LXXIX. EFFECT OF ADDITION OF SALTS ON THE ISOELECTRIC POINT OF PROTEINS. I

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THE question whether the isoelectric point (I.P.) of proteins is a fixed, invariable value or not is of great importance in the study of equilibria in biological systems, as a consequence of the fact that the physical state, osmotic pressure, viscosity, coagulation, gelation, imbibition, sorption of other colloids, *etc.* of colloidal solutions depend on how far they are from the I.P., *i.e.* on the value of the charge present on the surface of the colloidal particles.

If it be accepted that, according to Hardy [1899] and Michaelis [1923], the colloidal particles are discharged at the I.P., *i.e.* that no potential difference exists between the disperse and the dispersed phases, then the normality of anions and cations should be equal ($[a'] = [a'']$) for ampholytes at the I.P. The I.P. would thus depend, apart from the nature of the colloid, on the presence of various substances in the system; those substances which can affect the ratio $[a'']/[a']$ must alter the p_H of the I.P.

Michaelis [1923] has expressed the opinion that the p_H of the I.P. is invariable. On the other hand, it has been shown [Gerngross and Bach, 1923; Kuntzel, 1929; Ostwald *et al.*, 1925; Pauli and Valko, 1929; Wilson, 1922], that the p_H of protein solutions at the I.P. may vary considerably according to the state of purity of the protein, or to the nature and concentration of salts present; the mechanism of action of mineral salts would appear to depend on inter-ionic forces and on the formation of complex salts. The explanation of the action of salts is simpler in the case of lyophobic than of lyophilic; in the former case the degree of dispersion and the stability of the sol are functions solely of the charge of the particles, whilst in the latter case two factors may exist, *viz.* the affinity of certain undissociated parts of the colloid (Loeb's hydrophilic groups) for the medium, in addition to the charge of the ionised particles. The difference between lyophilic and lyophobic lies therefore chiefly in the nature of the forces affecting affinity for the medium, whilst those factors which are connected with the charge are, apart from its actual origin, probably the same in both cases. The theoretical aspect of the question of the variability of the I.P. is thus the same for both types of colloid, and for this reason the same factors should in both cases affect the p_H of the I.P.

The action of salts on the charge of hydrophobic colloidal particles may

depend, in the case of anti-ions (Pauli's Gegenionen), on the concentration and the valency of the anti-ion, on the extent to which it is able to polarise the colloidal surface, on the dimensions, *i.e.* the degree of hydration of the anti-ion, on its heteropolar structure, and on changes in hydration, and hence of solubility, of the product of association; in the case of iso-ions (Pauli's Nebenionen) on the activity factor of the salt (thus the anti-ion K^+ has a different activity according to the anion with which it is associated), on the formation of difficultly soluble salts and complexes, on the increase in charge of the colloidal particles, due either to the partial neutralisation of one of the charges of ampholytes, or to adsorption of iso-ions, or, finally, to changes in the lyophilic or lyophobic character of the colloid.

It is our opinion that the above postulates may, except for high concentrations of salt, be applied also to lyophilic colloids. Experiments will be described in this paper in which the action of salts on isostable types of protein, such as gelatin and serum-albumin, and on isolabile caseinogen, is studied under various conditions.

EXPERIMENTAL.

The experimental material taken was crystallised serum-albumin (Merck), purified caseinogen, and purified gelatin [Przyłęcki, 1930]. These substances were dissolved in such amounts that the final concentrations in the systems studied, after addition of all reagents, were 0.25, 0.5, 1.0 and 2.0 %. The p_H of the systems was adjusted by adding the requisite amount of 0.02 *N*, 0.1 *N* or *N* sodium hydroxide or hydrochloric acid. p_H was determined potentiometrically, using a hydrogen electrode; in certain cases it was determined before and after addition of salt, and before and after boiling. The salts used were Merck's chemically pure reagents and were added before boiling or addition of alcohol to the systems.

The action of heat was studied in the following way. Solutions of different salts were placed in a number of test-tubes of approximately equal diameter, and an equal volume of water was placed in the control test-tubes. Water was added to 5 cc. in all test-tubes, 10 cc. of serum-albumin solution were added, and the contents of all tubes were thoroughly mixed. The test-tubes were then placed in a holder, which was immersed in boiling water and left for 10 or 20 min. before removal of the systems from the water-bath. In some cases the degree of coagulation was roughly determined by filtering and comparing the intensity of the biuret coloration given by the filtrates.

The effect given by salts at laboratory temperature was studied on acid and alkaline solutions of caseinogen, prepared respectively by dissolving caseinogen in 0.03 *N* hydrochloric acid and 0.02 *N* sodium hydroxide. Different concentrations of various salts were added to 1 % solutions of this protein, and the production of opalescence, turbidity, or precipitation was noted.

