CCLXXXI. THE COMBINATION OF COAGULATED PROTEIN PARTICLES WITH ANIONS.

By MONTAGUE MAIZELS.

From the Department of Pathology, University College Hospital.

(Received November 1st, 1934.)

IN a previous communication [Maizels, 1934] the permeation of anions into erythrocytes was studied and it was shown that those substances which penetrated most rapidly were hydrophobe; thus the permeation of the following ions was in accordance with the lyotropic series $I > CNS > NO_3 > Cl > SO_4 > PO_4$. In the case of the aliphatic organic acids permeation was found to be directly proportional to the length of the carbon chain and inversely proportional to the number of polar groups, suggesting that rapidity of permeation was associated with a high degree of solubility in non-polar solvents. On the other hand it was recognised that adsorption like solubility in non-polar solvents is also an expression of hydrophobe qualities, and as substances which penetrate erythrocytes most rapidly are approximately those which have the greatest power of peptising starch and collagen [Katz, 1933] it was thought possible that speed of permeation might depend to some extent on the concentration of capillary-active substances at the surface of the erythrocyte.

In the present investigation, an attempt has been made to measure at varying p_H the relative combination of anions with such protein particles as are produced by coagulating haemoglobin with alcohol, and to compare these values with the relative permeation of anions into erythrocytes.

Method.

Washed erythrocytes are laked with distilled water, the stromata removed by centrifuging and the haemoglobin in the supernatant fluid precipitated with three volumes of alcohol. After 2 hours, the alcohol is removed and the precipitate thrice washed with water, the sediment being centrifuged and triturated with a glass rod between each washing. After the final washing, the suspension is distilled for a short time *in vacuo* at 40° to remove traces of alcohol as far as possible and is then ready for use.

The suspension is centrifuged in tubes drawn out to sealed graduated and calibrated capillaries for 30 mins. at 4500 r.p.m. and the volume of the packed deposit noted. The latter is then resuspended in 150 times its volume of salt solutions of varying composition and p_H , and after 10 mins. the suspension is again centrifuged. Any change of volume is noted and the anion content of the deposit estimated by methods described elsewhere [Maizels, 1934]. The errors in these methods are as follows: standard chloride solutions show slight losses, usually about 2 %, and seldom exceeding 5 %. Error for iodide ± 2 % and for other methods ± 5 %.

Equilibrium with the external solution is reached in less than 10 mins. It may be noted that after centrifuging for 30 mins. at 4500 r.p.m., continued centrifuging for 15 mins. at 9000 r.p.m. caused a further decrease in volume of 7 %. However, it was found more convenient to centrifuge at the lower rate, and the packed deposit at 4500 r.p.m. was found to have a water content of between 77 and 81 % by volume, varying with different batches of material. For this reason experiments are not exactly reproducible, although results obtained on any single batch are comparable. Thus when particles centrifuged from distilled water are resuspended in KCl solution at p_H 5.2 the volume change varies between 96 and 108 % (usually about 100 %), and the corresponding anion taken up by unit volume of the original deposit between 194 m.eq. and 214 m.eq., while at p_H 7.2 the volume lies between 95 and 101 % and the Cl between 130 and 138 m.eq. However, in spite of these limitations, certain conclusions have been reached which have been embodied in the text.

Further it is clear that the anion present in the centrifuged deposit is not all combined with protein, because the latter constitutes only 22 % of the deposit, the residue consisting of free and bound water in which a proportion of anion must be present.

Anion combined with protein particles suspended in solutions of single salts.

The amounts of anion taken up by the deposit at p_H 5.2 and 7.2 are shown in Table I. Only one salt was present in the suspending solution, the concentration being 175 m.eq. In the Table, V indicates the volume of deposit centrifuged down from salt solution, relative to the volume centrifuged from distilled water; A is the amount of anion present in the final deposit and TA is the amount of anion taken up by unit volume of original standard deposit (centrifuged from distilled water) that is $V \times A$.

Table I.

External anion 175 m.eq. Time 10 mins. T. 24°.

