XLV. THE PENETRATION OF ERYTHROCYTES BY ANIONS.

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THE unequal penetration of ions into erythrocytes has been the subject of numerous observations. Thus the mature cell although impermeable to cations, contains large quantities of potassium, and this in spite of its being suspended in plasma which contains little potassium and much sodium. Again, though erythrocytes are permeable to anions the latter show varying degrees of penetration, and even anions which permeate freely, frequently show an anomalous distribution; the concentration of normal cell-bicarbonate is higher than would have been anticipated from a simple Donnan equilibrium, while Hastings and Van Dyke [1931] have shown that a similar excess of bromide occurs, if bromide is added to the fluid in which erythrocytes have been suspended.

The present investigation is concerned with the entry into erythrocytes of various acids or anions and attempts to correlate certain physical properties with their ability to penetrate the cell membrane.

The relative permeation of anions may be determined from the amount of one anion entering the cell in a given period of time compared with the amount of a second anion entering during the same period. Alternatively, one can expose erythrocytes to a solution containing two salts and estimate their relative concentrations in the cells. The second method has been adopted chiefly because differences in anion permeation, which are barely perceptible with cells suspended in solutions of single salts, are greatly magnified in solutions of the mixed salts, the more penetrating ion greatly depressing the entry of the less penetrating ion. Chloride was adopted as the standard anion and the second anion B was estimated indirectly and sometimes both directly and indirectly. The indirect method is based on the following assumptions:

(1) *If erythrocytes are suspended in KCl solutions, chloride diffuses across the cell membrane and equilibrium is reached within five minutes at 23°. The validity of this assumption is shown by the figures in Table I.*

Table I.

External p_H 5.5 Ext. Cl 205 m.eq.			Ext. p_H 9 Ext. Cl 88 m.eq.		
Time	% of original volume (V)	Cl (m.eq.)	Time	(V)	Cl (m.eq.)
0 min.	100	50	0	100	50
1 "	94	141	1	140	22
5 "	102	165	5	122	20
60 "	102	170	60	122	20

(2) *The amount of chloride entering erythrocytes from pure KCl solutions depends only on p_H .* This statement is approximate. The erythrocyte contains

haemoglobin and potassium which are unable to penetrate the cell membrane, and according to the Donnan equation, if the external Cl falls, some decrease of cell-Cl must also occur. The actual decrease of Cl however is barely detectable by ordinary analytical methods, and if at the end of an experiment unit volume of cells has changed to V and the final content of Cl is x , then the total Cl taken up by unit volume of cells, Vx , remains practically constant and is independent of the external concentration (Table II). When the external concentration of chloride falls very low, lysis of the erythrocyte occurs, but if the external osmotic pressure is sustained by the addition of glucose, lysis is prevented and it will be seen that the chloride concentration of the erythrocytes is fairly well maintained.

Table II.

(5 minutes' suspension at 23°.)

Ext. Cl	p_H	V	Cl	Vx	p_H	V	Cl	Vx
205	7	88	105	92	5.1	108	182	196
175	7	99	92	91	5.1	129	160	205
146	7	108	79	85	5.1	133	150	200
124	7	124	70	87	5.1	155	125	194
88	7	150	59	88	5.1	—	—	—
22*	—	—	—	—	5.1	88	176	155

* Plus glucose 9 %.

If the slowly penetrating glucose is replaced by a slowly penetrating anion like phosphate, the phosphate acts in the same way as the glucose; very little cell-base combines with phosphate, the bulk of it existing as chloride.

(3) *When erythrocytes are placed in a mixture of salt solutions the sum of all the anions entering the cell in five minutes is the same as the amount of chloride entering erythrocytes suspended for the same time in pure KCl solution of the same p_H .* This follows from the preceding observation. If the second anion B permeates more slowly than Cl, Cl enters to excess to satisfy the combining power of cell-base, but with the passage of time, more and more of B diffuses into the cell displacing chloride until true equilibrium is reached. Similarly, if B permeates the more readily, excess of B enters the cell and is subsequently displaced by Cl. In other words the anion distribution during a quasi-stationary state is used as an index of penetrating power.

If therefore at a given p_H , unit volume of cells takes up X m.eq. of Cl from pure KCl solution and if from a solution containing equivalent amounts of KCl and KB, cells take up Y m.eq. of Cl, then the cell content of B, Z m.eq., should equal $X - Y$ m.eq. and the concentration of B relative to Cl, R , may be expressed as Z/Y or $(X - Y)/Y$.

The preceding paragraph itself involves two assumptions:

(a) that at a given external p_H , a constant difference exists between the internal and external p_H , whatever the nature of the external salts may be;

(b) that at a given p_H inside the erythrocyte, the cell has the power of taking up constant amounts of anions whatever the nature of the diffusing salts.

Neither of these assumptions is strictly correct, but with regard to the second the following may be said. The rate of entry of a substance into a cell depends on its ability to penetrate the cell membrane and on the amount of the substance that the cell can take up. If B is an acid it can exist inside the cell as an anion, as an undissociated acid or as a complex combination with haemoglobin. It follows that if B has a low dissociation constant, or if it forms complexes with haemoglobin comparable with the formation of carbhaemoglobin by CO_2 , then its concentration gradient will be relatively greater than that of Cl and it will tend to enter the erythrocyte in greater

amounts; but its power of penetrating the cell membrane will not necessarily be greater than that of Cl.

