

globule of water having a radius of equilibrium which is a function of the humidity of the air.

<sup>1</sup> McClelland, J. A. and Nolan, P. J., *Proc. Roy. Irish Acad.*, A 33, 1916 (29); Nolan, P. J., *Ibid.*, A 33, 1916 (10); McClelland, J. A. and Nolan, P. J., *Ibid.*, A 35, 1919 (1).

## THE RELATION BETWEEN THE ISOELECTRIC POINT OF A GLOBULIN AND ITS SOLUBILITY AND ACID COMBINING CAPACITY IN SALT SOLUTION

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Globulins are, by definition, soluble in solutions of the salts of strong acids and strong bases<sup>1</sup> and tuberin, the protein of the potato, has accordingly been classified as a globulin.<sup>2</sup> In the juice that can be squeezed from the potato, tuberin is dissolved on account of the high concentration of electrolytes.<sup>3</sup> It is precipitated by dialysis of the juice, and readily dissolves in solutions of neutral salts. The effect of neutral salts in precipitating tuberin is, however, variable with the hydrogen ion concentration. The nature of this variation, and to some extent its causes and its bearing on the classification of proteins, is considered in this paper.

The tuberin that was employed in these experiments was precipitated by dialyzing potato juice that had been freed from starch by filtration through pulp, in a collodion bag against tap water. After a period of days the tuberin had largely precipitated and settled. The contents of the collodion bag were then evenly suspended, transferred in 100 cc. samples to flasks and brought to different hydrogen ion concentrations by the addition of the amounts of sodium hydroxide and hydrochloric acid recorded in table I. The systems containing tuberin and acid, or tuberin and base in identical concentration were next divided, and added to vessels containing different amounts of sodium chloride. The systems, therefore, varied in two respects; in the first place in the amount of free, and, therefore, of combined protein, acid or base; in the second place in the amount of salt that they contained. After they had stood at constant temperature for about 6 hours, and it was believed that equilibrium had been established, the hydrogen ion concentrations of the systems were electrometrically determined, and the solubility of the tuberin in them was estimated by determining nitrogen in an aliquot part after the precipitated tuberin had been removed by centrifuging. In certain of the systems it will be noted that all of the protein dissolved. In these cases, therefore, the data do not represent solubility; that is solutions saturated with respect to protein.

The results obtained in this preliminary investigation were not, it is true, altogether regular. Abnormal values of nitrogen and also of hydro-

gen ion concentration appear in the data. None the less, certain conclusions can quite safely be drawn, the more so since they are borne out by several other experiments.

TABLE I  
EFFECT OF NaCl UPON SOLUBILITY AND UPON ACID COMBINING CAPACITY OF DIALYZED TUBERIN

N/10 HCl added to 100 cc. dialysed potato protein cc.	NaCl ADDED TO 100 CC. OF DIALYZED POTATO PROTEIN. VOLUME INCREASED BY ADDED ELECTROLYTES TO 120 CC.											
	0 gm.	0.25 gm.	0.5 gm.	1.0 gm.	2.0 gm.	5.0 mg.	0 gm.	0.25 gm.	0.5 gm.	1.0 gm.	2.0 gm.	5.0 gm.
	pH						Tuberin dissolved (mg. nitrogen in 100 cc.)					
50	1.58	1.58	1.58		1.56	1.46	5.96	5.79	5.79	5.79	5.79	5.79
20	2.67						5.70	5.70	5.45	5.11		
5	3.28	3.33	3.37	3.50	3.49	3.45	5.45	5.28	5.34			
3	3.93	3.97	4.02	4.09	4.03	4.13	4.77	5.28	4.17			
2	4.29	4.34	4.41	4.44		4.34	4.09	4.17	4.51	4.09		
1	5.22	5.13	5.13				3.40	3.83	4.09	4.43	4.68	
0	5.71	5.68	5.92	5.75	5.48	5.36	2.47	4.09	3.66	5.02		
NaOH.												
0.5	6.40	6.23	6.02	6.28	6.10	5.84	3.58	4.51	4.85	5.19		
1	6.95	6.76	6.78				6.52	4.98	4.17	4.77	5.96	5.19
3	8.99	8.77	8.71	8.77	7.95		5.87	5.02	5.21	5.70	5.87	
5	9.41	9.06	9.23	9.25	9.09	8.98	5.87	5.70	5.87	5.62	5.87	
10	10.39	10.09	10.03	10.26	9.72	9.83	5.96	6.04	5.79	5.70	5.87	5.79

#### I. THE EFFECT OF SODIUM CHLORIDE UPON THE SOLUBILITY OF TUBERIN

*Solubility of Tuberin in Salt Solution.*—The tuberin that had been precipitated by dialysis, in the way that has been described, must not be regarded as pure protein, but rather as a natural protein compound. It contained phosphorus and may have contained calcium. It was, however, largely redissolved by salt.

