An Electrochemical Study of Salmine Salts.

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The conductivities at 25° of aqueous solutions of the protamine salmine sulphate and salmine hydrochloride are reported, and an approximate value (87·7 ± 0·7) is derived for the mobility of the salmine cation. The basic groups of salmine are not all of identical strengths, and its electrometric titration curve shows two distinct breaks.

A complete amino-acid analysis of the protamine salmine has been given by Tristram (*Nature*, 1947, **160**, 637), and Mills (*Biochem. J.*, 1952, **50**, 707) has reported closely concordant results. The strongly basic nature of salmine is due to the free guanidine groups of the arginine residues, and Tristram's work points to there being 40 of these in an open-chain polypeptide containing 58 residues in all and having a molecular weight of 8000. A study of the conductivities of some salmine salts seemed likely to be of interest, since salmine occupies an intermediate position between highly charged complex ions such as that studied by James (*Trans. Faraday Soc.*, 1951, **47**, 392) and typical colloids.

Experimental

The conductivity equipment and experimental technique were as described by Davies (*J.*, 1937, 432). All measurements were made at 25° ± 0·01°. Two conductivity cells were used: a Hartley–Barrett quartz cell (cell constant = 0·046280 ± 0·03%), and a hard-glass cell with sealed-in electrodes (cell constant = 0·12251 ± 0·03%). The cells were standardised against potassium chloride solutions of concentrations up to about 1×10^{-3}M by using the interpolation formula (Davies, *loc. cit.*) $\Lambda = 149{\cdot}92 - 93{\cdot}85C^{\frac{1}{2}} + 50C$. Gahl and Greves (*Univ. Calif. Pub. Physiol.*, 1926, **5**, 289) have recommended the use of smooth platinum electrodes for measurements of the conductivity of protein solutions. In the present work, however, platinum-black electrodes were used and gave perfectly reproducible resistance readings.

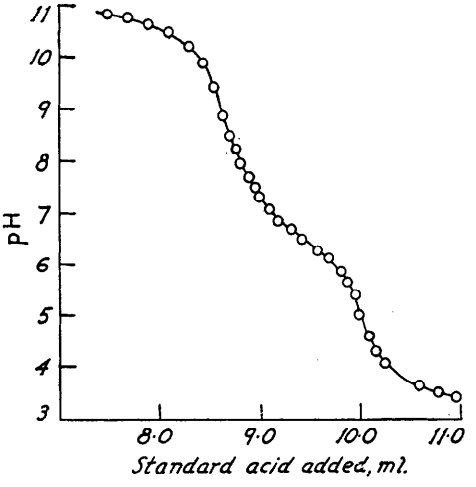

Fig. 1.

Potentiometric titration curve of salmine with hydrochloric acid.

For the measurement of pH, a glass-electrode system was used. The reference electrode was a dipping silver–silver chloride electrode contained in a potassium chloride liquid-junction tube fitted with a ground-glass cap. It was made by Brown's method (*J. Amer. Chem. Soc.*, 1934, **56**, 646). The pH cell was immersed in an oil thermostat at 25° ± 0·01°, and, during runs, was swept free of air with purified nitrogen. A "Cambridge" bench-model pH meter was used, and calibrated with a 0·05M-potassium hydrogen phthalate solution which, at 25°, has a pH of 4·005 (Acree, Hamer, and Pinching, *J. Res. Nat. Bur. Stand.*, 1944, **33**, 287). In order to reduce interference effects, the current to the thermostat was switched off whilst readings were being taken.

Salmine Sulphate.—A commerical sample of salmine sulphate, when titrated conductimetrically with baryta solution, gave an equivalent weight of 1400 which compared very unfavourably with the value 200, determined by Tristram (*loc. cit.*). This indicated a considerable amount of impurity in the sample, probably including nucleic acid with which salmine is combined in the natural state.

The salt was purified by a modification of the methods described by Kossel ("The Protamines and Histones," 1886). The crude salmine sulphate was dissolved in hot water (100 ml. per g. of salt) and, on cooling, the least soluble fraction of the salt separated as a yellow oil. The supernatant liquid was evaporated under reduced pressure to about half its volume and transferred to a separating funnel to collect the main bulk of the salt, again as an oil. This

middle fraction, the purest, was further purified by treatment with a solution of sodium picrate which precipitated the salmine as salmine picrate. The well-washed precipitate was dissolved in an excess of 2N-sulphuric acid, the solution was freed from picric acid by shaking it with toluene, and the salmine sulphate was precipitated by addition of alcohol. Final purification was effected by three times dissolving it in a little hot water and precipitating with absolute alcohol. It was dried by washing it with alcohol, then with ether, and keeping over phosphoric oxide in a vacuum-desiccator. Stock solutions were made up by weight from conductivity water, and, owing to its hygroscopic nature, the dried salt was weighed out in stoppered weighing bottles which were dropped into the stock flasks. The equivalent weight of the purified salmine sulphate, determined gravimetrically by precipitation of the sulphate as barium sulphate, was 231·2.