Now X equals the total anion capacity of cell-base expressed in terms of chloride equivalents; Y represents the amount of Cl combined with cell-base in the presence of anion B and $X - Y$ represents the amount of B combined with cell base also expressed in terms of chloride equivalents. Under these circumstances, it is thought that the value $(X - Y)/Y$ more truly indicates the relative permeation of B and Cl than Z/Y , for Z represents cell-B, dissociated, undissociated and complexly combined.

Further with regard to both the assumptions (a) and (b) it may be said that these approximate statements receive justification from Table III, where, with the exception of the phosphate-chloride system, total cell-anion (B and Cl) is nearly constant at each p_H level.

Table III.

(5 minutes' suspension at 23°.)

Ext. B = 87.5 m.eq.

Ext. Cl = 87.5 m.eq.

Ext. anion (B)	p_H	B + Cl m.eq.	p_H	B + Cl m.eq.	p_H	B + Cl m.eq.
Chloride	7	92	6	150	5.1	205
Iodide	7	98	6	159	5.1	211
Phosphate	7	87	6	133	5.1	182
Iodoacetate	7	92	6	160	5.1	212
Oxalate	7	86	6	146	5.1	198
Salicylate	7	96	6	149	5.1	—

It is thought that the low figure provided by the phosphate-chloride mixtures in acid ranges is due to some specific action of the anion on the cell membrane. Such decrease of permeation also invalidates assumption 3 and may be suspected where increased acidity causes a decrease of cell volume accompanied by agglutination.

It must be clearly understood that though the value $R = (X - Y)/Y$ approximately indicates the relative concentrations of B + Cl in erythrocytes it would only indicate the relative permeation of B + Cl if the cells were naturally chloride-free. But, in fact, R does not show the relative permeation of B and Cl because at the inception of any experiment erythrocytes have a natural chloride content of about 50 m.eq., and chloride therefore has an advantage over B, which is normally absent from the cell. If one were examining a system at equilibrium this initial Cl content would be unimportant, but since the comparison of anion permeation depends on the relative anion concentration before equilibrium has been reached, the initial Cl content is significant.

Although one cannot accurately compare the permeation of B relative to Cl one can however compare the ratios B/Cl, C/Cl, D/Cl and so obtain a more accurate idea of the relative permeations of B, C and D. Further, if the ratio B/Cl is equal to 1 or is greater than 1, it may be said that B permeates more readily than Cl, while if R is very much less than 1, one may infer that B permeates less readily.

It would, of course, be easy to remove chloride from erythrocytes by a preliminary soaking in potassium sulphate solution and to suspend the chloride-free cells in a mixture of KB and KCl, but it has been found that the permeability of cells twice suspended in salt solutions differs greatly from that of cells only once suspended, an observation which agrees with the experiments of Brinkman and Van Dam [1920].

Method.

Normal human blood was centrifuged in two tubes drawn out into sealed graduated and calibrated capillaries for thirty minutes at 4000 r.p.m. The volume of erythrocytes was noted and the cells in each tube then suspended in one hundred times their volume of the same test-fluid at 23°. This fluid usually contained 87.5 m.eq. of the test-anion (B) and 87.5 m.eq. of chloride. After 5 minutes the tubes were respun, the p_H of the clear fluid determined colorimetrically and the change in volume of the erythrocytes noted. The containing tube was carefully washed free of all trace of salts with distilled water, which was introduced with a capillary pipette down as far as the upper face of the erythrocyte column. The cells were then analysed. Sometimes it was necessary to examine the stability of anion distribution in erythrocytes, and under these circumstances the cells were suspended for periods varying from one to sixty minutes, and the relative anion contents were then compared.

It is clear that errors in reading the p_H of an unbuffered solution are much greater than if the solution is buffered. In fact, however, the external solution is never completely unbuffered, because a trace of bicarbonate diffuses into it from the suspended erythrocytes. To limit p_H errors as far as possible, two solutions of each indicator were used, one at each end of the corresponding p_H range. The method in the case of slightly alkaline KCl solution was as follows. A few drops of slightly acid phenol red were added as a layer on the surface of the clear supernatant fluid in one of the centrifuge-tubes, while to the fluid in the duplicate tube the slightly alkaline phenol red was added. As each indicator diffused downwards, its colour changed slightly, the orange solution becoming slightly redder and the red solution becoming slightly yellow. The weakest clearly perceptible colour in the supernatant fluid was matched with an inverted p_H colour-tube, comparison being made with a layer of suitable thickness in the tapering portion of the standard tube. It was thought that by these means a fair idea of p_H might be obtained.

Chloride was estimated by Claudius's method [1924]; phosphate by the method of Briggs [1922]; iodide and iodoacetate by the method of Kendall [quoted by Harington and Randall, 1929] and lactate by Mendel and Goldscheider's method [1925].

Salicylate was estimated by Benedict and Theis's colorimetric method [1924] slightly modified. Diazotisation was allowed to proceed for fifteen minutes. It was found desirable to prepare numerous standards so that the colour of the unknown did not differ by more than 20 % from that of the standard. Duplicate experiments on diffusion of salicylate into erythrocytes gave results which agreed within 10 %.

