XXXIV. ORGANIC ANIONS OF HUMAN ERYTHROCYTES

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IN their efforts to simplify the two phase physico-chemical system of the blood, Henderson [1928] and other authors [Van Slyke *et al.* 1923; Dill *et al.* 1937] assumed that anions in normal human erythrocytes other than Cl^- , HCO_3^- and Hb^- (designated by Henderson X^-) were usually insignificant in amount. This assumption was supported by analyses which seemed to show that any excess of base over $\text{Cl}^- + \text{HCO}_3^- + \text{Hb}^-$ was negligible. It is now clear, however, that the values assigned to Hb^- were too high either because it was assumed that the buffering powers of human and horse haemoglobins were similar or because the data were derived from haemolysed erythrocyte solutions instead of from solutions of pure Hb. Maizels & Paterson applied Adair's [1925] titration curves to the data of Henderson [1928] and of Dill *et al.* [1937], and found that the base was actually greater than $\text{Cl}^- + \text{HCO}_3^- + \text{Hb}^-$; they confirmed these observations [1937] experimentally by analysing a series of erythrocyte solutions and calculating Hb^- from their own electrometric titrations of Hb.

It thus became necessary to assume the presence of another anion which, following Henderson, they called X^- , and which further appeared to be roughly inversely proportional to the Hb content per litre of original cells, being much greater in the haemoglobin-deficient cells of certain anaemias than in normal erythrocytes. In the latter, X^- was about equal to Hb^- , while in anaemic cells it might be twice as great as Hb^- . Further, knowing the Hb content per 1000 ml. cells it was found possible to foretell the probable value of X^- to within $\pm 15\%$ (Fig. 1).

It will be realized that the amount of X^- depends ultimately on the titration curve of Hb^- and although the figures found by Adair [1925] and by Maizels & Paterson [1937] are probably correct, in order to remove any ambiguity on this score an effort was made to demonstrate the presence of X^- in the absence of Hb by analysing the dialysate of laked cell solutions. A number of experiments were done but as the fuller and more satisfactory experiments carried out subsequently are described later in this paper, the results of the original experiments will only be summarized here.

The dialysate of laked cell solutions contained a marked excess of base over Cl^- and HCO_3^- , so that the presence of X^- , apparently responsible for 30% of total base-binding, in dialysates was confirmed. The titration curve of the dialysate showed marked buffering between pH 6 and 7 and in this resembled the titration curve of phosphate and the phosphoric esters [Meyerhof & Suranyi, 1926] but while the buffering power of phosphate greatly decreases at $\text{pH} > 7$, the buffering power of the dialysate was maintained until well beyond pH 9 and this part of the curve recalled the titration curve of glutathione [Pirie & Pinhey, 1929]. Analysis of the dialysates showed in fact that organic phosphorus and SH— groups were present. It therefore seemed reasonable to assume that phosphoric esters and glutathione might account in part at least for X^- . Support for this view was forthcoming from results already available in the literature

which may be summarized as follows: human erythrocytes contain about 70 mg./100 ml. glutathione [Platt, 1931] which would bind 1.1 equiv. base per mol. at the pH of the erythrocyte (7.5 at 20°), and about 50 mg./100 ml. acid-soluble P [Kay *et al.* 1927; Kerr & Daoud, 1935; Rapoport, 1937] which binds 2.4 equiv. base per atom P at pH 7.5 [Farmer & Maizels, 1938]; approximately 25 % is easily hydrolysable phosphate, probably pyrophosphate corresponding in whole or in part to adenosine triphosphate [Kerr & Daoud, 1935], which binds 1.3 equiv. base per atom P, while the residue consists of inorganic phosphate, hexosephosphate and other phosphates binding little < 2 equiv. base per atom P at pH 7.5. At a conservative estimate then cell P should bind 1.9 equiv. base per atom P so that theoretically cell P and glutathione should bind together 32 m. equiv. base per 1000 ml. cells.