The solubility of the tuberin was increased by the addition of sodium chloride to the systems that were otherwise unchanged. It was increased more by sodium chloride in the systems which contained small amounts of sodium hydroxide. However, when a small amount of acid was added the power of sodium chloride to increase the solubility was greatly weakened. This "effect of minute quantities of acid on the solubility of a globulin in salt solutions" appears to be quite general. It was studied by Osborne and Campbell in 1897<sup>4</sup> and will be discussed later in this paper.

In our experiments, which were extended over a wider range of hydrogen ion concentration, the effect of sodium chloride steadily decreased as the acidity increased, until at hydrogen ion concentrations in the neighborhood of  $10^{-4} N$ , the solubility of the protein was nearly independent of the salt concentration.

*Solubility of Tuberin in Acid Solution.*—Not only was tuberin dissolved by salt solutions, in the absence of acids, but it was also dissolved by acid in the absence of salt. In solutions of lower hydrogen ion concentration than  $10^{-4}$  *N*, the dialyzed tuberin compound was only slightly soluble;\* less so than in salt solutions. At greater hydrogen ion concentrations than  $10^{-4}$  *N*, tuberin was largely dissolved in the systems to which no sodium chloride had been added. Its solubility in solutions of this degree of acidity was, however, decreased by sodium chloride. Indeed in the presence of sufficient salt the tuberin was nearly completely precipitated. The precipitation of many other globulins besides tuberin from acid solution by neutral salts has been observed. Osborne<sup>5</sup> studied the precipitation of edestin under these circumstances, and Hardy<sup>6</sup> that of *β*-globulin. This phenomenon is also apparently quite general.

*Solubility of Tuberin at pH 4.3 Independent of Salt.*—Between the systems in which sodium chloride decreased the solubility of tuberin and the nearly neutral ones in which sodium chloride increased solubility, there existed a narrow range where sodium chloride had very little effect upon the solubility of tuberin. This range coincided with a slightly higher hydrogen ion concentration than  $10^{-4}$  *N*.

These results are reported in table I. If represented graphically, the solubility of tuberin plotted as ordinates, and hydrogen ion concentration as abscissae, the curves representing the solubility of tuberin in the presence of different amounts of sodium chloride converge, and intersect each other within a narrow range of hydrogen ion concentrations, slightly lower than  $10^{-4}$  *N*, probably nearest to  $0.5 \times 10^{-4}$  (*pH* 4.3).

*The Isoelectric Point of Tuberin.*—In a previous investigation the method of cataphoresis was employed in determining the isoelectric points of the proteins of "Certain Vegetable Juices."<sup>3</sup> Potato juice was brought to different hydrogen ion concentrations and the migration of tuberin in an electric field determined. The direction of migration was found to change at a hydrogen ion concentration slightly lower than  $10^{-4}$  *N*. At greater acidities tuberin migrated to the cathode, at more nearly neutral reactions to the anode. At hydrogen ion concentrations slightly lower than  $10^{-4}$  *N*, tuberin migrated in neither direction. At this reaction, therefore, it was isoelectric.

*The Ionization of Tuberin.*—The ionization of a protein compound can be inferred from the direction of its migration during cataphoresis. In neutral potato juice and potato juice less acid than  $10^{-4}$  *N*, tuberin bore a negative charge. Presumably therefore tuberin as it existed in the juice of the potato was ionized as an acid. This compound decomposed on the addition of the stronger acid HCl, and tuberin was freed at its isoelectric point. In the neighborhood of its isoelectric point tuberin was, moreover, less ionized than at greater or at lesser acidities and was also

\* This requires further study.

combined with fewer of the electrolytes that existed in potato juice or were formed by the addition of acid to it. For this reason the flow of current during cataphoresis was greatest at the isoelectric point (3, p. 155). At hydrogen ion concentrations greater than the isoelectric point, where tuberin migrated to the cathode, and bore, therefore, a positive charge, it was evidently ionized as base and combined with acid.