Oxalate was estimated by permanganate titration of the protein-free filtrate from cells treated with zinc hydroxide; a blank titration was done on normal cells.

Errors in the method are least marked in buffered solutions and are most evident in neutral unbuffered solutions. This arises from difficulties in reading p_H and from the relatively low chloride content to be estimated.

Errors in unbuffered solutions at p_H 7 are as follows:

Errors in tube volume	± 0.5 %.
Errors in Cl estimation	± 1.5 m.eq. in Y.
Errors in p_H reading	± 0.15 $p_H = \pm 7$ m.eq. in X.

Another possible source of error arises from differences in the haemoglobin content of unit volume of cells. In normal blood such differences are slight.

Table IV. Summary of Results.

Group	No.	Anion B	p_K	pH 5.1				pH 6.0				pH 7.0						
				% of origi- nal vol.	Chlo- ride m.eq.	B m.eq.	B/Cl calcu- lated	B/Cl esti- mated	% of origi- nal vol.	Chlo- ride m.eq.	B m.eq.	B/Cl calcu- lated	B/Cl esti- mated	% of origi- nal vol.	Chlo- ride m.eq.	B m.eq.	B/Cl esti- mated	B/Cl calcu- lated
Inorganic	1	Chloride	—	129	205	—	1.00	—	114	150	—	1.00	—	98	92	—	1.00	—
	2	Phosphate	—	126	173	32	0.18	0.05	109	126	24	0.19	0.06	103	80	12	0.15	0.06
	3	Iodide	—	116	94	111	1.18	1.20	110	79	79	1.11	1.27	96	41	51	1.24	1.39
	4	Sulphate	—	122	126	79	0.63	—	115	112	38	0.34	—	103	76	16	0.21	—
	5	Nitrate	—	134	102	103	1.01	—	106	68	82	1.20	—	97	44	48	1.09	—
Organic	6	Thiocyanate*	—	134	84	94	1.12	—	103	60	90	1.50	—	93	44	48	1.09	—
	7	Formate	—	136	115	90	0.78	—	112	80	70	0.87	—	100	54	38	0.70	—
	8	Acetate	—	144	79	126	1.60	—	114	80	70	0.87	—	96	50	42	0.84	—
	9	Iodoacetate	—	136	80	125	1.56	1.75	113	81	69	0.85	1.00	98	52	40	0.77	0.77
	10	Propionate	—	140	82	123	1.50	—	112	80	70	0.87	—	100	54	38	0.70	—
	11	Butyrate	—	144	72	133	1.84	—	113	80	70	0.87	—	98	49	43	0.88	—
	12	Valerate	—	150	68	137	2.02	—	117	74	76	1.03	—	101	54	38	0.70	—
	13	Glycolate	—	130	117	88	0.75	—	110	94	56	0.60	—	97	64	28	0.44	—
	14	α -Hydroxypropionate	—	130	121	84	0.69	—	112	104	46	0.44	0.33	96	73	19	0.26	—
	15	β -Hydroxypropionate	—	136	95	110	1.16	—	108	94	56	0.60	—	96	66	26	0.39	—
	16	β -Hydroxybutyrate	—	137	92	113	1.23	—	113	87	63	0.72	—	101	67	25	0.37	—
	17	Glycerate	—	120	142	63	0.44	—	106	120	30	0.25	—	90	72	20	0.28	—
Ketonic acids	18	Thioacetate	—	128	70	135	1.92	—	110	68	82	1.20	—	104	60	32	0.53	—
	19	Pyruvate	—	130	106	99	0.94	—	112	92	58	0.63	—	100	66	26	0.39	—
	20	Levulate	—	141	78	127	1.62	—	114	80	70	0.87	—	98	60	32	0.53	—
	21	Oxalate	1.42	4.39	105	28	177	6.32	106	71	79	1.11	1.0	97	48	44	0.92	0.81
	22	Malonate	2.8	5.68	122	110	95	0.86	111	94	56	0.60	—	102	68	24	0.35	—
	23	Succinate	4.18	5.57	126	114	91	0.80	116	122	28	0.23	—	102	74	18	0.24	—
	24	Azelate*	4.62	5.6	115	72	106	1.47	108	74	76	1.03	—	102	75	17	0.23	—
	25	Maleate	1.93	6.58	114	110	95	0.86	115	106	44	0.41	—	106	75	17	0.23	—
	26	Fumarate	3.03	4.49	121	138	67	0.49	—	—	—	—	—	103	79	13	0.16	—
	27	Tartrate	3.0	4.39	126	164	41	0.25	—	—	—	—	—	—	—	—	—	—
Tricarboxylic acid	28	Citrate	—	—	—	—	—	—	116	126	24	0.19	—	—	—	—	—	—
	29	Benzoate*	—	4.2	123	72	106	1.48	—	106	60	1.50	—	96	49	43	0.88	—
	30	<i>o</i> -Hydroxybenzoate*	—	3.0	112	61	117	1.92	—	104	54	1.78	1.75	95	44	48	1.09	1.14
	31	<i>m</i> -Hydroxybenzoate	—	4.13	124	70	135	1.93	—	100	54	1.78	—	—	—	—	—	—
	32	<i>p</i> -Hydroxybenzoate	—	4.55	122	70	135	1.93	—	102	55	1.73	—	—	—	—	—	—
	33	<i>p</i> -Nitrobenzoate*	—	3.47	110	60	118	1.97	—	108	57	1.64	—	95	50	42	0.84	—
	34	<i>p</i> -Aminobenzoate	—	—	126	66	139	2.10	—	104	58	1.58	—	99	44	48	1.09	—
	35	α -Naphthoate*	—	4.7	110	56	149	2.67	—	102	48	2.13	—	93	44	48	1.09	—
	36	Phthalate	2.92	5.41	102	64	141	2.21	—	117	84	0.66	—	96	64	28	0.44	—
	37	Cinnamate*	—	4.46	108	59	119	2.02	—	—	—	—	—	—	—	—	—	—
	38	Mandelate	—	3.37	128	94	111	1.18	—	106	86	0.75	—	94	58	34	0.59	—
	39	Diphenylacetate	—	3.96	—	—	—	—	—	103	59	1.54	—	—	—	—	—	—
Alicyclic	40	Hexahydrobenzoate	—	—	—	—	—	—	118	72	78	1.08	—	98	48	44	0.92	—
	41	Quinate	—	3.54	118	156	49	0.31	—	107	123	27	0.22	95	75	17	0.23	—