The experiments performed in the presence of alcohol were carried out

in tall 200 cc. cylinders, in which 10 cc. of serum-albumin, caseinogen or gelatin solutions were placed, and 2 cc. of salt solution and water were added to all systems. Equal volumes of 90 % alcohol were then successively added to all systems, the final alcohol concentration of which was 85 %. The molar concentration of the salts present was for these systems calculated for 12 cc. of aqueous solution, without taking the volume of added alcohol into account.

A. SERUM-ALBUMIN.

Effect of boiling in acid solutions.

The dependence of critical concentration (C_K) on the valency of iso- and anti-ions and on p_H was examined in the following way. Hydrochloric acid was added to 1 and 1.5 % solutions of serum-albumin (this will in future be designated as S.A.), to give a series of p_H 4.2, 3.9, 3.2, 2.6, 2.2, 1.4 and less than 1, and all systems were placed in boiling water. Coagulation was observed at p_H 4.5, and opalescence at p_H 4.2–3.9, clear solutions at p_H 3.2–1.4; at $p_H < 1$ the solutions again became turbid, and in 0.5–0.7 *M* hydrochloric acid coagulation again occurred.

Table I.

C_K for 1 % serum-albumin heated at 100°.

Salt	p_H 4.1 concentration		p_H 3.2 concentration		p_H 2.2 concentration	
	Molar	Normal	Molar	Normal	Molar	Normal
$K_3Fe(CN)_6$	<0.00005	<0.00015	0.00016	0.0005	0.0007	0.0021
Na_2SO_4	0.0024	0.0048	0.0094	0.0188	0.020	0.040
$MgSO_4$	0.0072	0.0144	0.031	0.062	0.150	0.300
$ZnSO_4$	—	—	0.045	0.090	—	—
$CuSO_4$	—	—	0.0576	0.1132	—	—
$\frac{1}{3}Fe_2(SO_4)_3$	0.017	0.034	0.060	0.120	—	—
$MgCl_2$	0.056	0.112	0.200	0.400	0.390	0.780
$CaCl_2$	0.053	0.106	0.180	0.360	—	—
$ZnCl_2$	0.110	0.220	0.270	0.540	—	—
$NaCl$	0.074	0.074	0.280	0.280	0.560	0.560
KCl	0.068	0.068	—	—	—	—

The addition of salts provoked coagulation at certain definite concentrations (C_K), which varied according to the p_H of the solution, being greater the further this was removed from the I.P. (cf. Table I).

Table I a.

Ratios of C_K of various salts.

Salts	p_H 4.1	p_H 3.2	p_H 2.2
$Na_2SO_4/MgSO_4$	1/ 3.0	1/ 3.3	1/ 7.5
$Na_2SO_4/ZnSO_4$	—	1/ 4.7	—
$MgSO_4/ZnSO_4$	—	1/ 1.45	—
$Na_2SO_4/CuSO_4$	—	1/ 6.1	—
$MgSO_4/MgCl_2$	1/ 8	1/ 6.5	1/ 2.6
$Na_2SO_4/NaCl$	1/16.25	1/14.9	1/14.0
$NaCl/MgCl_2$	1/ 1.5	1/ 1.4	1/ 1.4
$MgCl_2/ZnCl_2$	1/ 2.0	1/ 1.35	—
$Na_2SO_4/MgCl_2$	1/23	1/21.3	1/19.5

In Table II are given the results expressing the relation between the p_H of the solutions, the concentration of hydrochloric acid and the C_K of sodium sulphate. It will be seen that the concentrations of acid and salt are directly proportional to one another, whilst such a relation between p_H and C_K holds only over a narrow range. In strongly acid solutions (0.5–0.7 N) coagulation takes place with or without the addition of salts, but in 0.4 N HCl coagulation occurs on the addition of very small quantities of salts ($MgCl_2$, $NaCl$, or Na_2SO_4). The curves connecting C_K of salt with p_H of solution exhibit a maximum at p_H 1.4–2.2.

Table II.

C_K of Na_2SO_4 at various p_H and concentrations of hydrochloric acid.						
Conc. of HCl M	0.009	0.0167	0.0227	0.0286	0.050	0.500
p_H	3.99	3.22	2.60	2.20	1.40	1
C_K Na_2SO_4 (M)	0.0046	0.0114	0.017	0.0205	0.040	0.003
$[HCl]/[Na_2SO_4]$	2.0	1.5	1.3	1.4	1.25	170.0

