Dill *et al.*, using both sets of data for cell buffering. From Table VI, it may be seen that according to Dill *et al.* $B-Cl-HCO_3$ equals 40·7 m.eq. per 1000 ml. cells. Using the data of the present writers: the buffering power of HbO_2 equals 0·148 m.eq. per g. and the isoionic point is at *p*H 6·65, hence the 337 g. Hb present in 1000 ml. cells will bind at *p*H 7·19, $0·148\times(7·19-6·65)\times 337$ m.eq. or 27 m.eq. base. Hence X equals 13·7 m.eq.;

this contrasts with Dill's own figure of −1 m.eq. but agrees with those of the present writers.

Next, we may apply the buffering data of Dill *et al.* to our own experiments. According to Dill *et al.* $BP_c = HbO_2 (-0{\cdot}5\ pH_c^2 + 10{\cdot}625\ pH_c - 48{\cdot}46)$. In this equation the value of BP_c is derived from HbO_2, which is expressed in milli-equivalents. The equation is an empirical one based on experimental results. There is no reason why one should be able to forecast the value of $Hb^- + X^-$ from HbO_2, but one is able to do this in normals simply because the ratio of Hb to Dw. or alternatively of Hb to unidentified cell buffers, is fairly constant. But in the anaemics, the ratio is not constant and the above equation breaks down completely. Thus, if one applies it to the normal figures shown in the last line of Table VI, we find that at *p*H 7·06, $B - Cl - HCO_3 = 32{\cdot}5$ m.eq. per 1000 ml. cells, $BP_c = 33{\cdot}2$ m.eq. and X^- in the sense of Dill *et al.* equals −0·7 m.eq. This agrees well with Dill's own figures. But if one applies the equation to a case of anaemia (Table V, No. 4) it is found that $B - Cl - HCO_3$ is 41 m.eq. per 1000 ml. cells and BP_c only 15. So that X^- in the sense of Dill *et al.* is still very large, +26 m.eq. per 1000 ml. cells.

On general grounds it seems unlikely that a substance which is certainly present in anaemic cells in quantity should be entirely absent from normal cells, and although we may have made X^- 10 % too high by taking the isoionic point of HbO_2 to be at *p*H 6·65 instead of at *p*H 6·6 or may have made X^- 10 % too low, by taking *p*H 6·65 instead of 6·7, there can be little doubt that in normals the value of X^- is at least equal to one-half that of Hb^-.

The buffering power of reduced anaemic and normal cell solutions

The buffering power of dilute laked cell solutions at 25° is set out in Table VII and compared with the buffering power of dilute Hb solution. Solutions contained about 0·5 % Dw. and the number of milliequivalents required to change the *p*H of 1 g. from 7·6 to 8·0 and from 8·0 to 8·4 are shown, together with the standard deviations of five experiments. Next to these figures, the buffering power, (Base, m.eq. per g. Dw.)/0·4, is shown in brackets. *p*H was estimated with the hydrogen electrode and haemoglobin in the solutions examined was therefore reduced.

Table VII

	*p*H 7·6–8·0 m.eq. base per g. Dw.		*p*H 8·0–8·4 m.eq. base per g. Dw.	
Pure Hb solution	0·052	(0·130)	0·047	(0·118)
Anaemic cell solution	0·054 ± 0·004	(0·135 ± 0·010)	0·048 ± 0·005	(0·120 ± 0·012)
Normal cell solution	0·053 ± 0·004	(0·132 ± 0·010)	0·047 ± 0·002	(0·118 ± 0·005)

It will be seen that the buffering powers of all three solutions over these ranges are similar. It is, however, hardly possible to arrive at any conclusion as to the buffering power of X, since the proportion of X is less than one-sixth that of Hb by weight and unless the buffering power of X were very different from that of Hb its influence on the general buffering would be masked.

Titration curves of dialysed cell solutions

Laked cell solutions appear to be practically transparent but after dialysis a heavy flocculus forms. The fresh solution contains the ghosts of the laked cells in suspension. But after the removal of salts and change in *p*H resulting from dialysis the ghosts flocculate. In the case of anaemic cell solutions, the flocculus is more bulky than that derived from normal cells.