* (At pH 5.5.)

From a consideration of the values obtained in parallel experiments it is considered that where the ratio of B/Cl is in the neighbourhood of unity, differences exceeding 20 % are significant, but if the ratio is about 0.3, differences of permeation should not be assumed unless the ratios differ by 50 % or more.

The estimated ratio of B/Cl is shown in Table IV immediately after the calculated ratio. The organic anions in the Table are arranged in an aliphatic and an aromatic series, and these are subdivided into the simple anions and those containing additional polar groups, *e.g.* OH, COOH *etc.*

Inorganic anions and thiocyanate.

(Table IV.) The order of permeation is thiocyanate > iodide > nitrate, chloride > sulphate > phosphate.

Phosphate-chloride system. Solutions containing KH_2PO_4 and K_2HPO_4 (175 m.eq.) were mixed to give the required p_{H} , and an equal volume of KCl was added. It was thought better to use solutions of the same equivalence rather than the same osmotic pressure. Since phosphate may be univalent or bivalent within the p_{H} range investigated, it is not possible to determine accurately the content of cell-phosphate from the values for cell-phosphorus without knowing the p_{H} of the cell. But as over 80 % of phosphate is univalent between p_{H} 5 and 6.2 no serious error is involved if one assumes that phosphate is mainly univalent when the p_{H} of the external solution is between 5 and 6. In any case the figures for cell-phosphorus are so low as to make it clear that phosphate permeates very much more slowly than Cl (Table V).

Table V.

5 minutes at 23°.

p_{H}	% of original volume	Cell-Cl m.eq.	Cell-P mg.	P m.eq.	<i>R</i>
4.8	130	147	62	20	0.13
5.1	126	173	26	9	0.05
6.0	109	126	21	7	0.06

It will be noted that phosphate permeates at about the same rate between p_{H} 5.1 and 6, but at p_{H} 4.8, where some degree of haemolysis occurs, the phosphorus content rises sharply. It will also be seen that the permeation of phosphate is greatly depressed, while chloride enters to excess to satisfy the combining power of cell-base. Thus cells in pure phosphate solution containing 570 mg. P take up 235 mg. of P in 5 mins. at p_{H} 5.1, while if the phosphate is diluted with KCl so that its P content becomes 285 mg., cell-phosphorus does not diminish proportionately to 117 mg., for actually the cells take up only 26 mg. of P.

Effect of p_{H} on phosphate permeation. After 5 mins. suspension at 23° cells take up very little phosphate and the content is practically the same at p_{H} 5 and 6 (24 mg. P), but if the cells are immersed for 1 hour in phosphate-chloride solution at 33° (containing 87.5 m.eq. Cl and 285 mg. P) they take up 85 mg. P at p_{H} 5.1 and 215 mg. P at p_{H} 6. In other words if the exposure of erythrocytes to phosphate-chloride mixtures is prolonged, a certain impermeability develops at p_{H} 5 relative to p_{H} 6. It is thought that this may be due to a specific coagulation effect of phosphate at low p_{H} , which greatly decreases the permeation of phosphate itself, while leaving the permeation of the chloride ion relatively little affected.

The unequal distribution of phosphate and chloride inside erythrocytes is unstable, for the longer the cells are exposed to mixtures of phosphate and chloride, the more nearly does the distribution of these anions in the cells become proportional to that in the external solution.

Iodide-chloride (No. 3). Iodide was estimated directly and chloride, Y , was calculated indirectly and was also estimated directly from the total halide content of the protein-free filtrate of erythrocytes treated with sodium tungstate and sulphuric acid, silver nitrate and nitric acid being added and the solution titrated with thiocyanate, without previous boiling. The chloride content of the cells was also estimated by Claudius's method, for it was found that after prolonged boiling with AgNO_3 , $\text{K}_2\text{Mn}_2\text{O}_8$ and HNO_3 the silver iodide was decomposed almost entirely, and a halide value was obtained which equalled the cell-chloride *plus* a small residue of iodide. Thus at p_{H} 6, iodide was 90 m.eq., giving a calculated Cl value of 60 m.eq. Total halide was estimated as 161 m.eq., giving an actual chloride value of 71 m.eq., which by the second method (acid incineration) was found to be 74 m.eq. At p_{H} 7, chloride was calculated at 35 m.eq. compared with estimated values of 41 and 39 m.eq.