Since this is about twice as great as the amount of base bound by Hb, it is extremely remarkable that workers on the acid-base equilibrium of the blood should hardly have taken the base-binding properties of phosphoric esters into account at all. Comments have indeed been made on the compensatory decrease in cell P which occurs in experimental acidosis [Haldane *et al.* 1924; Kay, 1924; Rapoport, 1937], but the only direct reference we have found is by Peters & Van Slyke [1931] who, in contrast with Van Slyke's statements of 1923, were able to say in 1931: "it has generally been assumed that proteins are the chief

Fig. 1.

buffers in cells; but it is quite possible that organic phosphates are more important". We shall see later that this suggestion is incorrect since, the importance of the organic phosphates lies primarily in their base-binding and not in their buffering powers. In view of the amount of base that could theoretically be bound by the acid-soluble P of cells, we have ourselves carried out a number of new analyses on normal and anaemic cells and have compared

the theoretical amount of base bound by cell phosphate and glutathione with the amount probably bound by X^- , the non-haemoglobin organic anions of erythrocytes, as determined from Fig. 1.

Methods

Erythrocytes were separated for analysis by centrifuging blood which had been treated with phosphate-free heparin (Hoffmann-la Roche). Whole blood was kept on ice as far as possible, and despite the delay in adding trichloroacetic acid Table I shows that little hydrolysis of phosphoric esters occurred. Inorganic P and total acid-soluble P were determined according to Martland & Robison [1926]. Easily hydrolysable P was estimated by boiling the trichloroacetic acid filtrate for 7 min. with $N H_2SO_4$, while the glutathione was estimated iodimetrically in acid solution [Platt, 1931]. The results are summarized in Table I and are expressed in mg. or m. equiv. per 1000 ml. cells.

Table I

Case	Hb g. blood	Hb g. cells	Total acid-sol. P mg.	Hydro- lysable P mg.	Inor- ganic P mg.	Gluta- thione (Gl) mg.	P + Gl m. equiv.	X^- m. equiv.
1. Normal	156	335	485	93	17.4	680	32	14
2. Normal	148	333	465	125	12.1	810	31.5	16
3. Hyperpiesis	118	325	550	94	30.5	790	36.5	17
4. Normal	124	320	527	124	21.4	600	34	18
5. Normal	152	318	510	—	20.7	740	34	19
6. Hyperpiesis	114	312	510	—	20.1	730	33.5	21
7. Normal	150	312	540	115	18.8	980	36.5	21
8. Normal	146	308	462	103	16.9	800	31	21
9. Normal	136	302	510	122	23.0	840	34	23
10. Prostatectomy	141	301	457	100	17.0	970	31.5	23
11. Microcytic anaemia	88	280	680	115	22.5	1040	45.5	28
12. Microcytic anaemia	59	260	592	114	18.1	910	39.5	31
13. Microcytic anaemia	70	259	691	128	22.6	1050	46	31
14. Microcytic anaemia	70	230	702	130	22.3	1140	47	35
15. Microcytic anaemia	52	229	675	—	17.0	1130	45.5	35
16. Microcytic anaemia	36	203	625	118	16.1	1350	43	38
17a. Macrocytic anaemia	50	283	610	110	15.2	1130	41.5	?
17b. Macrocytic anaemia	63	267	692	100	19.2	1220	46.5	?
18. Acholuric jaundice	134	343	450	106	21.5	600	29.5	10

The cases fall into three groups: 10 without anaemia, 6 with hypochromic microcytic anaemia and 1 with macrocytic anaemia. The two main groups are placed in order of their Hb content per 1000 ml. cells. The analyses of normal cells correspond with those of other workers; in microcytic anaemia both acid-soluble P and glutathione are increased but the correlation between these increases and Hb either in unit volume of cells or whole blood is not close. The possibility of increases in P and glutathione being associated with reticulocytosis was considered but since reticulocytosis in the first 16 cases never exceeded 2% this could not have been a cause. Again, in case 17, 0.25% of reticulocytes were present at the first examination (a) and 25% at the second (b), but glutathione was only increased by 10%. Finally, the last case in the series had 4.8% reticulocytes but total P and glutathione were the lowest recorded. At present the relation of high glutathione values to anaemia therefore remains obscure. The function of the increased phosphoric esters is a little more clear; in microcytic anaemia Hb and base bound by Hb decrease while cell water increases; phosphoric esters will help to maintain base-binding and, being osmotically active, will support the osmotic pressure of the cell contents. On the other hand, phosphates are not good buffers at $pH > 7$ and they probably play little part in