The salts studied can be classified into a number of groups, according to the valency of the anion; thus potassium ferricyanide in concentrations of less than 0.001 M produces coagulation at 16°, but is slightly less active at 100°, whilst the bivalent sulphate ion has a much weaker action, and using chlorides ($NaCl$, $MgCl_2$, $CaCl_2$) C_K is so high that the dehydration factor has to be taken into account in addition to the ionic effect. The following series of C_K is obtained for ter-, bi-, and univalent anions:

p_H 4.1	$Fe(CN)_6''' : SO_4'' : Cl' = 1 : 32 : 520$
p_H 3.2	
p_H 2.2	
p_H 4.1	$SO_4'' : Cl' = 1 : 16.3$
p_H 3.2	
p_H 2.2	

Apart from the above relations, which are in agreement with Hardy's and Schulze's anti-ion valency rule, a very interesting relation between the nature of the iso-ion and the C_K of the anti-ion is apparent. If we compare the value of C_K for sodium, magnesium, and ferric sulphates we see that at p_H 4.1, for example, it is lowest for sodium sulphate (0.0024 M), three times as great for magnesium sulphate (0.0072 M), and over seven times as great for ferric sulphate (0.034 M). For solutions the p_H of which is further removed from the I.P. these differences increase, until at p_H 2.2 we have the ratio $Na_2SO_4 : MgSO_4 = 1 : 7.5$. The same phenomenon holds, although to a less marked extent, for salts possessing univalent anions; thus at the same p_H C_K is 1.4 times as great for magnesium as for sodium chloride. The action of bivalent cations was further studied using copper and zinc sulphates, and zinc and cadmium chlorides. The results obtained with zinc and copper salts are of the same order as those obtained using alkaline earth salts, whilst cadmium chloride has a considerably higher C_K than have other salts possessing a bivalent cation.

Only very small changes in p_H are noticed as a result of coagulation of

protein. Thus, in one case, the p_H of a control system containing serum albumin, acid and water was 3.02 both before and after boiling, and the p_H of the same system with sodium sulphate was 3.26 before boiling, and 3.30 after.

Table III.

Influence of dilution of albumin on the C_K of sodium sulphate.

% albumin	2	1	0.5	0.25
C_K	0.0094 <i>M</i>	0.0078 <i>M</i>	0.0062 <i>M</i>	0.0054 <i>M</i>

If coagulation is brought about by the presence of definite concentrations of salt, dilution of the whole system before boiling should inhibit coagulation. This was found to be the case; the addition of an equal volume of water at the same p_H as that of a system in which coagulation occurred after heating for a few minutes at 100° prevented coagulation. In connection with these experiments, it was noticed that the C_K of salts diminishes with the dilution of albumin (Table III).

Dependence of C_K for sodium sulphate on the peptising acid.

The results given in Table IV show that the C_K of sodium sulphate is considerably higher for hydrochloric acid than for sulphuric acid solutions of albumin (0.020 and 0.012 *M* respectively). This difference is due partly to the coagulating effect of the sulphate ions originating from the sulphuric acid originally present in the second case: it is, however, of interest that the total sulphate ion concentration is greater at coagulation for such systems than for hydrochloric acid solutions, and the difference between the action of the SO_4^{--} from sulphuric acid and from sodium sulphate increases with greater original concentration of sulphuric acid (Table IV).

Table IV.

C_K of sodium sulphate in acidified systems, containing 50 cc. of 1 % serum-albumin and 30 cc. of 0.1 *N* acid.

Solutions are	<i>M</i> conc. of Na_2SO_4 in the presence of	
	H_2SO_4	HCl
Clear	0 — 0.005	0 — 0.0142
Opalescent	0.005 — 0.012	0.0142 — 0.023
Coagulated	Over 0.012	Over 0.023

Simultaneous action of different anti- and iso-ions.

The addition of sodium sulphate up to a definite concentration to solutions of serum-albumin in the presence of sub-critical concentrations of potassium ferricyanide leads to coagulation, even in unheated solutions. This effect is due to summation of the action of different multivalent anti-ions, and the C_K of the second salt in the presence of the first is greater, the greater the dilution of the first below its C_K (Table V).

Table V.

Coagulative effect of Na_2SO_4 in the presence of sub-critical concentrations of ferricyanide at p_{H} 2.

M concentration of Na_2SO_4	No $\text{K}_3\text{Fe}(\text{CN})_6$	0.0004 M $\text{K}_3\text{Fe}(\text{CN})_6$	0.0006 M $\text{K}_3\text{Fe}(\text{CN})_6$
0	Clear	Clear	Slight opalescence
0.017	"	Opalescent	Flocculation
0.025	"	Intense opalescence	"
0.050	"	Flocculation	"

The addition of magnesium or aluminium chloride to systems containing sodium sulphate, keeping the p_{H} and volume constant, has a contrary effect, raising the C_{K} of the latter salt. The magnitude of this effect depends on the p_{H} of the systems, on the concentration of salt, and on the valency of the iso-ion (Table VI). Thus, adding various quantities of magnesium chloride

Table VI.

Peptising effect of various iso-ions in systems containing 0.030 M Na_2SO_4 at p_{H} 2.2.