It is clear that iodide attains a greater concentration in erythrocytes than does chloride, and this is the more remarkable when it is recalled that before iodide diffusion has begun chloride is already at its natural level of 50 m.eq. Moreover the unequal distribution is relatively stable for periods up to one hour; at p_{H} 5.9 cell-chloride and iodide were respectively 67 and 93 m.eq. while after 1 hour they were 69 and 96 m.eq.

Sulphate-chloride (No. 4), sulphate permeates less readily than chloride; this is least evident at p_{H} 5.1 and very marked at p_{H} 7.

Organic anions.

A large number of organic anions have been examined. Most of them have been standardised by titration, but in some cases salts of known purity were used. In the following, calculated (as opposed to estimated) values of B are shown in brackets.

All the organic anions appear to penetrate more readily at low p_{H} .

Lower fatty acids. This group, from acetate to valerate, appears to permeate more readily than chloride at p_{H} 5.1 and at about the same rate or very slightly more slowly between p_{H} 6 and 7. Table IV shows some indication that permeation becomes more rapid with increasing length of the carbon chain.

Formate (No. 7) is exceptional, for while between p_{H} 6 and 7 it has the same ratio as the acetate-valerate series, it permeates much more slowly at p_{H} 5.1.

Acetate-chloride (No. 8) (sodium acetate; KCl and HCl). At p_{H} 5.1, R is 1.60; at p_{H} 4.9, however, some haemolysis occurs, and R falls to 0.83. Presumably the damage to the cell membrane impairs its semipermeability so that the superior penetration of acetate is no longer in evidence.

Differences in permeation between acetate and chloride at p_{H} 5 and 6 are clearer if acetate and chloride are mixed in the proportion of 1 : 7 and cells are suspended for 5 and 60 mins. At p_{H} 6, at which acetate and chloride permeate at about the same rate, the distribution of acetate and chloride remains unaltered. At p_{H} 5.1, the superior permeation of acetate is shown by the low initial chloride at 5 mins. and its rapid increase during the subsequent 55 mins. (Table VI).

In order to confirm the superior permeation of acetate, an iodoacetate-chloride system was used, although the insertion of an iodine atom in the CH_3 group may well alter the permeation of the organic anion. However, the comparative similarity of the observed iodoacetate value and the calculated acetate

Table VI.

	External acetate 22 m.eq. p_H 5.1		External chloride 153 m.eq. p_H 6	
	Cell-chloride	Cell-acetate	Cell-chloride	Cell-acetate
5 mins.	107	(98)	129	(21)
60 mins.	170	(35)	131	(19)

figure lends support to the belief that the latter is approximately correct (Table IV, Nos. 8, 9).

Hydroxyaliphatic acids. The following were examined: glycolate, lactate, β -hydroxypropionate and β -hydroxybutyrate; these substances also permeate most rapidly at p_H 5.1 and more slowly at higher p_H . The addition of a hydroxyl group decreases penetration, and this decrease is much greater when the OH is in the α -position than when it is in the β -position. Indeed at p_H 5.1 the permeation of the β -hydroxy-anion is greater than that of Cl, but at p_H 7 it is probably less.

Lactate. The calculated values for lactate were checked by the veratrol colorimetric method. At p_H 5.05, lactate was calculated to be 89 m.eq. and estimated as 84 m.eq.; at p_H 5.7, lactate calculated was 44 m.eq. and estimated to be 38 m.eq.

The superior permeation of β -hydroxy- over α -hydroxy-anions is shown by a comparison of the B/Cl ratio at 5 and 60 mins. In the lactate mixture, the chloride which permeates first is displaced by the organic anion and decreases with time. In the β -hydroxypropionate mixture, chloride increases towards an equable distribution of anions.

Table VII.

	(Ext. Cl 87.5 m.eq., Ext. B 87.5 m.eq.)					
	Lactate p_H 5.1			β -Hydroxypropionate p_H 5.05		
	Cl	B	R	Cl	B	R
5 mins.	118	(87)	0.74	96	(114)	1.19
60 mins.	112	(93)	0.83	107	(103)	0.96

Glycerate (No. 17). This dihydroxy-acid permeates much more slowly than the preceding anion.

Thiolacetate (No. 18). The substitution of sulphur for the oxygen in the polar group increases permeation.

Ketonic acids. These acids like the hydroxy-acids also permeate less quickly than the corresponding fatty acids, and similarly when the carbonyl group is near the $-\text{COOH}$ group permeation is slower than when it is further removed from the carboxyl group. Compare pyruvate with laevulate, which has the CO group in the γ -position (Nos. 19 and 20).

Amino-acids. The dissociation constants of these substances render them unsuitable for examination.

The dicarboxylates. These fall into two groups; oxalate which permeates more quickly than Cl, and the other dicarboxylates which on the whole permeate more slowly. The latter include the saturated malonate and succinate and the unsaturated maleate and fumarate. It also includes the hydroxy-anion tartrate.