cell buffering in normal or anaemic cells: assuming that 1000 ml. normal cells contain 320 g. Hb binding 20 m. equiv. base at pH 7.25, and that there are also present 500 mg. acid-soluble P binding 30 m. equiv. base at pH 7.25, then it would require 4 m. equiv. base to shift a solution of the Hb alone to pH 7.35 and only 0.6 m. equiv. to effect a corresponding change in the phosphoric esters alone. In anaemia on the other hand the buffering power of the phosphoric esters is a little more significant.

If cell phosphate bound its full theoretical amount of base at cell pH it would combine with nearly twice as much base as Hb itself. Moreover, since plasma contains very little phosphoric ester, the acid-soluble phosphates within the erythrocyte should act as non-permeating anions whose effect on the distribution of permeating ions should far outweigh that of haemoglobin. For this reason equations dealing with the distribution of water and electrolytes between cells and plasma, which refer solely to Hb as the major non-permeating ion are likely to have no more than comparative significance.

Base bound by X^- and the phosphoric esters

If the phosphoric esters and glutathione in the cells were to bind their full theoretical quota of base, then X^- , that is $B^+ - Cl^- - HCO_3^- - Hb^-$, should be equal to or greater than the equivalence of the phosphoric esters + glutathione. Actually, comparison of the last two columns in Table I shows that the reverse is the case and this can only arise in one of four ways: (a) less than 1.9 equiv. base is bound per atom P; (b) an unidentified excess of base is present in the erythrocytes; (c) the value of $B^+ - Cl^- - HCO_3^- - Hb^-$ is too small owing to some analytical error; (d) phosphoric esters and/or chloride exist in some form in which they fail to bind base. Consideration of these possibilities leads to the following conclusions. (a) It is probable that P does in fact bind at least 1.9 m. equiv. base per atom at cell pH . This was confirmed in the following way: a trichloroacetic acid filtrate of erythrocytes was neutralized with ammonia using methyl orange as an indicator. Basic lead acetate and alcohol were added to precipitate the phosphates. The deposit was well washed, first with dilute ammonia solution to remove acetate, trichloroacetate, Na and K, and then with alcohol and water to remove ammonia. Lead was removed with H_2S and the filtrate evaporated *in vacuo* at 40° . The residue was taken up in water and found to be free from ammonia and sulphur. The P content of this solution was estimated and it was then titrated electrometrically at 38° . It was found that each atom of P corresponded to 1.86 equiv. base at pH 7 and 2.03 equiv. at pH 7.5. (b) There is no evidence of the presence of any unidentified excess of base, but if such were present, then it should be recovered by electrodialysis. (c) The values for base and chloride recorded by Maizels & Paterson [1937] were obtained by well-tried methods which gave consistent results agreeing with the figures of other workers. Of these, the data of Dill *et al.* [1937] may be quoted. These workers, using entirely different methods, give the following average figures of twelve individuals: base, 11.0; Cl, 5.6; HCO_3 , 1.75 m. equiv. per 100 ml., whence $X^- + Hb^- = 3.6$ m. equiv./100 ml.

