and decomposes at about 165°. The same or a similar change takes place much more rapidly when the crude product is left exposed to the air, more than half of it becoming insoluble in alkalies in the course of a few days. Acetone solutions rapidly reduce permanganate and here also the product is a white powder which is insoluble in alkalies and which decomposes at about 165°. The amount of permanganate that was reduced varied slightly in different experiments but always indicated that two atoms of oxygen were involved in the process. All attempts to get the exceedingly insoluble white product in a form that would give consistent analytical results were unsuccessful.

Ring-opening.—The foregoing account deals with transformations of the orthoxazine derivative in which the ring is not involved. In all these operations there are indications that the ring does not completely escape. Direct experiments showed that the ring can be opened by digesting the substances with alcoholic solutions of acids and bases, and even by protracted boiling with Grignard reagents. The product in all of these cases is the deep yellow furan ketone (XIII).

Summary

1. The paper gives an account of the transformations of two orthoxazine derivatives which have an hydroxyl or an oxo group in the 5 position.

2. One of these is relatively stable, the other extremely unstable. It is shown that the stability of orthoxazine derivatives, like that of other heterocyclic compounds depends upon the number, character and distribution of the groups in combination with the atoms constituting the ring.

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THE ISO-ELECTRIC POINTS OF VARIOUS PROTEINS

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Having samples of many vegetable proteins,[1] most of which had been prepared in this Laboratory in connection with other studies, it was thought that an excellent opportunity was offered for making a comparative study of these proteins with respect to one of their most interesting physicochemical properties, namely, their iso-electric points. Aside from obtaining information regarding the iso-electric points of the individual proteins, such a study, in which the work was done by the same workers using the same method, and under constant experimental conditions, would

[1] For the sources and preparation of the proteins used in this study see Jones, Gersdorff and Moeller, *J. Biol. Chem.*, **62**, 183 (1924).

give data which might well serve as a basis for correlating them, both with other known properties of the same proteins and with proteins of the same or of a different class.

Among the chemical methods already in use for determining the iso-electric points of proteins, based on the principle of solubility, probably the most widely applied is that of Michaelis[2] or some modification of this method. In this method, a solution of the protein is added to a series of buffer solutions of various hydrogen-ion concentrations. The Sörensen value (*P*H) of the buffer producing the largest precipitate is considered the iso-electric point of that protein, since its solubility is at a minimum at that point.

Objection may be raised to the use of alkali for dissolving the protein on the ground that the character of the protein may thus be changed. Furthermore, it is somewhat difficult to decide in which tube the greatest precipitation occurs. In the method here described we have tried to overcome these difficulties by producing a solution of the protein in buffers directly, and precipitating the protein in the clear filtrate with tungstic acid. The Sörensen value of the buffer in which was produced the least turbidity is taken as the iso-electric point of the protein. We are aware that the method employed may not be entirely free from objections. However, it was not our purpose in the work described in this paper to make a critical study of methods, but to employ a convenient method which would give us results sufficiently accurate for the comparative study of the proteins undertaken. In this connection it is of interest to note the close agreement between the results we have obtained and those which others have obtained for the same proteins by using different methods.

Method

The details of the method used follow.

1. Approximately 0.1 g. (roughly measured) of protein is placed in each of 14 test-tubes.

2. To each tube is added about 10 cc. of the appropriate buffer solution, making a series ranging from *P*H 4.0 to *P*H 6.8. The tubes are then shaken several times during the course of an hour.

3. The samples are filtered clear through small dry filter papers (double, to insure a clear filtrate), and 2 cc. of each filtrate is measured into a test-tube; 0.5 cc. of 10% sodium tungstate solution is added to each tube and then enough 0.667 *N* sulfuric acid to bring the final Sörensen value in each sample to 2.6, the point of maximum precipitation.[3,4]

The tube showing the faintest turbidity is chosen, and the Sörensen value of the buffer used is taken to represent the iso-electric point of the protein. The buffer

[2] Michaelis and Davidson, *Biochem. Z.*, **30**, 144 (1910). Michaelis and Pechstein, *ibid.*, **47**, 260 (1912).

[3] Folin and Wu, *J. Biol. Chem.*, **38**, 81 (1919).