Salmine Hydrochloride.—A method described in the literature (Kossel, *op. cit.*) for the preparation of salmine hydrochloride involves the addition of the calculated amount of barium chloride to a solution of salmine sulphate. This is not satisfactory for conductivity work, so solutions of salmine hydrochloride were prepared by passing solutions of the purified sulphate through a column of the strongly basic ion-exchange resin Amberlite IRA-400 in the hydroxyl form. The resulting basic solution was converted into that of the hydrochloride by potentiometric titration with hydrochloric acid (glass-electrode). A typical titration curve is shown in Fig. 1. It has two points of inflexion, and the second was taken as that at which complete conversion into salmine hydrochloride occurred. End-points were determined from the data by Kolthoff and Lingane's method (*J. Amer. Chem. Soc.*, 1936, **58**, 1531). The equivalent weight of salmine sulphate calculated for the second end-point was 237. In view of the uncertainty in removing the last traces of free base from the resin column, this agrees well with the gravimetric equivalent.

Conductivity and pH figures for salmine sulphate and hydrochloride in the concentration range 1×10^{-5} to 1×10^{-3} g.-equiv./l. are given in Tables 1—4.

TABLE 1. *Conductivity of salmine sulphate solutions.*

$\kappa_{H_2O} = 2{\cdot}58 \times 10^{-7}\ \Omega^{-1}$.

Expt. No. 1			Expt. No. 1 (corr.)			Expt. No. 2					
10^4C	$10^2C^{\frac{1}{2}}$	Λ	10^4C	$10^2C^{\frac{1}{2}}$	Λ	10^4C	$10^2C^{\frac{1}{2}}$	Λ	10^4C	$10^2C^{\frac{1}{2}}$	Λ
0·08674	0·2945	194·1	0·08354	0·2912	110·1	0·19132	0·4374	101·1	5·1117	2·261	62·20
0·17504	0·4184	196·6	0·14344	0·3787	102·1	0·3610	0·6008	93·74	7·469	2·733	58·16
0·3088	0·555	198·5	0·2499	0·4999	97·54	0·5532	0·7438	88·63	10·045	3·169	55·12
0·7394	0·8599	192·6	0·5884	0·7671	86·92	1·0944	1·0461	80·39	12·455	3·529	53·01
1·8044	1·3433	185·8	1·4374	1·1990	76·90	2·0544	1·433	72·72			
2·9892	1·7290	181·6	2·3866	1·5450	72·01						
6·0581	2·4614	175·5	4·798	2·1910	63·31						

TABLE 2. pH *of salmine sulphate solutions.*

Expt. No. 1						Expt. No. 2					
10^4C	$10^2C^{\frac{1}{2}}$	pH	10^4C	$10^2C^{\frac{1}{2}}$	pH	10^4C	$10^2C^{\frac{1}{2}}$	pH	10^4C	$10^2C^{\frac{1}{2}}$	pH
0·08118	0·2849	5·832	1·2527	1·1193	4·493	0·2948	0·5429	6·070	6·752	2·598	5·800
0·2029	0·4504	5·323	1·9893	1·4105	4·293	0·6834	0·8267	6·013	9·660	3·108	5·768
0·3367	0·5802	5·068	3·038	1·743	4·118	1·3505	1·1620	5·958	12·322	3·510	5·740
0·4244	0·6514	4·953	8·669	2·944	3·590	2·524	1·589	5·898	15·262	3·907	5·727
0·6240	0·7899	4·807				3·828	1·956	5·858			

TABLE 3. *Conductivity of salmine hydrochloride solutions.*

$10^6\kappa_{H_2O} = 0{\cdot}228$ (Expt. 5); 0·205 (Expt. 6).

Expt. No. 5			Expt. No. 6			Expt. No. 6 (corr.)		
10^4C	$10^2C^{\frac{1}{2}}$	Λ	10^4C	$10^2C^{\frac{1}{2}}$	Λ	10^4C	$10^2C^{\frac{1}{2}}$	Λ
0·1118	0·3344	154·0	0·1193	0·3452	163·3	0·1144	0·3401	157·2
0·2411	0·4910	152·2	0·3582	0·5985	157·1	0·3500	0·5916	150·7
0·5209	0·7217	147·6	0·7193	0·8481	152·2	0·7028	0·8383	145·6
0·9622	0·9809	143·6	1·175	1·084	147·9	1·148	1·072	141·4
1·546	1·244	139·0	1·800	1·342	143·8	1·759	1·326	137·4
2·240	1·497	135·2	2·556	1·599	140·2	2·498	1·580	133·7
3·962	1·991	128·7	3·503	1·872	137·1	3·423	1·851	130·3
7·062	2·658	121·7	5·117	2·262	132·4	5·000	2·238	125·7
8·439	2·905	119·5	6·662	2·581	129·3	6·509	2·583	122·4
10·23	3·198	117·2	8·065	2·840	127·1	7·880	2·810	120·2
			9·412	3·068	125·3	9·196	3·040	118·3