If Hb^- is calculated according to the data of Dill *et al.*, $X^- = -0.07$ m. equiv., but employing the more accurate data of Adair [1925] or of Maizels & Paterson [1937] for the buffering power of reduced Hb, then Hb^- may be calculated as follows:

$$Hb^- = Hb \times (pH - i.p.) \times B_r$$

where Hb equals the amounts of haemoglobin present in g.; pH its pH ; i.p. the isoelectric point of reduced Hb (6.81 at 38°) and B_r the buffering power of

reduced Hb per g., which is 0.138 m. equiv. per pH unit between 6.8 and 7.3. Then in the experiments of Dill *et al.*, $\text{Hb}^- = 33.5 \times (7.167 - 6.81) \times 0.138 = 1.7$ m. equiv. per 100 ml. cells, and $\text{X}^- = 3.6 - 1.7 = 1.9$ m. equiv. per 100 ml. blood. This agrees well with the corresponding figure given by Maizels & Paterson, and there is therefore no reason to ascribe the apparent excess of base to analytical error. (d) There remains the fourth possibility that phosphoric esters and/or chloride within the cell bind less base than they do in simple aqueous solution. This might possibly occur through some form of complex association and at present represents the most likely explanation; experimental evidence on the point is however so far lacking. One further point in this connexion does indeed emerge from Table I: although X^- and acid-soluble phosphate are both increased in hypochromic microcytic anaemia, the increase in X^- is relatively the greater and this suggests the possibility that the increase in X^- may be accomplished by the diversion of phosphate from a complex to a simple form.

In the attempt to obtain direct evidence as to the state of phosphoric esters within the cell, laked cell solutions were dialysed with the object of determining whether X^- and the phosphoric esters dialysed at the same rate and whether the apparent excess of total phosphate over X^- was to be observed in the dialysate as well as in the dialysed solutions.

X^- and phosphoric esters in dialysed cell solution and dialysate

Human erythrocytes were washed 5 times with isotonic KCl solution, packed by centrifuging and the supernatant KCl removed with a capillary pipette. The cells were then mixed with half their volume of distilled water, vigorously shaken with a similar volume of ether and recentrifuged. A compact ethereal scum rose to the surface, containing lipins, cell stromata and other substances. The subjacent clear laked cell solution was removed and freed from ether by evaporation *in vacuo* at room temperature; it was then dialysed in cellophane bags against one-third its volume of water, the systems being mixed by inversion every 2 hr. Dialysis was carried out in two cases for 18 hr. and in one case for a week. In no case was true equilibrium attained. Finally, the dialysed cell solution and dialysate were analysed. Details of the methods employed are given in the appendix together with a full statement of all the data obtained in Exp. 2. The results of all three experiments are summarized in Table II, where the following are set out:

(a) No. of hr. of dialysis; (b) vol. of cell solution and of dialysate; (c) Hb in g. per 1000 ml.; (d) electrometric measurement of pH; (e) Hb^- in m. equiv. per 1000 ml. $\text{Hb} \times (\text{pH} - \text{I.P.}) \times B_r$; (f) Cl in m. equiv. per 1000 ml.; (g) HCO_3^- in m. equiv. per 1000 ml.; (h) base bound by glutathione (Gl^-) per 1000 ml. at the experimental pH (calculated from the titration curves of Pirie & Pinhey [1929]); (i) total base (this is one of the most crucial as well as the most difficult estimations; it was carried out by three different methods: (i) electrolytically; (ii) as the sum of $\text{K} + \text{Na} + \text{Mg} + \text{NH}_4$; (iii) as sulphate by the benzidine precipitation method; (j) X^- in m. equiv. per 1000 ml. (in this case,

$$\text{X}^- = \text{Base}^+ - \text{Cl}^- - \text{HCO}_3^- - \text{Hb}^- - \text{Gl};$$

CO_2 and glutathione are quite small and may well be neglected; they are, however, included for the sake of completeness); (k) inorganic phosphate; (l) total acid-soluble P mg. per 1000 ml.; (m) total acid-soluble phosphate in m. equiv. per 1000 ml.; it has been assumed that 1 atom acid-soluble P binds 1.9 m. equiv. base at pH 7.2; if all cell phosphate is fully ionized then base bound by X^- should be as great as that bound by total phosphate or greater.