[4] Merrill, *J. Biol. Chem.*, **60**, 257 (1924).

solutions used were Sörensen's phosphate mixture and Walpole's acetate buffer.[5] The former covers the range *P*H 3.5–4.9, and the latter *P*H 5.2–6.9, at intervals of about 0.2 *P*H, thus limiting the inaccuracy of our method to less than 0.2 *P*H.

As a check on the first determinations, the residues were re-extracted with fresh portions of the same buffers, and the tests repeated as described above. Invariably the same values were obtained in both cases, although a marked decrease in solubility of the protein was noted. This might be due to the denaturing effect of the buffer.

The following experiment makes this explanation appear probable. A sample of protein was extracted with a buffer having *P*H 4.9 for one hour. The residue was then extracted again for one hour with a buffer having *P*H 3.8. A second sample was then extracted directly with a buffer having *P*H 3.8. Two-cc. portions of each of the filtrates were taken, and the tungstic acid reagent was added. The ratio of turbidities of the solutions was approximately 1:10:50, respectively. Now, if there had been no denaturation of the protein as a result of the first extraction, the quantity of protein dissolved by the double extraction should have been equal to or greater than that from the single extraction with buffer *P*H 3.8.

Experimental Data

In the first column of Table I are given the values obtained by our method. In the second column are included the iso-electric points of a few well-known proteins as determined by other investigators. The glutelins have not been included in this study because their chemical individuality and their method of preparation are not satisfactorily established, and their number is, in any case, too small to permit drawing general conclusions.

Table I

Iso-Electric Points of Proteins

	*P*H	*P*H
Albumins		
Lactalbumin	4.55	...
Ovalbumin	..	4.8[6,7]
Serum albumin of dog, rabbit, guinea pig, oxen, turtle and man	..	4.7[8]
Albumin from Georgia velvet bean	4.2	...
Albumin from Jack bean	4.2	...
Albumin from locust bark	5.5	...
Albumin from wheat bran	4.2	...
Globulins		
Stizolobin from Chinese velvet bean	5.35	...
α-Globulin from Georgia velvet bean	5.5	...
β-Globulin from Georgia velvet bean	5.35	...
α-Globulin from cottonseed	5.5	...
β-Globulin from cottonseed	5.35	...

[5] Clark, "The Determination of Hydrogen Ions, Etc.," Williams & Wilkins Co., Ed. 2, **1922**.

[6] Sörensen, *Compt. rend. trav. lab. Carlsberg*, **12,** 149 (1915–1917).

[7] Michaelis, "Die Wasserstoffionen Konzentration," J. Springer, Berlin, **1914**.

[8] Michaelis and Nakashima, *Biochem. Z.*, **143,** 484 (1923).

Table I (*Concluded*)

	PH	PH
Globulin from locust bark	5.5	...
Conphaseolin from navy bean	5.2	...
Phaseolin from navy bean	4.5	...
α-Globulin from adsuki bean	5.2	...
β-Globulin from adsuki bean	4.7	...
Canavalin from Jack bean	5.2	...
Concanavalin from Jack bean	5.5	...
Glycinin from soy bean	4.7	...
α-Globulin from lima bean	5.5	...
β-Globulin from lima bean	5.2	...
Legumin from pea	5.35	...
Vicilin from pea	5.2	...
Vicilin from lentil	4.7	...
Globulin from squash	5.35	...
α-Globulin from tomato seed	5.2	...
β-Globulin from tomato seed	4.9	...
Globulin from cantaloupe seed	5.5	...
Globulin from walnut	5.2	...
Globulin from coconut	5.2	...
Globulin from wheat bran	5.5	...
Globulin from almond	5.5	...
Globulin from flaxseed	5.35	...
Globulin from filbert	5.5	...
Arachin from peanut	5.35	...
Conarachin from peanut	4.9	...
Edestin from hemp seed	..	5.6[9]
Serum globulin of horse, sheep, man, guinea pig	..	5.44[10]
Prolamins		
Gliadin from wheat	6.45	6.5[11]
Prolamin from wheat bran	6.2	...
Gliadin from rye	6.6	...
Zein from corn	6.2	...
Kafirin from kafir	5.9	...
Miscellaneous		
Casein	4.85	4.74[10]
Hemoglobin	..	6.77[12]
Hemoglobin	..	6.78[13]
Gelatin	..	4.7[14]
Gelatin	..	4.7 and 7.7[15]