Solutions are	M conc. of added salt			
	NaCl	MgCl_2	FeCl_3	AlCl_3
Flocculated	0.057–0.57	0 –0.042	0 –0.020	0 –0.010
Opalescent	—	0.042–0.083	0.020–0.035	0.010–0.021
Clear	—	Over 0.083	Over 0.035	Over 0.021

to a 1 % albumin solution at p_{H} 2.65, the maximum elevation of C_{K} for sodium sulphate occurs in 0.1 M MgCl_2 , whilst at p_{H} 3.6 the maximum C_{K} is in 0.05 M MgCl_2 . In general, the peptising action of magnesium chloride diminishes as the p_{H} of the solution differs more and more from the i.p. The action of aluminium chloride is considerably greater than is that of magnesium chloride, since the latter salt under optimal conditions increases the C_{K} of sodium sulphate 2.5 times, and the former 6.7 times (Table VII). At the same

Table VII.

Peptising action of MgCl_2 and AlCl_3 .

M conc. of MgCl_2	C_{K} of Na_2SO_4 at		M conc. of AlCl_3	C_{K} Na_2SO_4 at p_{H} 3.0 (M)
	p_{H} 3.6 (M)	p_{H} 2.65 (M)		
0	0.0064	0.0161	0	0.010
0.025	0.014	—	0.007	0.0175
0.050	0.020	0.031	0.010	0.022
0.100	0.0177	0.037	0.015	0.026
0.150	—	0.030	0.030	0.055
0.200	0.0155	—	0.055	0.067
0.250	—	0.024	—	—

time we see that the C_{K} of magnesium chloride is greater than that of aluminium chloride (0.08 and 0.05 M respectively, at p_{H} 3.2). Similar results for the peptising effect of these two salts were obtained for systems coagulated in the cold by the addition of ferricyanide (Table VIII).

Table VIII.

Peptising action of iso-ions in 2 % serum-albumin systems containing ferricyanide at p_H 2.0 and 20°.

<i>M</i> conc. of ferri-cyanide	Effect on solutions of addition of					
	H ₂ O	0.03 <i>M</i> HCl	0.3 <i>M</i> HCl	0.6 <i>M</i> NaCl	0.09 <i>M</i> MgCl ₂	0.05 <i>M</i> AlCl ₃
0.0004	Clear	Clear	Opalescence	Opalescence	Clear	Clear
0.0008	Opalescence	Opalescence	Turbidity	Turbidity	"	"
0.0012	Flocculation	Flocculation	Flocculation	Flocculation	Opalescence	Opalescence
0.0016	"	"	"	"	"	"
0.0020	"	"	"	"	Turbidity, then flocculation	"
0.0024	"	"	"	"	Flocculation	Flocculation
0.0030	"	"	"	"	"	"

Effect of boiling in alkaline solution.

The action of the salts NaCl, MgCl₂, CaCl₂, MgSO₄, AlCl₃ and FeCl₃ on serum-albumin solutions at p_H 9.2 was examined. Aluminium and ferric chlorides in higher concentrations were to a small extent precipitated by alkaline solutions. Normal sodium chloride did not coagulate alkaline albumin even on boiling, whilst coagulation took place in 0.026–0.027 *M* calcium or magnesium chlorides without heating. The C_K of magnesium sulphate is 0.033 *M*, i.e. 27 % greater than for calcium chloride. The C_K of aluminium and ferric chlorides could not be determined with any accuracy, and we do not attach any special significance to our figures (Table IX).

Table IX.

C_K of various salts for 1 % serum-albumin solutions at p_H 9.2.

NaCl	CaCl ₂	MgCl ₂	MgSO ₄	AlCl ₃	FeCl ₃
0.640 <i>M</i> (opalescence)	0.026 <i>M</i>	0.027 <i>M</i>	0.033 <i>M</i>	0.0045 <i>M</i>	0.006 <i>M</i>

The C_K of magnesium chloride was determined alone and in the presence of sodium chloride or sulphate and potassium ferricyanide. All systems were at p_H 9.2, and contained 1 % serum-albumin, and 0.03 *M* MgCl₂, and were, in the absence of other salts, in a state of flocculation. The addition of 0.014 *M* Na₂SO₄ or of 0.0016 *M* K₃Fe(CN)₆ prevented flocculation; the systems exhibited, however, intense opalescence. As the concentration of salts is augmented, the solutions become increasingly clear, so that in the presence of 0.03 *M* Na₂SO₄ or 0.003 *M* K₃Fe(CN)₆ they are practically quite clear (Table X).

Table X.

C_K for MgCl₂ at p_H 8.5 (*M*).