Table II. T. = 38°

No. of hr. dialysis	Exp. 1		Exp. 2		Exp. 3	
	Cell soln.	Dialysate	Cell soln.	Dialysate	Cell soln.	Dialysate
	18		18		168	
Vol. ml.	50.4	34	53	38	63.5	33
Hb colorimetric g./1000 ml.	11.4	0	17.7	0	13.4	0
Hb gasometric g./1000 ml.	—	—	18.1	—	—	—
pH	7.29	7.68	7.19	7.65	7.19	7.57
Hb ⁻ m. equiv./1000 ml.	7.5	0	9.7	0	7.0	0
Cl ⁻ m. equiv./1000 ml.	21.2 ± 0.5	25.8 ± 0.4	33.1 ± 0.2	40.2 ± 0.2	32.3 ± 0.5	37.5 ± 0.5
HCO ₃ ⁻ m. equiv./1000 ml.	0.2	0.14	0.2	0.1	0.1	0.1
Gl ⁻ m. equiv./1000 ml.	0.64	0.85	0.58	0.66	0.57	0.67
Total base:						
Electrolytic m. equiv./1000 ml.	32.1 ± 0.4	31.9 ± 0.2	48.4 ± 0.6	47.5 ± 0.5	48.1 ± 0.5	52. ± 1.0
K + Na + Mg + NH ₄ m. equiv./1000 ml.	—	—	48.0 ± 1.0	47.6 ± 0.2	—	—
as SO ₄ m. equiv./1000 ml.	—	—	48.4 ± 1.2	—	—	—
X ⁻ m. equiv./1000 ml.	2.6 ± 1.0	5.1 ± 0.6	4.7 ± 1.2	6.4 ± 0.7	8.1 ± 1.0	13.8 ± 1.5
P inorganic mg./1000 ml.	24.7	12.2	15.0	6.9	59.5	54.3
P acid-sol. mg./1000 ml.	144.3 ± 1.3	70.1 ± 0.9	224 ± 1.0	79.9 ± 0.9	185 ± 3.0	140 ± 1.5
Total acid-sol. phosphate	8.8 ± 0.1	4.3 ± 0.06	13.7 ± 0.06	4.9 ± 0.06	11.3 ± 0.2	8.6 ± 0.1
—RHPO ₄ ⁻ m. equiv./1000 ml.						
X ⁻ /—RHPO ₄ ⁻	0.3	1.2	0.34	1.3	0.7	1.6

The following conclusions may be drawn from Table II.

(1) The dialysate is much more alkaline than the dialysed cell solution; this is to be expected since the cell solution contains the non-penetrating anions Hb⁻ and organic phosphates.

(2) Chloride, glutathione, base and X⁻ diffuse rapidly.

(3) Acid-soluble phosphate diffuses slowly through cellophane; at 18 hr. the dialysate contains less than one-half the amount of acid-soluble P present in the cell solution, while after 7 days' dialysis, P in the dialysate is only two-thirds of that in the cell solution. It will also be noted that the ratio of inorganic to total acid-soluble P rises as the period of dialysis increases and this is doubtless because hydrolysis by cell phosphatases is prolonged.

(4) When all allowance is made for experimental errors, it is still true that after 18 hr. dialysis, the theoretical amount of base that could be bound by total phosphate in the cell solution is twice as great as that bound by X⁻, while in the dialysate it is slightly less. The observations gain strength from the fact that the same methods, often in quadruplicate, were used on both solutions which differed only in that one contained haemoglobin while the other did not. Further, of all the values given in Table II, there is only one assumed figure and that is the pH at which reduced haemoglobin is isoelectric. This is usually taken to be 6.81; if in fact the isoelectric point were lower, then the ratio of X⁻ to phosphate would be even less; if on the other hand the isoelectric point were higher, then X⁻ and phosphate would tend to become more nearly equal, though X⁻ would still be much less than phosphate unless the isoelectric point of haemoglobin at 38° were at pH > 7. Rejecting the latter remote possibility, one is forced to conclude that at least half of the phosphate in cells is not binding base but is complexly combined. The only possible alternative to this view is the unlikely one that chloride and not phosphate is complexly combined.