Anion	p_H 5.2			p_H 7.2		
	V	A m.eq.	TA m.eq.	V	A m.eq.	TA m.eq.
Chloride	100	204	204	100	135	135
Iodide	90	220	198	98	149	146
Phosphate	106	197	209	—	—	—
Thiocyanate	86	228	196	95	140	133
Oxalate	100	203	203	100	132	132
Salicylate	88	228	201	97	161	156

It will be observed that at p_H 7.2 the various salts cause practically no change in volume, the total anion taken up lying between 132 and 156 m.eq.; the hydrophobic ions iodide and salicylate give higher figures, while chloride and oxalate give lower. To this statement thiocyanate, which is hydrophobic, forms an apparent exception which will be considered later. At p_H 5.2 the total amount of anion combined (196–209 m.eq.) is greater than at p_H 7.2 but the deposit shows little or no swelling; on the contrary the hydrophobic ions iodide, thiocyanate and salicylate cause an actual shrinkage, so that, although the total anion content is greater than at p_H 7.2, the volume of the deposit is less than that of the original deposit centrifuged from distilled water.

It was also found that addition of glucose to the suspending solution made no difference to the amount of anion combined.

Anions combined with protein particles suspended in solutions containing equivalent amounts of two salts.

The deposit centrifuged down from distilled water was resuspended in a solution containing 87.5 m.eq. of KCl and 87.5 m.eq. of a second salt "KB." Chloride was always taken as the standard for comparison and the ratio of B to Cl present in the deposit, B/Cl or R , is shown in Table II.

Table II.

Anion B	V	p_H 5.2				V	p_H 7.2			
		Cl m.eq.	B m.eq.	TA m.eq.	R		Cl m.eq.	B m.eq.	TA m.eq.	R
Phosphate	105	100	98	208	0.98	(103	65	62	131	0.95)*
Iodide	90	77	148	202	1.93	100	67	78	145	1.16
Thiocyanate	86	78	150	196	1.93	95	66	77	136	1.16
Iodoacetate	103	88	117	210	1.33	103	65	66	135	1.01
Oxalate	98	90	123	209	1.37	101	69	65	135	0.95
Salicylate	86	67	166	200	2.48	96	66	90	150	1.36

* At p_H 8.8.

In this series also, when one of the pair is strongly hydrophobic (iodide, thiocyanate or salicylate) a shrinkage of volume occurs at p_H 5.2 compared with the volume at p_H 7.2 or compared with the original volume from distilled water. When both ions are more hydrophilic, on the other hand, little change of volume is observed at either p_H . At p_H 5.2 the sum of $Cl+B$ is roughly constant and approximately equal to the amount of Cl or B which would be present in the deposit if it were exposed to a solution containing the corresponding salt alone, at a concentration of 175 m.eq. But in the case of mixtures containing chloride and a strongly hydrophobic ion, the chloride content is low while that of the hydrophobic ion is relatively very high, while where neither ion is strongly hydrophobic the amounts of the two ions present in the deposit are more nearly equal. At higher p_H (7.2 or more), the amounts of Cl and B are about equal if neither ion is strongly hydrophobic (chloride with phosphate or oxalate) and together total about 133 m.eq. But here again if one of the pair is strongly hydrophobic the concentration of chloride remains at about 66 m.eq. while the concentration of the hydrophobic ion is higher so that the sum of $Cl+B$ is greater. (Chloride with iodide or salicylate.) It follows that at p_H 7.2 R is 1 where both anions are hydrophilic and is >1 if one of the ions is strongly hydrophobic. In the case of chloride-thiocyanate R is >1 , but the total anion taken up is, as in the case of pure thiocyanate, relatively low.

It has been noted that the total anion taken up at p_H 5.2 by unit volume of the original standard deposit is roughly constant (196–210 m.eq.). It will appear later that this constancy may well be fortuitous; nevertheless, if the amount of Cl taken up from a mixture of KCl and KB is known, an approximation for B and hence for R can be determined. Thus the value for Cl is estimated for each new batch of coagulated protein suspended in a solution of pure KCl at p_H 5.2. If this value is X , and if the amount of Cl taken up by the deposit suspended in a mixture of KCl and KB is Y , then B/Cl or R equals $(X - Y)/Y$. These calculated values are shown in Table III, together with the values for R already set out in Table II. No significance can be attached to differences less than 0.2. The value R is also compared with r , the ratio obtaining in the case of erythrocytes exposed to similar salt mixtures. The changes in volume of protein deposit in various salt mixtures are also shown in Table III. It has

been noted previously that protein in KCl solution at p_H 5.2 may swell or shrink slightly and, since these changes in volume vary with each batch of protein deposit, the values for different solutions are more comparable if they are referred not to the original volume of deposit centrifuged from distilled water, V_1 , but to the volume of deposit from the same batch, centrifuged from KCl solution at p_H 5.2, V_2 .