C_K MgCl ₂	In presence of NaCl			In presence of Na ₂ SO ₄		
	MgCl ₂ alone	0.15	0.30	0.55	0.0135	0.020
	0.025	0.0263	0.0257	0.022	0.0335	0.035
						0.032

Precipitation by alcohol.

The relation between the C_K of sodium sulphate, the p_H of the system, and the concentration of hydrochloric acid was determined in the presence of 85 % ethyl alcohol. The results, given in Table XI, show that a proportionality exists between the concentration of acid and the C_K of sodium sulphate.

Table XI.

Comparison of C_K of Na_2SO_4 for boiled systems and for 85 % alcoholic solutions.

p_H	C_K for systems heated at 100°	C_K for 85 % alcohol systems	C_K 100°	[Na_2SO_4]/[HCl]	
	(M)	(M)	C_K alcohol	100°	Alcohol
3.2	0.0096	0.0016	6.0 : 1	1.6	9.6
2.6	0.0145	0.0027	6.1 : 1	1.5	10.0
2.0	0.0223	0.0042	5.3 : 1	1.75	11.0
1.6	0.031	0.0057	5.5 : 1	1.6	10.0
1.4	0.042	0.009	4.7 : 1	1.5	8.4

Comparison between the C_K of sodium sulphate in systems coagulated by heat and by alcohol.

C_K was determined for sodium sulphate in acid serum-albumin solutions, in the presence of 85 % alcohol, and heated at 100°. The value of C_K is at p_H 3.2 six times greater in the latter case than in the former, but the difference diminishes as more acid solutions are taken, so that at p_H 1.6 the ratio is 5:1. Acidification of the solutions to a certain extent augments C_K for both systems.

B. CASEINOGEN.

C_K was determined for sodium sulphate and potassium ferricyanide in caseinogen solutions at p_H 2, in unheated and heated systems and in the presence of 85 % alcohol. The results, given in Table XII, show that C_K is 0.0076 M in unheated systems, 0.0064 M at 100°, and 0.005 M in the presence of alcohol. The ratio of the C_K values in heated and alcoholic systems is 1.27, in unheated and heated systems 1.19, and in unheated and alcoholic solutions 1.52; these ratios differ considerably from those obtained for serum-albumin.

A second series of experiments on the relation between p_H and C_K showed that the C_K of sodium sulphate increases with the charge on the colloidal particles.

Table XII.

C_K of sulphate ions for caseinogen solutions acidified with hydrochloric acid to p_H 2 (M).

At 15°	Heated 10 min. at 100°	In 85 % alcohol
0.0076	0.0064	0.005

Determination of the C_K of sulphate ion added as a mixture of hydrochloric and sulphuric acids and of hydrochloric acid and sodium sulphate showed that in the former case it has a value of over 0.08 M , whilst in the latter it is 0.0067 M .

C. GELATIN.

Systems heated at 100°.

The addition of salts giving rise to univalent or bivalent ions (0.001–0.2 *M*) to gelatin solutions at p_H 1–12 does not produce coagulation, either in heated or unheated systems. Potassium ferricyanide coagulates in 0.0005–0.001 *M* solutions, according to the p_H , whilst ferrocyanide is without action.

Action of salts in the presence of alcohol.

The results for systems containing 80 % alcohol (Table XIII) are in every way similar to those obtained for serum-albumin. The C_K of sodium sulphate in systems containing 0.5 % gelatin and 50–90 % alcohol falls at first rapidly with increasing concentration of alcohol, from 0.08 *M* in 50 % to 0.0008 *M* in 85 % alcohol, above which concentration no further change is observed (Table XIV). The value of C_K rises with increasing acidity from 0.00045 *M* in 0.005 *M* HCl to 0.009 *M* in 0.06 *M* HCl (Table XV).

Table XIII.

C_K of salts in gelatin solutions containing 80 % alcohol (dilution by alcohol taken into consideration) (*M*).

p_H 7.30			p_H 1.75			
KCl	MgCl ₂	AlCl ₃	NaCl	Na ₂ SO ₄	MgSO ₄	K ₃ Fe(CN) ₆
0.017	0.0025	0.00025	Over 0.15	0.003	0.0014	0.00015

Table XIV.

Relation between C_K and concentration of alcohol.

C_K of Na ₂ SO ₄ (<i>M</i>)	% alcohol	C_K of MgSO ₄ (<i>M</i>)	% alcohol
0.0008	85	0.011	71
0.0016	80	0.014	69
0.0032	71	0.016	66
0.0047	66	0.020	62
0.0064	62		
0.008	59		
0.032	55		
0.040	54		
0.080	52		

Table XV.

C_K of Na₂SO₄ at various p_H .

p_H	C_K of Na ₂ SO ₄ (<i>M</i>)
3.8	0.00045
3.0	0.0012
2.5	0.0021
2.2	0.0027
1.5	0.009

Action of iso-ions.