Although the systems are not in equilibrium, it is still of interest to compare the concentrations of ions in the cell solution and in the dialysate. Concentrations are obtained by dividing the known ion contents by the amount of water present

in the corresponding phase. In the case of cell solutions a figure has been subtracted from the measured total content of water equal to 0.2 ml. bound water per g. Hb (Table III).

Table III

	Exp. 1	Exp. 2	Exp. 3
$[\text{H}_2\text{O}]$ cell soln./ $[\text{H}_2\text{O}]$ dialysate	0.85	0.86	0.85
$[\text{H}^+]$ cell soln./ $[\text{H}^+]$ dialysate	2.45	2.82	2.40
$[\text{Base}]$ cell soln./ $[\text{Base}]$ dialysate	1.19	1.18	1.09
$[\text{Cl}^-]$ dialysate/ $[\text{Cl}^-]$ cell soln.	1.04	1.05	1.00
$[\text{Gl}]$ dialysate/ $[\text{Gl}]$ cell soln.	1.12	0.98	1.00
$\sqrt{[\text{Acid-sol. P}]} \text{ dialysate} / \sqrt{[\text{Acid-sol. P}]} \text{ cell soln.}$	0.64	0.56	0.80
$[\text{X}^-]$ dialysate/ $[\text{X}^-]$ cell soln. and	1.7 (1.3)	1.2 (1.1)	1.4 (1.2)
$\sqrt{[\text{X}^-]} \text{ dialysate} / \sqrt{[\text{X}^-]} \text{ cell soln.}$			

The phosphate ratio has been calculated on the assumption that P is divalent and the X^- ratio is shown both for a univalent and for a divalent anion.

It will be seen that the dialysate is much more alkaline than the cell solution and this has already been attributed to the excess of absolutely or relatively non-permeating anions, Hb and phosphate in the cell solution. But while $[\text{H}^+]$ ratios are similar in all three experiments, the phosphate ratios are very different, for in Exps. 1 and 2, internal phosphate is twice external phosphate, while in Exp. 3, after a whole week's dialysis total acid-soluble phosphates within and without the cellophane bag are much more nearly equal. Hence changes in phosphate distribution are not accompanied by changes in the distribution of H^+ , which suggests that phosphate in whole or in part is not acting as a relatively non-permeating anion and is in fact not acting as an anion at all. For, were phosphate in the cell solution acting as an anion a decrease in the phosphate ratio would bring about a decrease in the pH difference between cell solution and dialysate.

It will further be noted that $[\text{Cl}^-]$ is greater in the dialysate and $[\text{B}^+]$ greater in the cell solution; this is doubtless brought about by the excess of Hb in the cell solution. While $[\text{B}^+]$ cell solution/ $[\text{B}^+]$ dialysate and $[\text{Cl}^-]$ dialysate/ $[\text{Cl}^-]$ cell solution are in rough conformity, neither ratio agrees at all with the $[\text{H}^+]$ ratio. Apart from the fact that all the systems are still far from equilibrium, there seems no explanation for this discrepancy. Phosphate has of course diffused very slowly and even after a week's dialysis is still greater in the cell solution.

Finally, we may note that while the dialysate/cell solution ratios of Cl, glutathione and X^- on the one hand, and the cell solution/dialysate ratio of base on the other, are in rough agreement, the phosphate ratios show no correspondence at all. Since the correspondence of $[\text{Cl}^-]$ ratios is based on the assumption that Cl^- in cell solution is fully ionized, it is reasonable to believe that this assumption is correct; the phosphate ratios alone disagree with the other ion concentration ratios, while if one assumes that chloride and not phosphate in the laked cell solution is complexly combined, then both the phosphate and chloride ratios would be out of keeping with the ratio of base concentration.