Table III.

Cl=87.5 m.eq. B=87.5 m.eq. p_H 5.2. T.=24°.

B	V_1	V_2	R	r
Iodide	90	90	1.9	1.18
Thiocyanate	86	88	1.9	1.12
Nitrate	95	95	1.15	1.01
Sulphate	100	100	1.0	0.63
Phosphate	105	101	1.0	0.05
Formate	101	103	0.9	0.78
Acetate	98	100	0.95	1.6
Propionate	98	100	0.95	1.5
Butyrate	98	99	0.95	1.84
Valerate	102	98	1.0	2.02
<i>n</i> -Hexanoate	83	83	2.0	—
α -Hydroxypropionate	101	103	0.85	0.69
β -Hydroxypropionate	101	100	0.85	1.16
Iodoacetate	103	102	1.35	1.75
Oxalate	98	98	1.35	6.32
Succinate	107	104	0.9	0.80
Azelaate*	92	90	1.7	1.47
Tartrate	101	101	1.0	0.25
Benzoate	94	88	1.9	1.48
Salicylate	86	86	2.5	2.07
Mandelate	98	95	1.4	1.18
α -Naphthoate†	90	90	2.2	2.13
Diphenylacetate†	91	91	2.3	1.54

* At 30°.

† At p_H 5.8.

It will be seen that the strongly adsorbed anions cause a marked decrease in volume and are present in greater amounts than chloride so that R is >1 , and the less adsorbed anions cause no change in volume, or else a slight increase, and are present in the deposit in amounts which are presumably about equal to that of chloride. The first group includes iodide, thiocyanate, *n*-hexanoate, azelaate and the aromatic acids; the contrast between the two groups is well shown on comparing the values of R for the two pairs azelaate and succinate, valerate and *n*-hexanoate.

DISCUSSION.

In the following discussion it is necessary to consider

- (1) the relation of the anion in the free water of the deposit to that combined with the actual protein particles,
- (2) the cause of deposit shrinkage in acid solutions containing hydrophobic ions, and
- (3) the comparison of anion ratios obtaining in the case of protein deposit and erythrocytes.

(1) It has been seen that the ion content of the protein deposit is equal to the sum of the anion combined with the actual protein micellae and that present in the intermicellar water. If one assumes that all this water (some 78 %) is free, then the total anion in the deposit water would be 0.78×175 m.eq. or 136 m.eq., and this value must be subtracted from the total anion present in

the deposit in order to obtain the amount of anion actually combined with the protein particles. Thus, in Table I, 100 ml. of deposit containing 22 ml. of protein and 78 ml. of water combine with 204 m.eq. of chloride at p_H 5.2. The amount of chloride in the free water is 136 m.eq. leaving 68 m.eq. combined with 22 ml. of protein, corresponding to a concentration of 310 m.eq. In the case of iodide at p_H 5.2, 100 ml. of deposit shrink to 90 ml. containing 22 ml. of protein and 68 ml. of water. In this case the total anion is 198 m.eq., the anion in the free water is 119 m.eq. and the amount of anion in the protein corresponds to a concentration of 359 m.eq. In this way it may be shown that the amount of anion taken up by the protein of the deposit is about 300 m.eq. for hydrophilic ions and about 350–380 m.eq. if the deposit is exposed to a hydrophobic ion or to a pair of ions one of which is hydrophobic and the other hydrophilic. Hence, if all the deposit water is free, it follows that the amount of anion taken up by the actual protein particles varies considerably. Nevertheless, the amount of anion taken up at p_H 5.2 by unit volume of the original deposit is more or less constant. This probably arises in the following way: deposits centrifuged from solutions containing hydrophobic ions take up more ion in combination with the protein particles but contain less free fluid, so that there will be less intermicellar solution, and hence less anion, in the fluid portion of the deposit. In the case of deposits exposed to solutions containing only hydrophilic ions, the anion combined with the protein is less while the free water anion is greater. In other words at p_H 5.2 the sum of anions present in the deposit equals the sum of anion combined with protein particles (which is greater with hydrophobic ions and less with hydrophilic ions) *plus* the anion in the free intermicellar fluid (which is less with hydrophobic ions and greater with hydrophilic ions) and this sum is approximately 200 m.eq. in the case of most ions, whether hydrophobic or hydrophilic.