The following investigations were carried out on dilute cell solutions containing about 0·5 % dried weight. *p*H was measured at 25° by means of the hydrogen electrode and haemoglobin present in solutions was accordingly reduced.

The *p*H of the dialysed anaemic cell solution is from 0·1 to 0·2 less than that of the normal cell solution after dialysis. This is shown in Table VIII, where the results from an anaemic and normal pair dialysed in the same vessel are shown on one line.

It will be noted too that the *p*H of the dialysed anaemic and normal cells is also considerably less than that of a pure haemoglobin solution, pointing to the presence of some substance of relatively low isoionic point. To make this point more conclusive, a pure dialysed specimen of haemoglobin was diluted and redialysed for 6 days in the same vessel with samples of laked cell solutions. The *p*H of the dialysed anaemic cells was 6·7; that of the normal cells 6·8 and that of the haemoglobin 7·13. These differences of *p*H cannot be ascribed to the presence of CO_2 in the solutions, since the latter were all dialysed in a common vessel. It is thought that the relative acidity of the laked cell solutions is due to the substance X, which is still present after dialysis.

Table VIII

$T = 25°$

Anaemia			Normal		
No.	Hb g. per 1000 ml. original cells	*p*H dialysed solution	No.	Hb g. per 1000 ml. original cells	*p*H dialysed solution
1	194	6·60	11	318	6·77
3	236	6·61	12	320	6·85
4	240	6·69	8	306	6·80
5	259	6·61	15	340	6·83
6	261	6·62	10	311	6·82

The buffering values of dilute (0·5 % Dw.) laked cell solutions, that is the number of milliequivalents of base added to 1 g. of dried cell material divided by the change in *p*H resulting is shown in Table IX. They cover 0·4 ranges between *p*H 6·8 and 8·4. The standard deviations for 6 experiments are shown after the average values.

Table IX

	*p*H 6·8–7·2 m.eq.	*p*H 7·2–7·6 m.eq.	*p*H 7·6–8·0 m.eq.	*p*H 8·0–8·4 m.eq.
Haemoglobin solution	0·130	0·136	0·130	0·118
Dialysed anaemic cell solution	$0·140 \pm 0·012$	$0·140 \pm 0·012$	$0·130 \pm 0·010$	$0·114 \pm 0·007$
Dialysed normal cell solution	$0·133 \pm 0·012$	$0·136 \pm 0·010$	$0·128 \pm 0·010$	$0·119 \pm 0·012$

The figures suggest that the buffering power of pure Hb is a little less than that of dialysed anaemic cell solution between *p*H 6·8 and 7·6.

Certain other deductions may be drawn from the preceding data; the buffering power of laked cell solution is practically constant over the range *p*H 6·8 to 7·6, so that, knowing the initial *p*H of a dialysed cell solution, one can calculate the amount of base bound at 7·12, the isoionic point of Hb, and this should equal the value of X^- at *p*H 7·12.

Further, knowing the base bound by dialysed cell solution at any given pH and the amount of base bound by haemoglobin at the same pH one can calculate the value of X^- at that particular pH from the formula

$$X^- = \beta_c (pH_f - pH_i) - \beta_{Hb} (pH_f - 7{\cdot}12) \times Hb/Dw.$$

β_c is the buffering power of the laked dialysed cell solution. Between pH 6·8 and 7·6 it equals 0·1345 m.eq. per g. Dw. for normal cells, and 0·140 m.eq. for anaemic cells. pH_f is the particular pH at which X^- is to be calculated. pH_i is the pH of the cell solution after dialysis. β_{Hb} is the buffering power of haemoglobin and Hb/Dw. represents the proportion of haemoglobin of Hb to dried weight. In this way, a value for X^- may be derived quite independently of estimations of base and chloride and the two values may be used to check one another (Table X). The data for X^- "calculated" may be obtained from Tables III and VIII; the figures for X^- "found" are given in Table III.