The action of iso-ions is illustrated by experiments in which the C_K values of sodium and magnesium sulphates in the presence of alcohol were compared.

In 66 % alcohol the value of C_K was 0.0047 M for sodium, and 0.016 M for magnesium sulphate, whilst in 71 % alcohol the corresponding values were 0.0032 M and 0.011 M (Table XIV).

Table XVI.

Peptising action of $MgCl_2$ in various concentrations of alcohol.

Salts present	Concentration of alcohol necessary for flocculation %
0.0047 M Na_2SO_4	66.5
0.008 M Na_2SO_4	60.0
0.008 M Na_2SO_4 + 0.1 M $MgCl_2$	73.0
0.016 M Na_2SO_4 + 0.1 M $MgCl_2$	70.5
0.024 M Na_2SO_4 + 0.1 M $MgCl_2$	69.5
0.032 M Na_2SO_4 + 0.1 M $MgCl_2$	66.5

When magnesium chloride is added in addition to sulphate, the C_K of the latter rises, from 0.011 M in 71 % alcohol to 0.016 M in the presence of 0.05 M $MgCl_2$ (Table XVI); this effect may be ascribed to the more powerful peptising effect of the magnesium iso-ion in the presence of the feebly coagulating chloride ion. The peptising effect of magnesium ions is not evident in systems containing over 80 % of alcohol; thus in 83 % alcohol 0.002–0.003 M $MgSO_4$ coagulates gelatin, and the addition of magnesium chloride (0.01–0.05 M) does not affect the results obtained.

Similar results were obtained for systems containing sodium sulphate, magnesium chloride and alcohol. The C_K of sodium sulphate is greatly increased by the presence of 0.1 M $MgCl_2$ in low concentrations of alcohol, but less so in higher concentrations; thus in 66 % alcohol C_K is raised from 0.0044 to 0.032 M , whilst in 71 % alcohol the corresponding values are 0.0032 and 0.007 M , and in 85 % alcohol peptisation due to magnesium ions is entirely absent, only the feebly coagulating effect of chloride ions being superimposed on that of sulphate ions.

DISCUSSION.

The stability of protein solutions depends on two factors—the affinity to water of the undissociated part of the protein molecule, and the charge of the colloidal particles. Proteins have been classified by Wo. Ostwald [1920] into isostable and isolabile; at the I.P. the affinity of the first for water exceeds their critical hydration value [Kruyt and de Jong, 1928], so that stable solutions of such proteins may be obtained without the addition of peptisers. Isostable proteins may be further classified into thermo-isostable and thermo-isolabile, according to the behaviour of their solutions on heating; should the affinity for water fall below the critical value at higher temperatures thermo-isolabile proteins coagulate. The affinity to water of isolabile proteins is even at the ordinary temperature less than the critical, so that the stability of sols of such proteins is a function of the charge on the particles. No sharp division exists between isostable and isolabile proteins; the thermo-isolabile group

represents a transition form between the two groups and certain of the thermo-isolabile proteins may at the I.P. be considered to be in a state of slow coagulation.

Isostable proteins may be obtained in the isolabile state, either by heating at above the critical temperature, in the case of thermo-isolabile proteins, or by the addition of such organic solvents as alcohol, acetone, ether, chloroform, *etc.* In the case of systems rendered isolabile by the addition of alcohol, two interesting observations, evidencing the relationship between the critical concentration of alcohol and the affinity of the given protein for water, are to be noted. These are the behaviour of purified protein at the I.P., and the difference between the action of electrolytes on protein solutions at p_H values other than the I.P. of the given protein and at the same p_H in the presence of alcohol. At the I.P. the C_K of alcohol increases with the isostability of the given protein, so that, *e.g.*, gelatin at the I.P. is coagulated by about 48 % alcohol, whilst serum-albumin is coagulated by 36 % alcohol.

At p_H values other than that of the I.P. the properties of various proteins with respect to the behaviour of their aqueous solutions on the addition of salts approach more nearly to one another as the concentration of alcohol in the system rises. Thus at p_H 2.5 aqueous gelatin is precipitated by 0.6 *M*, serum-albumin by 0.5 *M*, and caseinogen by 0.0076 *M* Na_2SO_4 . In heated solutions, the corresponding values of C_K are respectively 0.55, 0.031 and 0.0064 *M*; here the C_K of sodium sulphate for serum-albumin is very close to that for caseinogen. Finally, in 85 % alcohol, the values of C_K are respectively 0.006, 0.0057 and 0.005 *M*, showing that in these conditions the properties of these apparently so different proteins have approached closely to one another.