TABLE 4. pH *of salmine hydrochloride solutions.*

Expt. No. 6						Expt. No 6 (corr.)					
10^4C	$10^2C^{\frac{1}{2}}$	pH	10^4C	$10^2C^{\frac{1}{2}}$	pH	10^4C	$10^2C^{\frac{1}{2}}$	pH	10^4C	$10^2C^{\frac{1}{2}}$	pH
0·5772	0·7597	5·482	3·843	1·961	4·860	0·5640	0·7510	5·90	3·755	1·940	5·61
1·216	1·103	5·260	5·448	2·334	4·740	1·188	1·090	5·81	5·323	2·310	5·55
2·013	1·419	5·068	7·086	2·662	4·650	1·967	1·407	5·73	6·924	2·630	5·51
3·018	1·737	4·943	9·890	3·145	4·527	2·949	1·719	5·66	9·663	3·110	5·45

RESULTS AND DISCUSSION

The high Λ and low pH values obtained in Expt. 1 (Tables 1 and 2) may be due to two factors. Either the salmine sulphate has a small amount of free sulphuric acid present as an impurity, or considerable hydrolysis takes place. If the effect is due to free acid, then the pH of the solution should vary linearly with log C, and examination of the

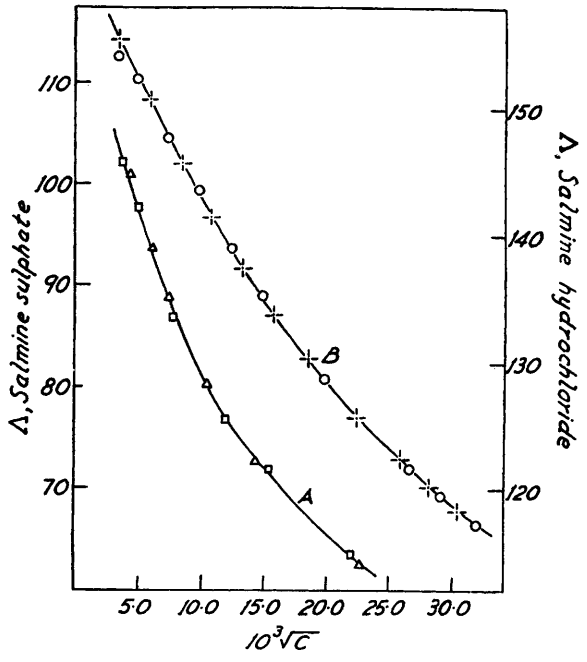

FIG. 2.
Conductivity data at 25°.
A = Salmine sulphate [□, Expt. 1 (corrected); △, Expt. 2.]
B = Salmine hydrochloride [+, Expt. 6 (corrected); ○, Expt. 5].

results in Table 2 shows this to be accurately true. The conductivity values were corrected for the free acid in solution by calculating, from the pH measurements, the contribution made by the sulphuric acid to the total specific conductance at each concentration studied. $\Lambda_{H_2SO_4}$ was obtained from the Onsager equation :

$$\Lambda = \Lambda_0 - [1{\cdot}932\ \Lambda_0 q/(1 + \sqrt{q}) + 110{\cdot}7]c^{\frac{1}{2}}$$

where $q = 2\Lambda_0/3(\Lambda_0 + \Lambda_0^{H^+}) = 0{\cdot}3676$. Errors in this approximate Onsager correction will be very small compared with the total conductivity.

Justification of the corrections is provided by the results of Expt. 2. In this, the free acid supposedly present in the salmine sulphate was neutralised with ammonium hydroxide in one step of the purification process. Fig. 2, in which conductivities are plotted against $C^{\frac{1}{2}}$, indicates the excellent agreement between Expt. 2 and Expt. 1 (corrected), confirming that the acidity in Expt. 1 was largely due to free acid.

The pH of the stock solution of salmine hydrochloride used in Expt. 5 was found to be 4·78. This agreed very well with the pH at the equivalence point (4·75, Fig. 1). In Expt. 6, the high Λ and low pH values indicated that excess of acid was present; a linear plot of pH against log C confirmed this. The conductivity data were corrected for the

contribution made by free hydrochloric acid to the total specific conductance at each point, Λ_{HCl} being calculated from the Onsager equation:

$$\Lambda = \Lambda_0 - 157{\cdot}68C_{HCl}^{\frac{1}{2}}$$

Fig. 2, in which conductivities are plotted against $C^{\frac{1}{2}}$, indicates the good agreement between Expt. 5 and Expt. 6 (corrected) even though different samples of purified salmine sulphate were used in the preparation of their solutions.