Table X

Normal			Anaemia		
No.	X^- calculated m.eq. per g. Dw.	X^- found m.eq. per g. Dw.	No.	X^- calculated m.eq. per g. Dw.	X^- found m.eq. per g. Dw.
11	0·052	0·046	1	0·085	0·119
12	0·045	0·045	3	0·083	0·111
8	0·050	0·060	4	0·072	0·112
15	0·047	0·036	5	0·084	0·097
10	0·047	0·047	6	0·080	0·102

When comparing the two values for X^-, the complexity of the data involved in their calculation must be realized: X^- found includes the summation of errors in base and chloride; X^- calculated involves the electrometric titration, not of a simple homogeneous solution, but of a liquid containing numerous minute bodies—the blood cell ghosts—in suspension. Errors in Hb^- are common to both values of X^-. Bearing these considerations in mind, it may be said that the agreement between X^- calculated and X^- found, in the case of normals, is as good as can be expected; the one being a little more or a little less than the other. But in the case of anaemic cells, X^- in the undialysed cells is consistently greater than in the dialysed cell solution and this suggests that in anaemic cells a portion of X^- is of sufficiently small molecular size to dialyse away through cellophane. In both normal and anaemic cells, the high values for X^- are confirmed.

The relation of haemoglobin to dried weight of erythrocytes

The proportion of haemoglobin to dried weight in cell solution is shown in Table XI.

Table XI

Hb g./ 1000 ml. cells average	Range	No. cases	Hb/Dw.	
			Average	Standard deviation
225	(194–250)	10	0·814	±0·022
260	(250–276)	8	0·834	±0·012
314	(301–319)	12	0·871	±0·018
327	(320–347)	10	0·875	±0·012

Although great accuracy is hardly possible when measuring the Hb and Dw. of small quantities of material (about 0·05 g.) the figures in Table XI do suggest that the non-Hb portion of dried cell material is greater in anaemic cells.

It will be seen that even in normal cells, Hb is only 87 % of the Dw.; of the remaining 13 %, 4–6 % consists of other proteins—possibly the stroma protein

described by Jorpes [1932] which is isoelectric at about *p*H 5·4; about 3 % of inorganic salts, and perhaps about 2 % of lipoids: both stroma protein and lipoid would bind base at *p*H 7 to 7·4.

In the series of Dill *et al.* [1937] 1000 ml. cells contain 733 ml. water. If the specific gravity of cells be taken as 1·090, then 1000 ml. cells will contain 357 g. dried weight. The haemoglobin content in this series was 336 g. so that Hb/Dw. is 94 %. On the other hand, according to Erickson *et al.* [1937] the specific gravity of cells is 1·090; assuming the water content to be 73 %, dried weight is 360 g. per 1000 ml. cells. These authors give the haemoglobin content as 300 g. per 1000 ml. cells so that Hb/Dw. is 84 %.

It is thought possible that the increased amounts of non-haemoglobin material found in anaemic erythrocytes may be correlated with the fact that such cells are smaller and thinner than normal, so that the ratio of cell membrane or stroma to haemoglobin will be increased.

Summary

In the course of work on the ion content of erythrocytes the titration curve of a dilute solution of human haemoglobin (0·552 %) was determined. The isoionic point at 25° lay between 7·12 and 7·15. The molecular buffering power (M.W. Hb = 67,000) was 9·1 eq. between *p*H 7·2 and 7·6; 8·7 eq. between 7·6 and 8·0 and 7·9 eq. between 8·0 and 8·4.

In the case of erythrocytes, if these are washed and dialysed the *p*H of normal cell solutions is about 0·3 less than that of pure haemoglobin solution, whilst the *p*H of anaemic cell solution is about 0·4 to 0·5 less. This is probably due to the presence of substances of relatively low isoionic point and of such a nature as not to diffuse easily through cellophane.

In the erythrocyte, part of the cation is combined with Cl and HCO_3 and the rest, about one-third of the total, is combined with Hb^- and X^-. The latter consists of unknown and probably complex ions, which in normal cells bind nearly as much base as does Hb^- and which in anaemic cells binds nearly twice as much. X^- cannot be washed out of intact cells with KCl solution and, in normal cells, little if any dialyses away through cellophane. In anaemic cells it is possible that a proportion of X^- diffuses through a cellophane membrane, but not through the normal cell membrane.

The results show that in anaemic erythrocytes, Cl and especially base and therefore X^- are relatively increased. Further, it may be shown from Table III (product of the figures in columns 3 and 6 divided by the figure in column 9) that the amount of base per unit volume of original cells is absolutely increased in anaemia, but as the water content is also increased [Maizels, 1936] the osmotic pressure of cells is not appreciably altered.