As the concentration of alcohol rises, the charge on the colloidal particles has to be increased in order to maintain solution. Coagulation may be effected at any p_H using the same concentration of alcohol as at the I.P., by adding a critical concentration of electrolyte. As the concentration of alcohol rises, dehydration increases, with consequent diminution in C_K of the electrolyte, which varies from 0.08 *M* in 50 % to 0.0018 *M* in 90 % alcohol. This phenomenon might be interpreted variously. Thus it might be supposed that even in high concentrations of alcohol gelatin still possesses a certain affinity for water, and that hydration remains as one of the stabilising factors. The stabilising effect of hydration may, however, be in itself insufficient to prevent coagulation without the additional action of a charge on the particles, the neutralisation of which would therefore lead to coagulation. In connection with this theory it would be necessary to assume that the particles are not completely discharged at the C_K , and that, as a result of a certain residual affinity for water, stabilisation is the resultant of the summation of two factors. For this reason the greater the degree of dehydration, the more the C_K of sodium sulphate approaches that for systems the stabilisation of which depends exclusively on the charge. Various other explanations might be put

forward; in the present state of knowledge of the subject, however, these would be somewhat speculative. These explanations would include possible variations in the thickness of the electrical double layer, decrease in the charge, changes in the dielectric constant and in the activity factor of the salts, and augmented association.

The observations recorded in this paper indicate that the dispersion of proteins in colloidal solution is due to two factors, which have to be reduced to a certain minimum for coagulation to supervene. This view is based on the following grounds. For isolabile proteins their charge is the decisive factor for the stabilisation of the sol. Since the charge is a consequence of the structure of both isolabile and isostable proteins, there is no reason for supposing that the latter class are not charged in solution. The C_K of various salts for isolabile proteins are very similar in ordinary and in dehydrated solutions (boiled, or in the presence of alcohol); in such solutions the C_K for iso-labile and -stable proteins are very close to one another, suggesting that such factors as alcohol or heat convert isostable proteins into a form similar to the isolabile. This process depends either on the action of the factors applied on groups on which depends the affinity of the given protein for water, or on changes in the concentration or physical state of water (*e.g.* increased mobility at 100°). At the same time the nature of the factors to which the charge on the particles is due does not undergo change, with the exception of variation in the thickness of the double layer and in its composition, and for this reason the dehydrated form of isostable proteins is well adapted to the study of variations in the charge.

This depends on the ratio of anions to cations; when these are present in equivalent concentrations, the charge is zero. If it be accepted that the charge is connected with the f_a of the dissociated salt and with the degree of dissociation of the colloid, as well as, within certain p_H limits, with zwitterion dissociation, the action of ions will depend on the degree of association and the f_a of the salts produced. These factors are the same as those taking part in homogeneous systems; in protein solutions the charge on the particles is further affected by surface forces, and, above all, by the thickness of the double layer.

The observed facts might be given a different interpretation from that advanced above; it might be considered that ions, in particular anti-ions, form salts with protein ions, and that the solubility of certain of the salts thus formed is less than that of the free protein. The coagulative action of salts would then depend not on their action on the charge on the particles but on the formation of difficultly soluble salts. This explanation is, however, improbable, in view of the following facts. In a relatively dehydrated medium containing alcohol, the C_K of salts for different proteins have very similar values, in spite of the fact that the solubilities of these proteins and probably therefore of their salts are very different. Further counter-arguments are that the action of ions is connected with their valency, that iso-ions act in the

opposite way to anti-ions, that the closer the system is to the i.p., the smaller is C_K , and that the C_K of sulphate ions is at the same p_H 4–6 times greater when they are added as sulphuric acid than when added as sodium sulphate.

All these observations are in agreement with the hypothesis that the action of salts is due to the action of their ions on the charge of the protein particles. Thus the action of the anti-ions conforms to the rule of Hardy and Schulze whereby the action of discharging anti-ions is a function of their valency. In our case, the ratio of the action of uni-, bi-, and tervalent anions is as 1:30:500; these ratios are similar to those found [Pauli and Valkó, 1929] for lyophobic gold sol (1:30:250). The connection between the action of anti-ions and the charge on the colloidal particles is shown by the observation that C_K rises as the p_H of the solution diminishes below the i.p., *i.e.* as the charge increases. The difference between the actions of sulphuric acid and sodium sulphate is due to their different actions on the double layer. Thus sulphuric acid up to a certain concentration augments the positive charge of protein, owing to the action of its cations, whilst the action of sulphate is due almost exclusively to its anions. The action of iso-ions is complex, and depends on the degree of dehydration of the colloid; where this is incomplete iso-ions inhibit the coagulative action of anti-ions, to an extent depending on their valency. The action of uni-univalent salts appears to be due chiefly to displacement by univalent or bivalent iso-ions from acid albuminates, whilst where multivalent iso-ions are added to systems containing multivalent anti-ions the increase in the C_K of the latter is due partly to reactions of double decomposition, resulting in products of a lower f_a ; thus the addition of magnesium chloride to sodium sulphate yields a certain amount of magnesium sulphate, the f_a of which is considerably lower than that of sodium sulphate. A further effect is that of attraction by iso-ions of anti-ions from the hydrosphere of the protein particles to the bulk of the solution, with consequent augmentation of the free charge of the protein complexes.