All these acids have two dissociation constants, and they were adjusted to give a constant concentration of 87.5 m.eq. when mixed with an equal volume of KCl solution. Table IV shows that all these acids permeate more readily at low p_H ; that they permeate less readily than oxalate; that succinate permeates more slowly than malonate, and that they all permeate more slowly than Cl at p_H 7;

that fumarate which is mainly bivalent at p_H 5.1 permeates less readily than maleate which is mainly univalent at this p_H . The table shows that the addition of hydroxyl or carboxyl groups to dicarboxylates makes it almost impossible for the anion to penetrate the cell membrane (*e.g.* tartrate (No. 27) and citrate (No. 28)). It must be noted however that azelate (No. 24), $(CH_2)_7(COOH)_2$ penetrates rapidly between p_H 5.5 and 6. It is thought that this may be correlated with its high solubility in organic solvents and its low dissociation.

Oxalate-chloride (No. 21). Oxalate was considered as a dibasic acid (p_K 1.42 and 4.39) at p_H 5.1. p_H was adjusted with oxalic or hydrochloric acid and in either case similar results were obtained.

Oxalate is unique in that it permeates more rapidly than chloride or any other anion at low p_H , and at p_H 7 it permeates more quickly than most anions and probably as quickly as chloride, for at p_H 7 cell-chloride remains practically unaltered while oxalate mounts from 0 to 39 m.eq.

The effect of length of exposure on the chloride-oxalate distribution at p_H 5.1 and 6.1 is as follows:

Table VIII.

Time	p_H 5.1			p_H 6.1		
	Cell-Cl	Cell-oxalate	R	Cell-Cl	Cell-oxalate	R
0 min.	50	0	0	50	0	0
$\frac{1}{4}$ min.	23	131	5.7	38	104	2.7
1 min.	26	139	5.4	—	—	—
5 mins.	30	168	5.6	69	70	1.0
60 mins.	83	126	1.5	—	—	—

So rapidly does oxalate diffuse that in a few seconds its concentration is six times as great as that of chloride. With the passage of time however cell-Cl rises and oxalate falls so that equality is approached but is not nearly attained in one hour. At p_H 6 equality of distribution is reached in 5 mins., but even here the superior permeation of oxalate can be shown by exposing cells to oxalate-chloride solution for only a few seconds, when it will be found that oxalate has increased to 104 m.eq. while chloride has decreased to 38 m.eq.

While superior permeation of oxalate explains its high initial cell concentration followed by its subsequent decrease, it does not explain the corresponding changes in cell-chloride, which falls from 50 to 23 m.eq. in a few seconds and then rises at 1 hour to 83 m.eq. This can only be due to an actual displacement of chloride from the cell followed by its return into the cell again.

For this power of displacing chloride there seems to be no adequate explanation.

The oxalate-chloride system also illustrates the depression of chloride permeation by the more mobile anion. Cells in pure KCl at 175 m.eq. at p_H 5.1 take up 205 m.eq. Cl. In a mixture containing 87.5 m.eq. each of oxalate and chloride, the cells instead of taking up 102 m.eq. Cl actually lose 30 m.eq. and give a Cl content of about 20 m.eq. Even if cells are placed in a mixture containing 155 m.eq. Cl and only 22 m.eq. of oxalate at p_H 5.1, they take up only 58 m.eq. of chloride and as much as 115 m.eq. of oxalate in 5 mins.

A similar unequal distribution of oxalate and chloride (and also of acetate and chloride in the case of acetate-chloride mixtures) is obtained if freshly collected unheparinised blood is added directly to the salt mixtures.

It should be noted with regard to oxalate that it must be numbered among those anions which decrease permeation and cause shrinkage of cell volume at

low p_H . Thus in oxalate-chloride mixtures, cell volume at p_H 5.5 is 111 %; at p_H 5.1, 105 % and at p_H 4.5, 102 %, while the corresponding total anion contents are respectively 168, 198 and 167 m.eq.

Aromatic anions.

Benzoate permeates rather more readily than chloride, but the following anions, *o*-, *m*- and *p*-hydroxybenzoate, nitrobenzoate and *p*-aminobenzoate, though containing a carboxyl group and an additional polar group, all permeate more readily than benzoate itself. This superior permeation is most marked at low p_H .

Phthalate (No. 36), which contains two carboxyl groups, permeates rapidly at p_H 5.1 but at p_H 7 its entry is slower than that of any of the preceding anions and probably slower than that of chloride itself.

Naphthoate (No. 35), which contains two benzene rings, penetrates with great speed and can be qualitatively recovered from the erythrocyte. Experiments with naphthoate, nitrobenzoate and cinnamate were carried out at p_H 5.5, 6 and 7, and acidity was adjusted by the addition of phosphate solution to equal 25 m.eq. in the final mixture. In this way precipitation of the acid at p_H 5.5 was avoided while, owing to its slow permeation, effects due to phosphate itself could be neglected.

Salicylate-chloride. Salicylate was determined directly and indirectly. The salicylate-chloride values are shown in Table IX (No. 30). The unequal distribution of these anions is not entirely stable, but even after erythrocytes have been suspended in salt mixtures for 30 mins. at 33° and after haemolysis has occurred, it is very evident that salicylate is still present in a concentration which is much higher relatively than Cl (Table IX). This is most clearly seen when the external solution contains 153 m.eq. of chloride and only 22 m.eq. of salicylate (Table IX).