Our thanks are due to Mr Gregory of the Department of Physiology, University College, London, who kindly carried out some of the gasometric analyses.

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APPENDIX I

Hydrogen ion concentration. The method described by Harington & Neuberger [1936] was slavishly followed. When titrating, complete drops were allowed to fall from the burette into the titrating vessel. ϵ for the calomel cell was determined by standardization with 0·01 *N* HCl in 0·09 *N* NaCl; at 25°, it is (E.M.F. −123) mV.; and at 38°, (E.M.F. −128) mV. $p\mathrm{H} = (\text{E.M.F.} - \epsilon)/59{\cdot}1$ at 25° and (E.M.F. $-\epsilon$) 61·7 at 38°. In three experiments of series III, E.M.F. of laked cell solutions were quite steady; in two it reached a maximum, followed by a slight gradual decrease in E.M.F. This may have been due to liberation of CO_2 in the electrode vessel. During equilibration of strong cell solution in the electrode vessel, a fine web of cell ghosts forms round the platinum electrode. No such web is visible in dilute solutions, and as weak and strong solutions give the same E.M.F. at a given temperature, it would appear that no interference occurs with the estimation of the hydrogen ion concentration.

APPENDIX II

Haemoglobin solution used in the titration curves (Table II)

The dried weight of this solution was 13·85 %; the haemoglobin content as determined by the Haldane haemoglobinometer was 12·9 g. per 100 ml. solution.

All the following data have been very kindly supplied by Dr G. S. Adair:

Haemoglobin by refractometer, 13·7 g., by oxygen capacity, 13·3 g. per 100 ml. solution. Assuming that the volume of 1 g. Hb is 0·75 ml. + 0·215 ml. bound water, then 100 ml. solution contain 13·2 ml. protein hydrate, or 13·7 g. Hb per 86·8 ml. free water. Therefore Hb equals 15·8 g. per 100 ml. free water, that is 0·00235 mol. per litre free water. (Molecular weight Hb = 67,000.)

The theoretical osmotic pressure of this solution is 40 mm. Hg.

But the solution is not ideal: a 0·004 *M* solution gives 1·44 times the ideal osmotic pressure [Adair, 1928; 1935]; and a 0·00235 *M* solution gives an osmotic pressure 1·26 times the ideal. So that the corrected theoretical osmotic pressure is 50·5 mm. Hg.

Observed osmotic pressure was 57·2 mm.

Therefore the excess was 6·7 mm., or 0·000395 *M*, since 0·01 *M* = 17 mm.

Correcting for incomplete ionization of haemoglobin salts, excess equals 0·00056 *M* [Adair, 1925; Stadie & Sunderman, 1931].

But haemoglobin was 0·00235 *M*, hence, excess O.P. corresponds to 0·00056/0·00235 or 0·238 mol. per mol. Hb. (This completes Adair's data.)

Now, base bound per mol. Hb = β (pH − isoelectric point). β according to the present writers equals 9·1 eq. per mol. Hb; the expression may be positive or negative.

Therefore, 0·238/9·1 = pH − isoelectric point = ± 0·026 pH.

Hence excess of O.P. corresponds to a shift in pH of ± 0·026. According to Adair, the Hb solution contained 1·2 vols. % CO_2 corresponding to a decrease in pH of 0·025. It is therefore probable that the osmotically active impurity in the Hb solution was CO_2. No determination of CO_2 in the solution was done after equilibration but it may be said that the isoionic point of Hb in dilute solution lies between 7·12 and 7·15 and probably nearer 7·12.

APPENDIX III

DETAILS OF EXPERIMENTS IN SERIES III

I. *Normal*

Hb g. per 1000 ml. blood, 149. Hb g. per 1000 ml. cells, 315.
Strong cell solution: Hb g. per 1000 ml., 51; Dw., 59; Hb/Dw., 0·87.
Weak cell solution: Hb g. per 1000 ml., 9·95; Dw., 11·5; Hb/Dw., 0·87.