SUMMARY

In normal human erythrocytes, Base^+ is equal to the sum of $\text{Cl}^- + \text{HCO}_3^- + \text{Hb}^-$ together with certain other complex organic anions symbolized by X^- . X^- binds about as much base as Hb^- , i.e. about 20 m. equiv. per 1000 ml. cells. Since erythrocytes contain phosphate (organic and inorganic) and glutathione, it is reasonable to assume that these substances may account for at least a part of X^- . Cell phosphate consists of diphosphoglycerate (65%), adenosine triphosphate

(25%) and other phosphates. These compounds in aqueous solution at pH 7.5 bind at least 1.9 m. equiv. base per atom of P. Since erythrocytes contain 500 mg. acid-soluble P per 1000 ml., this quantity of phosphate should bind 30 m. equiv. base, a quantity far in excess of X^- . It would therefore appear that much of the acid-soluble phosphate in cells is not ionized but is complexly combined.

If erythrocytes are dialysed, X^- is slightly greater than acid-soluble phosphate in the dialysate, but is very much less than acid-soluble phosphate in the dialysed cell solution. This shows that acid-soluble phosphate is fully ionized in the dialysate, but is only partially ionized in the laked cell solution.

In the erythrocyte of microcytic hypochromic anaemia, haemoglobin is decreased while X^- , glutathione, acid-soluble phosphate and water are all raised; this helps to maintain the base-binding and osmotic pressure of the anaemic cell. The function of cell phosphate as a buffer at the pH of the normal cell is relatively insignificant; in the anaemic cell at the same pH , it may be slightly more important.

APPENDIX

Methods

Haemoglobin. (a) Colorimetric, by Haldane's haemoglobinometer calibrated against a gasometrically standardized solution of blood. Duplicates should agree within $\pm 2\%$.

(b) Manometric [Peters & Van Slyke, 1931].

pH. This was measured in the hydrogen electrode after the method of Harington & Neuberger [1936]; the standardizing solution was 0.01 *N* HCl in 0.09 *N* NaCl and the values were sometimes confirmed with an acetate buffer. With these standard solutions reproducible E.M.F. were always obtained.

Chloride [Claudius, 1924]. Wet ashing is accomplished by boiling with HNO_3 in the presence of $KMnO_4$ as an oxidizing agent. The results obtained by this method have been compared with those obtained when H_2O_2 is substituted as an oxidizing agent and also with the results obtained on a protein-free filtrate of cell solution treated with sodium tungstate and sulphuric acid, where chloride is estimated without a preliminary wet ashing. The agreement between all three methods was very close.

Glutathione [Platt, 1931]. Glutathione is estimated by iodimetric titration in acid solution. The material is first reduced with zinc and hydrochloric acid.

CO_2 —manometric.

Total base [Adair & Keys, 1934]. (a) At Dr Adair's suggestion we have used cellophane instead of collodion to close the cathode tube, and have found it best to electrolyse for a first period of 24 hr., and titrate. We have found it advantageous to replace the titrated sulphuric acid by fresh acid and then electrolyse for a further 24 hr., after which the second lot of acid is titrated. The first electrolysis recovers about 95% of the base, and the second, the rest.

(b) Total base as $K+Na+Mg+NH_4$: Potassium was estimated by the method of Kramer & Tisdall [1921]; sodium, after Salit [1932]; magnesium by the technique described by Briggs [1924]. The test adopted for ammonia was nesslerization of a tungstate—sulphuric filtrate: it was negative in all cell solutions.

(c) Total base as sulphate, both directly [Fiske, 1922] and indirectly [Stadie & Ross, 1925].

Phosphorus was measured by the methods described by Martland & Robison [1926].

Protocol of Exp. 2

Hb. Colorimetric. 176 and 178 g. per 1000 ml.

Gasometric. 181 g. per 1000 ml.

pH. Standard solution: 0.01 *N* HCl in 0.09 *N* NaCl.

E.M.F. standard HCl + calomel half cell = 363 mV. at 38°.

E.M.F. HCl = $2.08 \times 61.7 = 128$ mV.

E.M.F. calomel half cell = 235 mV.

E.M.F. cell solution = (a) 678.5; (b) 678.5 mV. $pH = 7.19$.