Table IX.

p_H 5.7.					
Time	Temp.	External Cl	External salicylate	Cell-Cl	Cell-salicylate
$\frac{1}{2}$ min.	23°	153	22	56	91
5 mins.	23°	153	22	63	93
30 mins.	23°	153	22	82	68
60 mins.	33°	153	22	120	50

Three of the anions examined contained benzene rings attached to an aliphatic side-chain; of these cinnamate and diphenylacetate permeated quickly. But mandelate (No. 38) which contains a hydroxyl group in the aliphatic chain resembles the aliphatic anion glycollate in its penetrating power and not salicylate (No. 30) which contains the hydroxyl group attached to its benzene ring.

The permeation of the saturated alicyclic anions, hexahydrobenzoate and quinate, resembles that of the aliphatic acids, in that the former permeate quickly, while the latter hardly penetrates at all.

DISCUSSION.

The view is generally held that the erythrocyte consists of a semipermeable envelope holding in its interior water, haemoglobin, salts and possibly lipin. The envelope contains lecithin, cholesterol, probably haemoglobin and also a stroma protein, isoelectric at p_H 5.5, described by Jorpes [1932].

Erythrocytes are impermeable to cations at $p_H < 8$, and this indicates that the cell membrane must be positively charged [Mond, 1928]. On the other hand, cataphoresis shows that the surface is negatively charged [Höber, 1904]. But as Mond remarks, the cell membrane may consist of two elements; an electro-negative, giving the cell its cataphoretic properties, and a positively charged portion making the cell impermeable for cations.

The present investigation is concerned with the passage of anions through this complex envelope. The work of Henderson [1928] and of Van Slyke on electrolyte equilibria and the experiments of Höber [1910] on conductivity show that within the erythrocyte chloride is mainly ionised, and as it has been found that other anions quantitatively replace chloride, it may be presumed that these are also ionised. Further it has been found that erythrocytes take up anions from acid salt solutions, and as the process is accompanied by cell swelling, it seems probable that the bulk of cell-anion is distributed within the erythrocyte and not on its surface.

The difference of acid distribution within erythrocytes may depend on differing degrees of unionised complex formation or on differing ionic activities. In this case, one would expect the inequality of distribution to be stable. On the other hand unequal distribution may be due to differing rates of permeation through the cell membrane, in which event the inequality would be maximum at first and tend to diminish subsequently.

The only systems showing a stable inequality are iodide-chloride and thiocyanate-chloride, and the work of Hastings and Van Dyke [1931] on bromide-chloride distribution suggests that this may be due to iodide forming unionised compounds to a greater extent than chloride. In the case of salicylate-chloride, where the initial disparity in distribution diminishes to a fairly stable inequality, it is thought that both of the above mechanisms may be concerned. Most of the other systems show an unstable inequality which tends towards ultimate equality of anion distribution within the cell, and this is ascribed to differences of permeation through the cell membrane.

In view of the large size of some rapidly penetrating ions and the small size of others that permeate slowly, it seems unlikely that the pore theory of membrane permeability can hold with regard to the erythrocyte envelope, a probable alternative being that permeation rate depends on relative solubility in the cell membrane. It seems clear from Table IV that permeation does not depend on the solubility of anions in water, and the theory of Overton seems more applicable, namely that the penetration of substances into cells depends partly on their solubility in oily or lipoid substances. According to Jacobs [1924] the substances investigated by Overton fall into two groups—polar and non-polar. The latter (*e.g.* simple hydrocarbons) are unionised and soluble in organic solvents; these are said to penetrate cells rapidly; the former which contain groups like $-\text{OH}$, $-\text{NH}_2$ or $-\text{COOH}$ tend to be dissociated and water-soluble; these penetrate cell membranes less readily, and the more numerous the polar groups, the more slowly does permeation occur. Attempts to support or refute these theories are necessarily inconclusive in the absence of data regarding the solubility of substances in the cell membrane. However, the permeation of substances into erythrocytes has been examined from the standpoints of their dissociation and their partition coefficients in an ether-water system.

Reference to Table IV shows that the simple aliphatic acids with one polar $-\text{COOH}$ group permeate more rapidly than those with two polar groups, whether there be an $-\text{OH}$ or an additional $-\text{COOH}$ group, while substances like glycerate, tartrate and citrate (Nos. 17, 27 and 28) which have more than two

polar groups penetrate still more slowly. All these substances with numerous polar groups are more strongly dissociated and concentrate to greater extent than the corresponding fatty acids in the water phase of an ether-water system [Smith, 1921].

Overton suggests that organic substances permeate in the undissociated state, and it seems probable that the permeation of organic acids in general may depend on the dissociation constants of the acid and on its oil-solubility, varying according to the resultant of these two factors. Thus in the simple fatty acid group, the partition coefficient of formic acid is less than that of acetic acid, and the former is much more strongly dissociated, and it penetrates much more slowly at p_H 5.1, resembling the hydroxy-aliphatic acids in this. But at p_H 7, where both acids are well dissociated, the penetrations of formate and acetate are similar.