Base in weak solution:

1 ml. *N*/100 HCl	= 0·494, 0·489, 0·493, 0·493 ml. *N*/50 NaOH.
1 ml. HCl + 2 ml. solution	= 0·143, 0·145, 0·139, 0·143, 0·142, 0·143 ml. *N*/50 NaOH.
Base in 1 ml. solution	= 0·350 ± 0·006 ml. *N*/100 NaOH.
Base in 1 g. Dw.	= 0·304 ± 0·005 m.eq.

Chloride in weak solution:

0·485 ml. *N*/40 $AgNO_3$	= 1·19, 1·195, 1·19, 1·19, 1·19, 1·195 ml. KCNS.
0·485 ml. $AgNO_3$ + 2·95 ml. solution	= 0·535, 0·525, 0·535, 0·535, 0·525 ml. KCNS.
Chloride in 2·95 ml. solution	= 0·662 ± 0·007 ml. *N*/98·5 KCNS.
Chloride in 1 ml. cell solution	= 0·229 ± 0·002 ml. *N*/100 KCNS.
Chloride in 1 g. Dw.	= 0·199 ± 0·002 m.eq.

pH, CO_2 etc. of solutions:

ϵ calomel cell	Weak solutions at 25° 241 mV. E.M.F. readings		Strong solutions at 38° 234·5 mV. E.M.F. readings	
	Time min.	mV.	Time min.	mV.
	0	—	0	—
	7	682	5	655
	20	689·5	32	684
	30	689·5	45	684
pH	= (689·5 − 241)/59·1 = 7·59		= (684 − 234·5)/61·7 = 7·29	
Hb m.eq. per g. Dw.	= 0·136 × (7·59 − 7·12) × 0·87 = 0·055		= 0·138 × (7·29 − 6·81) × 0·87 = 0·057	
CO_2, vol. %	= 0·06		= 0·30	
X m.eq. per g. Dw.	= 0·304 − 0·199 − 0·055 − 0·002 = 0·048 ± 0·007		= 0·304 − 0·199 − 0·057 − 0·002 = 0·046 ± 0·007	

Anaemia IV

Hb g. per 1000 ml. blood, 81. Hb g. per 1000 ml. cells, 240.

Strong solution: Hb g. per 1000 ml., 36; Dw., 43; Hb/Dw., 0·84.

Weak solution: Hb g. per 1000 ml., 6·95; Dw., 8·2; Hb/Dw., 0·85.

Base in weak solution:

1 ml. $N/100$ HCl	= 0·486, 0·485, 0·490, 0·485, 0·484 ml. $N/50$ NaOH
1 ml. HCl + 2 ml. solution	= 0·154, 0·147, 0·139, 0·137, 0·139 ml. $N/50$ NaOH.
Base in 1 ml. solution	= 0·340 ± 0·011 ml. $N/100$ NaOH.
Base in 1 g. Dw.	= 0·415 ± 0·013 m.eq.

Chloride in weak solution:

0·485 ml. $N/40$ $AgNO_3$	= 1·205, 1·195, 1·195, 1·205 ml. KCNS.
0·485 ml. $AgNO_3$ + 2·95 ml. solution	= 0·565, 0·565, 0·560, 0·560 ml. KCNS.
Chloride in 2·95 ml. solution	= 0·638 ± 0·007 ml. $N/99$ KCNS.
Chloride in 1 ml. solution	= 0·218 ± 0·003 ml. $N/100$ KCNS.
Chloride in 1 g. Dw.	= 0·261 ± 0·003 m.eq.

p*H*, CO_2 *etc.:*

	Weak solution at 26°		Strong solution at 38°	
ϵ calomel cell	240·5 mV.		234 mV.	
Cell solutions	E.M.F. readings		E.M.F. readings	
	Time min.	mV.	Time min.	mV.
	0	—	0	—
	15	677	18	675·5
	20	678·5	24	677
	40	678·5	31	677
*p*H	7·41		7·18	
Hb m.eq. per g. Dw.	= 0·136 × (7·41 − 7·12) × 0·85 = 0·033		= 0·138 × (7·18 − 6·81) × 0·84 = 0·043	
CO_2, vol. %	= 0·09		= 0·55	
X m.eq. per g. Dw.	= 0·415 − 0·261 − 0·033 − 0·005		= 0·415 − 0·261 − 0·043 − 0·006	
	= 0·116 ± 0·016		= 0·105 ± 0·016	