The equivalent conductances of the salts are of the same magnitudes as those of simple electrolytes, but compared with these show, as might be expected, a much more rapid decrease with rising concentration. There is no method of estimating how much of this is due to ion-association and how much to normal inter-ionic effects, but for the hydrochloride it seems likely that the latter could account for the greater part of the decrease, and that ion-association is not of great importance at the high dilutions studied. In one

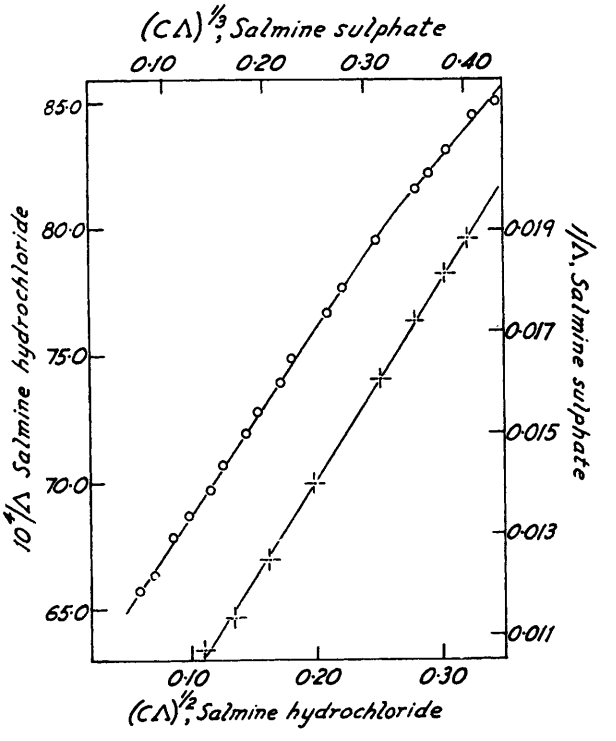

FIG. 3.

Conductivity data plotted according to Storch's equation.

○, Salmine hydrochloride;
⊹, salmine sulphate.

conductimetric experiment the acetate was formed from the hydrochloride ($C = 0{\cdot}001$) by titration with silver acetate; the conductivity change corresponded with the difference in mobilities of the chloride and acetate ions, so if ion-association occurs in the hydrochloride solutions it does so to the same extent in the acetate.

Below 0·0002N the hydrochloride curve is approximately linear with respect to $C^{\frac{1}{2}}$, and an extrapolation on this basis gives the rough value $\Lambda_0 = 163$. Gahl, Greenberg, and Schmidt (*Univ. Calif. Pub. Physiol.*, 1926, **5**, 307) and Hiyamoto and Schmidt (*J. Biol. Chem.*, 1933, **9**, 335) have found that satisfactory extrapolations are obtained with some salts of proteins when the data are plotted in accordance with Storch's equation (*Z. physikal. Chem.*, 1896, **19**, 13). A similar method is applied to our data in Fig. 3, where $1/\Lambda$ is plotted against $(\Lambda C)^{1/n}$, n having the value 2 for the hydrochloride and 3 for the sulphate. Good straight lines are obtained, leading to $\Lambda_0 = 164{\cdot}6$ for salmine hydrochloride and $\Lambda_0 = 167$ for the sulphate; the latter is rather less accurate owing to the longer extrapolation required for its determination.

If the mobilities of the chloride (76·3) and sulphate (80·0) ions are deducted, that of the fully ionised salmine cation becomes 88·3 from the hydrochloride, and 87 from the sulphate data. The agreement is satisfactory in view of the uncertainties of the extrapolations.

The existence of two end-points (Fig. 1) when basic salmine is titrated against hydrochloric acid is interesting. The effect was found with purified salmine from two different sources, and also with a pure sample kindly given by Dr. Tristram. The end-points are slightly sharpened by carrying out the titration in 0·1N-potassium chloride. The effect is not due to contamination by carbon dioxide, as other experiments with a column of Amberlite-400 have shown (Davies and Nancollas, *Nature*, 1950, **165**, 237). From the titration curves the ratio of hydrogen bound at the first point of inflection to that bound at the second was 0·86 in Expt. 5 and 0·84 in Expt. 6.

If there are 40 basic groups in salmine and all are neutralised at the second end-point, only 33 or 34 have reacted at the first point of inflection. This seems to show that in salmine there are 6 or 7 groups more weakly basic than the others. It has been suggested (Tristram, personal communication) that in salmine the hydroxyl groups of the six serine residues might influence the basic character of a similar number of guanidine groups. This suggestion must remain a tentative one, however, until the structure of salmine is known in greater detail.

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