*Solubility of Acid and of Basic Tuberin Compounds.*—The coincidence of the hydrogen ion concentration at which sodium chloride has least effect upon the solubility of tuberin with the isoelectric point allows of but one interpretation; sodium chloride affects tuberin differently as the migration of the protein varies. At hydrogen ion concentrations greater than  $10^{-4} N$ , where tuberin was ionized as base and combined with acid, sodium chloride decreased its solubility. When tuberin was least ionized, at its isoelectric point, sodium chloride had least effect upon its solubility. Especially when tuberin was ionized as acid did sodium chloride increase its solubility in the way that has led to its being classified as a globulin.

*Solubility of Other Globulins.*—This conception of the behavior of a globulin is not new. It was expounded by W. B. Hardy in his Croonian Lecture of 1905<sup>6</sup> on the basis of his studies of serum globulin.<sup>7</sup> Excepting in one point, which will presently be considered, his observations and conclusions agree with ours on tuberin. Neutral salts only increased the solubility of serum globulin at reactions more nearly neutral than its isoelectric point. At acidities greater than the isoelectric point, neutral salts precipitated serum globulin, as they did tuberin.

The earlier observations that have been mentioned indicate (1) that the solubility of many other globulins in salt solutions is decreased by small amounts of acid, and (2) that many globulins are precipitated from acid solution by small amounts of neutral salt. Up to the present the isoelectric points of most of these proteins have not been determined.\* The way in which they are ionized is, however, suggested by these criteria. This appears from a consideration of the effect of sodium chloride upon the dissociation of the compounds that tuberin forms at greater and at lesser acidities than correspond to its isoelectric point.

## II. THE EFFECT OF SODIUM CHLORIDE UPON THE ACID COMBINING CAPACITY OF TUBERIN

The chemical analysis of the effect of sodium chloride upon the ionization of the different tuberin compounds, whose solubility it differently affects, is carried one step further by the measurements of hydrogen ion concentration. The hydrogen ion concentration of a system containing protein and acid or protein and base is, as a first approximation, defined by the ratio of protein to acid, or protein to base,<sup>9,10,11</sup> and depends merely

\* Rona and Michaelis<sup>8</sup> give the isoelectric point of serum globulin as  $0.36 \times 10^{-8}$  and of edestin as  $1.3 \times 10^{-7}$ . These values depend in part, however, upon the points of maximum precipitation of the proteins.

on the ionization constant of the acid or base and the amphoteric constants of the protein. The effect of a neutral salt upon such systems has recently been exhaustively studied by Sørensen, Hoyrup, Hempel and Palitzsch.<sup>9</sup> It is illustrated by the data in table I. These are in substantial agreement with those recently published by Sørensen and his collaborators. They moreover derived an equation from the mass law which enabled them to calculate the hydrogen ion concentration of any ampholyte in the presence of an acid, and the salt of the acid and a strong base, if the dissociation constants of ampholyte, acid, and salt, and the concentrations of the components of the systems were known. In this equation "the influence of the salt only appears by the fact that the values of" "the degree of dissociation of the acid" and "of the salt of the acid together with the ampholyte" "vary with the concentration of the salt." (9, pp. 93 and 101.) In other words, the effect of a neutral salt with a common ion is to depress the dissociation of the acid, and of the salt of the acid with the protein. As a result the acid combining capacity of the protein is increased, and the hydrogen ion concentration decreased. If the protein is ionized as acid on the other side of its isoelectric point and is combined with base, the depression in the dissociation of the base, and of the salt of the protein and the base, causes the hydrogen ion concentration to increase. The effect of salt in depressing the dissociation of the protein compound, however ionized, thus causes the hydrogen ion concentration of protein systems to converge upon the isoelectric point of the protein. As a result Sørensen has concluded:

"at hydrogen-ion concentrations which are not situated at or in the neighborhood of the isoelectric point of the ampholyte the capacity to combine with acids

"(a) is independent of the ampholyte concentration;

"(b) increases with rising concentration of salt;

"(c) is positive at hydrogen-ion concentrations superior to that corresponding with the isoelectric reaction of the ampholyte, and

"(d) is negative—that is, the ampholyte is combined with surplus base—at hydrogen-ion concentrations inferior to that corresponding with the isoelectric point of the ampholyte.