The members of the acetate-valerate series dissociate to an equal extent, but there is a marked increase in ether- and olive oil-solubility as the length of the carbon chain increases. The apparent increase of permeation however is only slight, but probably changes in the B/Cl ratio are masked by pre-existing chloride in erythrocytes.

The α -hydroxy-acids, glycollate and lactate are strongly dissociated and concentrate in the water phase; these substances penetrate much more slowly; the β -hydroxy-acids on the other hand are weak acids and, although their partitions are similar to those of the α -acids, their penetration is more rapid. The same applies to pyruvic and laevulic acids. In the former, the ketonic group is next the carboxyl; it is well dissociated and penetrates slowly; in the latter a γ -ketonic group is associated with low dissociation and more rapid penetration, although the partition coefficients of pyruvic and laevulic acids are similar.

Both the hydroxy- and keto-acids permeate more slowly than the corresponding fatty acids at higher p_H .

The dicarboxylic acids, malonic and succinic, are moderately well ionised at p_H 5.1, they concentrate in the water phase and their permeation is slow. But azelaic acid, $(CH_2)_7(COOH)_2$, on the other hand is less well dissociated and has an ether-water partition of 9.5. At p_H 5.1 it permeates rapidly, but at p_H 7, its permeation is slow. In this series, oxalate is exceptional, and it is not possible to correlate this with its power to precipitate calcium, for there is no detectable calcium in the erythrocyte, while even if calcium precipitation resulted in phase reversal of the cell membrane, there is no reason why oxalate should permeate more rapidly than the water-soluble anion, chloride or the ether-soluble organic acids.

In the case of the aromatic series, even complex substances like naphthoic and diphenylacetic acids permeate rapidly, and the presence of two polar groups as in the hydroxy-, amino- and nitro-benzoic acids does not seem to interfere with permeation. All these substances concentrate in the ether phase and attain a relatively high concentration in the oil of an olive oil-water system, and all quickly penetrate the erythrocyte membrane. It may be noted that while the presence of an $-OH$ group in the benzene ring does not delay permeation, the substitution of $-H$ by $-OH$ in the aliphatic portion of phenylacetate greatly depresses permeation, which becomes comparable with that of the hydroxy-aliphatic acids: thus the permeation of mandelate is similar to that of lactate. It may be observed that the p_K , ether-water partition and permeation ratio at p_H 6 of salicylic acid are respectively 3.0, 5.0 and 1.78, while the corresponding data for mandelic acid are 3.37, 1.33 and 0.75.

The effect of p_H on permeation. The permeation of anions relatively to chloride decreases as p_H increases. This is due partly to the chloride which is normally present in erythrocytes, and the effect will be more apparent at high p_H , where the total anion taken up by the cells is less. But that the ratios obtaining at p_H 5.1 are not only due to this effect may be seen if the chloride figures at p_H 5.1 and 6 are compared. For not only does B increase as the total cell-anion increases, but in many cases cell-chloride remains unaltered and may even decrease, while in all cases the increase of B between p_H 6 and 5 is greater than the corresponding increase in chloride. This relative increase in permeation of anion B may be due to its being less dissociated at low p_H , but such an explanation could not apply to sulphate whose permeation relatively to chloride is also higher at p_H 5.1 than at 6. It might be thought that swelling of the cells would itself increase permeation by stretching the cell membrane, increase of permeation occurring *pari passu* with swelling. That this is not the case may be shown by suspending erythrocytes in hypertonic and hypotonic lactate-chloride solutions at p_H 7; in the latter solution, marked cell swelling occurs but the ratio of cell-lactate to chloride remains the same.

If one conceives the erythrocyte membrane as a negatively charged protein sieve whose holes are filled with lipid material, then it follows that the restraining effect of the sieve protein meshwork on the permeation of anions will be decreased at low p_H . Further at low p_H the cell swells but the protein element will contract as it approaches its isoelectric point and as the total area of the cell membrane increases, it follows that the lipid element must increase in area and decrease in thickness; this will differ from the effect of swelling resulting from hypotonicity where the protein and lipid elements will be extended equally.

It is also possible that p_H may affect permeation directly through the lipid portion. Condensed monomolecular films become expanded at low p_H [Adam, cited by Rideal, 1930] and such a change in the erythrocyte membrane may well alter its permeability. In this connection reference may be made to the high temperature coefficient of expansion of unimolecular fat films—a finding which is comparable in degree and range with the temperature coefficient of phosphate diffusion into erythrocytes [Maizels, 1932].

It is not suggested that lipid-solubility is the only factor in, or even the direct means of, cell permeation, for adsorption, as well as solubility in organic solvents, is an expression of hydrophobe qualities. It is also possible that adsorption and permeation may occur at the protein-water interface of the cell envelope. Indeed it is likely that water-soluble substances and water itself may penetrate by this route.

SUMMARY.

1. The order of permeation of inorganic ions into erythrocytes is (thiocyanate) > iodide > nitrate, chloride > sulphate > phosphate—a lyotropic series.
2. The permeation rate of anions relative to chloride increases with the external hydrogen ion concentration.
3. The presence of additional polar groups decreases the permeation of organic anions. This effect is less marked, if the polar group is not in the α -position to the carboxyl group, and it also becomes less marked with increasing length of the carbon chain.
4. Substances permeating rapidly are usually poorly dissociated, have a relatively high ether-water partition coefficient and are capillary-active.

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