E.M.F. dialysate = 707 mV. $pH = 7.65$.

Chloride. Solutions used: 0.5 ml. $N/40$ $AgNO_3$. 2 ml. of 2 in 15 dilution of cell solution or dialysate.

$AgNO_3$ = 1.200; 1.200; 1.205; 1.200; 1.200 ml. $N/96.2$ KCNS.

$AgNO_3$ + cell solution = 0.35; 0.35; 0.36; 0.36; 0.36; 0.36 ml. $N/96.2$ KCNS.

$AgNO_3$ + dialysate = 0.17; 0.16; 0.17; 0.16; 0.165; 0.16 ml. $N/96.2$ KCNS.

Chloride m. equiv. per 1000 ml. cell solution = 33.1 ± 0.2 .

Chloride m. equiv. per 1000 ml. dialysate = 40.2 ± 0.2 .

HCO_3 . Cell solution: $CO_3 = 0.4$ vols. $\%$ = 0.2 m. equiv. per 1000 ml.

Dialysate: $CO_3 = 0.18$ vols. $\%$ = 0.1 m. equiv. per 1000 ml.

Glutathione. Cell solution: 2 ml. of 1 in 2 dilution in trichloroacetic acid

= 0.58 ml. $N/1100$ I_2 = 0.53 ml. $N/1000$ I_2 .

1000 ml. cell solution contain: 0.53 m. mol. = 0.58 m. equiv. glutathione at pH 7.2.

Dialysate. 1 ml. = 0.61 ml. $N/1100$ I_2 = 0.55 ml. $N/1000$ I_2

= 0.55 m. mol. = 0.66 m. equiv. glutathione at pH 7.65.

Total base electrolytic. Solutions: 1 ml. $N/100$ H_2SO_4 . 1 ml. of 2 in 15 dilution of cell solution or dialysate.

1 ml. $N/100$ H_2SO_4 = 1.000; 1.002; 1.004; 1.002 ml. $N/100$ NaOH.

H_2SO_4 + cell solution = 0.365; 0.359; 0.351; 0.350 ml. $N/100$ NaOH.

H_2SO_4 + dialysate = 0.366; 0.374; 0.364 ml. $N/100$ NaOH.

Base m. equiv./1000 ml. cell solution = 48.4 ± 0.6 .

Base m. equiv./1000 ml. cell solution = 47.5 ± 0.5 .

Total base as K + Na + Mg + NH_4 . K m. equiv./1000 ml. cell solution

= 41.2; 41.0; 41.2; 41.0; 41.0

= 41.1 ± 0.1 .

K m. equiv./1000 ml. dialysate = 43; 43.1; 43; 43. = 43 ± 0 .

To these must be added ± 0.2 corresponding to variations in the standard solutions.

K m. equiv./1000 ml. cell solution = 41.1 ± 0.3 .

K m. equiv./1000 ml. cell solution = 43.0 ± 0.2 .

Sodium. Na m. equiv./1000 ml. cell solution = 3.7; 4.4. = 4.05 ± 0.3 .

Na m. equiv./1000 ml. dialysate = 3.2; 3.2. = 3.2 ± 0 .

Magnesium. Mg. m. equiv./1000 ml. cell solution = 3.1; 2.5 = 2.8 ± 0.3 .

Mg. m. equiv./1000 ml. dialysate = 1.4.

Ammonium. Absent in cell solution and dialysate.

Total base as sulphate.

Direct: Cell solution m. equiv./1000 ml. = 47.2; 48.3; 48.7; 47.9.

Indirect: Cell solution m. equiv./1000 ml. = 49.4; 47.5; 49.6; 48.4.

Total base as sulphate in cell solution

m. equiv./1000 ml. = 48.4 ± 1.2 .

Total acid soluble phosphorus. Three equal standards agreed exactly.

Cell solution mg./1000 ml. = 225; 225; 224; 225; 223.

Dialysate mg./1000 ml. = 80; 80.8; 79; 80.2.

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