"At hydrogen-ion concentrations corresponding with or situated in the neighborhood of the isoelectric point of the ampholyte, the conditions are not easily discernible; the capacity to combine with acids is in this case not independent of the concentration of the ampholyte, but is the greater the smaller the latter is." (9, p. 161.)

Whenever complicating colloidal phenomena do not interfere, therefore, the mass law demands that a neutral salt increase the hydrogen ion concentration of systems containing protein when ionized as acid, and decrease the hydrogen ion concentration when ionized as base. Salt should, accordingly, have least effect when the hydrogen ion concentration of the

system coincides with the isoelectric point of the protein. Sørensen's studies, therefore, add another property—acid combining capacity—in which the singular point in protein behavior coincides with the isoelectric point, and suggests another method which, in the absence of complicating phenomena, may be used to determine the isoelectric point.

The only apparent effects of sodium chloride upon the hydrogen ion concentration of systems containing tuberin and sodium hydroxide, or tuberin and hydrochloric acid, follow from this theory.\* When sodium chloride was added to such systems the acid binding capacity was increased, and the hydrogen ion concentration decreased, if tuberin was ionized as base at acidities greater than the isoelectric point. The hydrogen ion concentration was increased at more nearly neutral reactions. The hydrogen ion concentration of the system whose acid binding capacity was least affected by the addition of sodium chloride was slightly lower than  $10^{-4} N$ , and, therefore, in the neighborhood of the isoelectric point of tuberin.

*Dissociation of Tuberin in Salt Solution.*—The increased hydrogen ion concentration of tuberin systems less acid than  $pH$  4.3 to which sodium chloride was added shows that the dissociation of the tuberin compound was depressed, and that as a result tuberin combined with surplus base (the amount of base bound by tuberin, and by depression of the dissociation of all electrolytes being measured by the increase in free acid). We have seen that under these circumstances the solubility of tuberin increased.\*\*

Tuberin also combined with base in the systems to which sodium hydroxide was added. An increase in solubility also followed the formation of this tuberin compound, which may have been similar to that formed by sodium chloride. Tuberin never dissolved as completely, however, in neutral solutions to which no sodium chloride was added. Apparently this compound of tuberin is more soluble when little dissociated. That its dissociation products—like those of many other globulins which appear upon dialysis or dilution of their solutions—are very insoluble, is certain.

Small amounts of hydrochloric acid decomposed the compounds that were formed by the more weakly ionized acid, tuberin, at reactions less acid than its isoelectric point. We have seen that sodium chloride and sodium hydroxide favored the formation of this tuberin compound, and, therefore, increased its solubility. By decomposing it hydrochloric acid decreased the solubility of tuberin in salt solutions.

The observations of Osborne, that have already been referred to, indicate that the decreased solubility of many other globulins in salt solutions, to which small amounts of acid had been added, resulted from their acid

\* It is immaterial whether the change of hydrogen ion concentration caused or was caused by the change in state of the protein.

\*\* The tuberin preparation was probably not a simple sodium compound. It contained phosphorus, and may have contained calcium. The increase in solubility may, therefore, in part, have been associated with change in the protein compound.

isoelectric points, and the decomposition of their compounds with bases. If the isoelectric points of these globulins had not been acid, but had occurred at an alkaline reaction, their acid compounds would have been decomposed by bases, and as a result they would probably have been rendered less soluble by bases rather than by acids.

*Dissociation of Tuberin in Acid Solution.*—In the presence of hydrogen ion concentrations greater than the isoelectric point, tuberin ionized as a base and dissolved as an acid compound. The effect of sodium chloride in precipitating tuberin from such solutions is perhaps less clear. Hardy studied the identical effect upon serum globulin. He believed that proteins formed addition products with neutral salts through their amino groups, and that under these circumstances neutral salts displaced acid from combination with protein. If that were so the acidity of such systems would increase. We have seen that in the case of tuberin quite the contrary occurs. Tuberin chloride binds more rather than less acid in the presence of sodium chloride. For the present, therefore, it is simpler merely to assume that tuberin chloride is little soluble when little dissociated.