From the point of view of the zwitterion theory, the antagonistic effect of iso-ions in the coagulation of protein by addition of anti-ions may depend on the formation of salts of lower f_a between the carboxyl residues and the iso-ions, thereby augmenting the free positive charge due to ionisation of the amino-groups; this would apply to systems at the p_H of the i.p., or slightly below this p_H . This view is supported by Loeb's [1924] experiments, which showed that the free charge of denatured proteins is increased by the addition of salts yielding multivalent iso-ions and univalent anti-ions. According to Dorfmann [1930] multivalent iso-ions may further affect the degree of hydration of colloids, in this way inhibiting coagulation.

In systems dehydrated by the addition of alcohol the action of multivalent iso-ions diminishes with increasing alcohol content; no obvious explanation of this phenomenon is apparent.

It appears from the work of Loeb [1924, 1925] and of Kruyt *et al.* [1928, 1929], as well as from the results described in the present paper, that the chief

factor in the coagulative action of electrolytes on proteins is the reduction of the free charge on the colloidal particles below a certain critical minimum. A certain difference exists between proteins discharged at the isoelectric point and at other p_H , due to the fact that on the alkaline side of the I.P. the particles are discharged by and consequently associated with cations, and on the acid side by anions; the conception of the isoelectric state would apply only to purified proteins of zero free charge, whilst proteins discharged by electrolytes at other p_H values than that of the I.P. should rather be termed electrically neutral at the given p_H .

SUMMARY

1. Serum-albumin solutions coagulate on heating at 100° at p_H above 4.7 or below 1.
2. Various mineral salts produce coagulation at a certain critical concentration (C_K) for each p_H . The C_K is at a maximum at p_H about 2, and varies in accordance with the Hardy-Schulze rule. The ratios of C_K for ter-, bi-, and univalent anti-ions are as 1:30:500.
3. The value of the C_K of anti-ions also depends on the valency of the accompanying iso-ion.
4. The value of C_K of salts depends on the nature of the peptising acid; it is less in solutions acidified with acids giving rise to bivalent than to univalent anti-ions.
5. The coagulative action of two or more anti-ions at concentrations below the critical is additive.
6. Salts possessing a univalent anti-ion and a multivalent iso-ion exert in certain concentrations a peptising action.
7. Analogous results to the above are obtained at p_H values higher than those of the isoelectric point, at which cations are anti-ions.
8. Coagulation can be effected by the addition of salts to systems containing 85 % alcohol at any p_H . The Hardy-Schulze rule is followed in such systems, and the maximum values of C_K are at p_H 2.
9. The C_K of sodium sulphate for caseinogen hydrochloride at p_H 2 has practically the same value at 20° , 100° , and in 85 % alcohol systems (respectively 0.0076 M , 0.0064 M and 0.005 M).
10. The C_K of the sulphate ion added as sulphuric acid is considerably higher, *viz.* over 0.1 M .
11. Gelatin in 80 % alcohol can be flocculated at any p_H by critical concentrations of anti-ions, the values of C_K varying according to the Hardy-Schulze rule. The value of C_K varies inversely with the concentration of alcohol.
12. The C_K of sodium sulphate increases with the free charge on the gelatin particles up to a certain maximum, after which it again falls, as for serum-albumin.

13. The C_K of anti-ions increases with the valency of the iso-ions. The critical concentration of alcohol necessary to flocculate gelatin at a given concentration of sodium sulphate is higher in the presence of magnesium chloride than in its absence, and conversely, at a given concentration of alcohol, the C_K of sodium sulphate is considerably higher in the presence than in the absence of multivalent iso-ions.

14. The peptising action of multivalent iso-ions is not apparent in high concentrations (85 %) of alcohol, in which salts possessing a multivalent iso-ion and univalent anti-ions exert a coagulative action.

15. Isostable proteins can by heating at 100° or addition of alcohol be converted into proteins of the isolabile type. The C_K of sodium sulphate is at the same p_H practically the same for serum-albumin, gelatin, and caseinogen in presence of hydrochloric acid in 85 % alcohol.

16. Salts possessing multivalent anti-ions are able to discharge protein particles at any p_H , C_K being different at each p_H for each salt. The coagulum so obtained differs from that obtained at the isoelectric point.

17. The above observations may be readily explained if it be assumed that the state of dispersion of proteins is a function of their affinity for water and of the free charge on the particles; the reduction of the latter below a certain critical minimum results in conversion of protein sols into gels. Flocculation cannot be explained as being due to the formation of variously soluble salts.

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