Further, if all the intermicellar water is free, then the values for R in Tables II and III do not indicate the relative amounts of anion actually combined with deposit protein. Thus, in the case of salicylate-chloride at p_H 5.2, 100 ml. of deposit containing 22 ml. of protein lose 14 ml. of water, leaving 22 ml. of protein and 64 ml. of fluid. This fluid contains 56 m.eq. of chloride and 56 m.eq. of salicylate. The whole deposit contains 0.86×67 m.eq. of chloride and 0.86×166 m.eq. of salicylate. Subtracting the anion present in the free water from these values leaves 2 m.eq. chloride and 86 m.eq. of salicylate giving a ratio of 43. If the various ratios in Table II are recalculated in this fashion a value R_1 is obtained which differs from R in the following way: phosphate-chloride, $R=0.98$, $R_1=0.94$; iodide-chloride, $R=1.93$, $R_1=7.4$; thiocyanate-chloride, $R=1.93$, $R_1=6.6$; iodoacetate-chloride, $R=1.33$, $R_1=2.56$; oxalate-chloride, $R=1.37$, $R_1=2.5$; salicylate-chloride, $R=2.48$, $R_1=43$. At p_H 7.2 most anions are present in deposit at a concentration of about 135 m.eq. (Tables I and II) and only salicylate and iodide give contents which are substantially in excess of this figure. Since the water in the deposit could of itself account for 136 m.eq. it follows that at p_H 7.2 the hydrophilic ions are not combined with deposit protein to any extent, while iodide and salicylate are probably combined to a small extent (12–21 m.eq.). In the case of thiocyanate, which is also hydrophobic, only 133 m.eq. are present, but owing to shrinkage and loss of fluid from the deposit only 128 m.eq. of the salt are present in the free fluid, leaving 5 m.eq. combined with the protein.

Hence, if all the water in protein deposit is free, it may be said that hydrophilic anions do not combine with the actual protein in deposit at p_H 7.2, while the simultaneous presence of a hydrophobic ion practically excludes them from combination also at p_H 5.2.

But the assumption that all the deposit water is free is not necessarily correct, for a proportion may be free while the rest is bound by the protein particles (lyospheres). If the anion content of the bound water is less than that of the free water, then the sum of the various anions combined with the actual protein particles at p_H 5.2 would tend to be more nearly equal, while the ratio of anions combined with the particles, R_1 , would tend to approach R , the ratio for the whole deposit. Again, if all the water of the deposit were free, then the deposit anion could never fall below 136 m.eq. But if part of the deposit water were bound and had a lower anion content than the bulk of the suspending salt solution, then figures below 136 m.eq. might well be encountered. Experimentally, values in the neighbourhood of 130 m.eq. have been observed, but the differences from 136 m.eq. are too small and the methods employed insufficiently accurate to allow of conclusions being drawn.

(2) The causes of the volume changes observed in protein deposit may now be considered. The volume of a sediment depends on the amount of free and bound fluid it contains. Both these factors depend in turn on the nature of the fluid, while the proportion of the free fluid also depends on the size and shape of the constituent particles. If the nature of the fluid is such that the particles can glide freely over one another they assume a minimum volume, but if they adhere at corners and edges their disposition is less economical and the intermicellar fluid is greater [Von Buzágh, 1930]. Presumably, the degree of adherence varies inversely with the charge on the particles, the uncharged particles tending to adhere. In the case of coagulated haemoglobin particles the amount of anion taken up at p_H 5.2 is greater than at p_H 7.2; nevertheless very little increase of volume is noticed. Since combination with anion involves an increase in the hydration of the particle, the failure of the deposit to swell must be ascribed to a concomitant decrease in the free water it contains. It is probable that the particles are more charged at p_H 5.2 than at p_H 7.2, and are therefore more free to glide and more closely packed than at p_H 7.2 where they are more likely to adhere at corners and edges, and therefore to be more loosely packed. The actual decrease in volume which is observed at p_H 5.2, with protein deposit suspended in solutions containing a hydrophobic ion, may be due to the particles being more highly charged in the presence of hydrophobic ions, as compared with hydrophilic ions, in which case the free water of the deposit would be still further decreased. Further, the combination of a particle with a hydrophobic ion binds less water than is the case with a hydrophilic ion, for the hydration of the hydrophobic ion is less than that of the hydrophilic.