*Classification of Tuberin as a Globulin.*—The classification of tuberin as a globulin depends, as our observations indicate, upon the solubility in salt solutions of the compounds that tuberin forms when ionized as an acid, and upon the insolubility of the products of the hydrolytic dissociation of these same compounds. It would appear from Osborne's observations upon the behavior of many other globulins toward small amounts of acid, that they too have acid isoelectric points, and are, therefore, ionized as acids in neutral salt solutions. If the isoelectric points of these proteins had been alkaline, their solubility in salt solution would have been decreased by bases rather than by acids. But whether the relations that have been described are characteristic of all globulins, or merely of tuberin, it appears from them how intimately the classification of proteins according to solubility involves their isoelectric points.

I am greatly indebted to the Director of the Harriman Research Laboratory, the Roosevelt Hospital, New York, for courteously extending to me the facilities of his laboratory after the research that I had been conducting there for the Surgeon General's Office was concluded. This enabled me to consider these more theoretical aspects of the earlier problem.

<sup>1</sup> Recommendations of the Committee on Protein Nomenclature, *Amer. J. Physiol.*, **21**, 1908 (27).

<sup>2</sup> Osborne, T. B. and Campbell, G. F., *J. Amer. Chem. Soc.*, **18**, 1896 (575).

<sup>3</sup> Cohn, E. J., Gross, J. and Johnson, O. C., *J. Gen. Physiol.*, **1919**, II, (145).

<sup>4</sup> Osborne, T. B. and Campbell, G. F., *J. Amer. Chem. Soc.*, **19**, 1897 (482-487).

<sup>5</sup> Osborne, T. B., *Ibid.*, **24**, (28-39).

<sup>6</sup> Hardy, W. B., *Proc. Roy. Soc., London (B)*, **79**, 1907 (413-426).

<sup>7</sup> Hardy, W. B., *J. Physiol.*, **33**, 1905-6 (251-337).

<sup>8</sup> Rona, P. and Michaelis, L., *Biochem. Zs.*, **28**, 1910 (193-199).

<sup>9</sup> Sørensen, S. P. L., Hoyrup, M., Hempel, J. and Palitzsch, S., *C. R. trav. Lab. Carlsberg*, **12**, 1917.

<sup>10</sup> Henderson, L. J., Cohn, E. J., Cathcart, P. H., Wachman, J. D. and Fenn, W. O., *J. Gen. Physiol.*, **1**, 1918-19 (459).

<sup>11</sup> Loeb, J., *Ibid.*, **1**, 1918-19 (237).

THE CARBON DIOXIDE: HEAT RATIO IN CATTLE

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It was recently suggested to us by Dr. F. G. Benedict that if the ratio of the carbon dioxide excreted to the heat produced by cattle should prove to be sufficiently constant, this fact might be utilized as the basis for determinations of the energy metabolism of this species which would be approximately correct and which would require only a comparatively simple technique. In view of the great value which such a simplified method would have, especially for agricultural investigators, and of the further fact that a new form of respiration apparatus designed to be used in this manner has been constructed by Benedict in coöperation with the New Hampshire Agricultural Experiment Station, we have thought it worth while to study the results of our calorimetric experiments on cattle from this point of view and we present the results of that study herewith.<sup>1</sup>

These experiments were made with the Atwater-Rosa respiration calorimeter which has been in use here since 1901, the heat production as well as the elimination of carbon dioxide being measured, and are, so far as we are aware, the only direct determinations of the heat production of this species. The measured heat production has been compared with the carbon dioxide elimination in 188 24-hour periods.<sup>2</sup> The carbon dioxide included, of course, the considerable amount arising from the methane fermentation in the rumen. The following results were obtained for the heat expressed in kilogram calories divided by the carbon dioxide expressed in grams.

CARBON DIOXIDE : HEAT RATIO OF CATTLE

Range.....	2.1500 to 3.1500
Mean value.....	2.4947 ± 0.0085
Standard deviation.....	0.1713 ± 0.0060
Median.....	2.4938
Theoretical mode.....	2.4974
Error of single variate.....	± 0.12
Coefficient of variability.....	6.87%

In 51 of these periods we have also data for the carbon dioxide and heat production of the animals in the standing and lying positions respec-