(3) Returning now to the comparison of anion ratios B/Cl at p_H 5.2 obtaining in the case of protein particles and erythrocytes respectively, that is R and r in Table III, these may be divided into four groups:

(a) R and r are both much greater than 1. This group includes *n*-hexanoate, azelaate, benzoate, salicylate, α -naphthoate, diphenylacetate, and possibly iodoacetate. The ions are all very soluble in organic solvents and are strongly adsorbed. The relatively high concentration of these substances in protein deposit must be explained by their high adsorbability, while in the case of the erythrocyte it may be due either to adsorption or to their being more soluble than chloride in oily solvents, permitting them to penetrate the lipid portion of the membrane more rapidly.

(b) R is equal to or slightly greater than 1 and r is much greater than 1. Included here are acetate, propionate, butyrate and valerate. The ions are fairly soluble in organic solvents and are less strongly adsorbed. The differences between r and R in this case cannot be explained on grounds of adsorption, for

this factor is common both to protein deposit and erythrocytes, and the observation that these substances attain such a high concentration in the erythrocyte may be best explained by their solubility in organic solvents, which though less than that of the members of the first group, is still sufficiently great to ensure that they permeate more rapidly than chloride.

It might be thought that as the acetate-valerate series are more surface-active than chloride, their superior adsorbability should also be manifest in the case of protein particles, and indeed it is possible that such differences might be demonstrated by other methods, if more dilute solutions were used. But estimations of such dilute solutions are not possible by the present technique, and moreover, the large amount of intermicellar salt solution present obscures small differences in the salt content of the micellae themselves.

(c) r is slightly greater than 1, and R is much greater than r . The group includes iodide, thiocyanate and possibly nitrate and mandelate. The ions are less soluble in organic solvents but are strongly adsorbed. This suggests that while adsorption determines the great amount of these ions taken up by protein deposit, it is a less important factor in the case of permeation into the cell where r is only slightly greater than 1.

(d) R is approximately equal to 1, and r is very much less than R . The group includes phosphate, sulphate, formate, α -hydroxypropionate, succinate and tartrate. The substances attain a concentration in the protein deposit roughly equal to that of chloride, but they penetrate the erythrocyte much more slowly than chloride. In this case again adsorption can hardly explain the differences between R and r , and it seems probable that the slow penetration of this group of anions into erythrocytes may best be explained by their relative insolubility in organic solvents. It is thought that the lipid portion of the erythrocyte membrane imposes a barrier to permeation which is of course absent in the case of protein deposit. In support of this observation it may be said that while the permeation of phosphate (and most other anions) into erythrocytes has a high temperature coefficient [Maizels, 1934] the temperature coefficient of protein adsorption was too small to be estimated. Reference may also be made to the findings in the case of the α - and β -hydroxypropionates; both these acids concentrate in the aqueous phase of an ether-water system and are about equally adsorbed. Yet while they concentrate equally with chloride in protein deposit, the β -acid penetrates erythrocytes much more rapidly than the α -acid [Maizels, 1934]. This was correlated with the low dissociation constant of the former acid and it was suggested that acids were able to penetrate the lipid portion of the erythrocyte membrane more rapidly when in the undissociated state. The high values of R and especially of r in the case of oxalate-chloride remain unexplained in view of the fact that oxalate is hydrophilic.

CONCLUSIONS.

1. The amount of anion present in a centrifuged sediment of coagulated haemoglobin particles is greater at p_H 5.2 than at p_H 7.2.
2. If such protein particles are suspended in a solution containing both a hydrophobic and a hydrophilic ion, the amount of the hydrophobic ion combined is much greater than that of the hydrophilic ion.
3. If it is assumed that the bulk of the water in the sediment is free it follows that practically no hydrophilic ion is bound at p_H 7.2, while hydrophobic ions are combined to a small extent. In solutions of single salts at p_H 5.2 both

hydrophobic and hydrophilic anions combine with the protein, but if a hydrophobic and a hydrophilic ion are both present simultaneously in the suspending fluid, the hydrophobic ion practically excludes the hydrophilic ion from combination.

4. A comparison of the anion ratios occurring in a sediment of coagulated haemoglobin exposed to solutions containing chloride and a second ion at p_H 5.2 with the anion ratios found in erythrocytes suspended in similar solutions suggests that while adsorption determines the ratio in the case of protein particles, differences in the relative anion distribution within erythrocytes depend rather on the solubilities of these anions in the lipoid portion of the erythrocyte membrane.

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