[9] Michaelis and Mendelssohn, *Biochem. Z.*, **65**, 1 (1914).
[10] Rona and Michaelis, *ibid.*, **28**, 193 (1910).
[11] Tague, This Journal, **47**, 418 (1925).
[12] Michaelis and Bien, *Biochem. Z.*, **67**, 198 (1914).
[13] Ferry, *J. Biol. Chem.*, **57**, 819 (1923).
[14] Michaelis and Grineff, *Biochem. Z.*, **41**, 373 (1912).
[15] Wilson and Kern, This Journal, **44**, 2633 (1922).

Discussion

The most striking feature of the results given in Table I is the close agreement among the iso-electric points of proteins of any one group. Thus, albumins range in general from *P*H 4 to *P*H 5, globulins from *P*H 5 to *P*H 5.5, and prolamins from *P*H 6 to *P*H 6.5. A classification based on iso-electric points would group these proteins in the same way that they are grouped in the classification in use at present. There are exceptions, however, such as the locust bark albumin.[16] Also, among the globulins we found a few having iso-electric points on the border line. We have no conclusive evidence, however, that they are all chemically individual proteins. Evidence has been obtained indicating that glycinin from the soy bean consists of a mixture of at least two globulins. An account of this work will be reported later. It was found that without exception the α-globulins, which precipitate with ammonium sulfate before the β-globulins, have the higher iso-electric points.

There is, it would seem, some relation between the iso-electric point of a protein and its precipitation with ammonium sulfate. Thus, the most soluble proteins, albumins, have the lowest iso-electric points; the less soluble, α-globulins, precipitate with ammonium sulfate at a low saturation and have higher iso-electric points than do the albumins. This relation is also shown in the case of the locust bark albumin. This albumin, which is precipitated by ammonium sulfate at a lower concentration of this salt than are most albumins, was found to have an iso-electric point well above that of the other albumins studied. Thinking that ammonium sulfate might cause precipitation by influencing the hydrogen-ion concentration of the solution, we prepared solutions of this salt of various concentrations, and then tested their hydrogen-ion concentrations. The variations between 0.2 of saturation and complete saturation was only 0.3 *P*H, a magnitude entirely too small to account for the differences found between the iso-electric points of albumins and globulins.

In each experiment an approximate solubility curve was drawn from observation, judging from the turbidity produced by the tungstate reagent. Albumins were found to be more soluble in the buffers used on the alkaline side of their iso-electric points, while globulins, on the other hand, showed greater solubility on the acid side.

The iso-electric point found for casein (*P*H 4.85) agrees fairly closely with the higher figures found in the literature for this protein. The lower values in the literature are around *P*H 4.6. During the progress of our work a paper was published by Tague,[11] who determined the iso-electric points of wheat gliadin and glutenin by measuring electrochemically the

[16] The method of preparation, and properties of this protein, are described in a recent publication from this Laboratory. [Jones, Gersdorff and Moeller, *J. Biol. Chem.*, **64**, 655 (1925).]

changes of the buffer solutions. His figure for gliadin is in close agreement with ours.

The method used in our work is inapplicable to proteins that are very soluble, such as gelatin and egg albumin, or to those that have but small solubility in the buffer used, as we found to be the case with edestin and zein. To overcome the difficulty caused by the low solubility of zein in our buffer, this protein was first dissolved in dil. alcohol and then added to the buffer. In the case of edestin, a buffer would be required in which this protein is more soluble than it is in those used in the work here described. The principle of the method does not depend on the buffer used.

Summary

The iso-electric points of a large number of proteins have been determined by shaking buffers having a *P*H range from 4.0 to 6.8, with an excess of the protein investigated, and precipitating the dissolved protein in the clear filtrate with tungstic acid. The Sörensen value of the buffer filtrate in which was produced the least turbidity is taken as the iso-electric point of the protein.

The results obtained show that those proteins having the greater solubility in aqueous solutions have the lower iso-electric points, and that a correlation exists between the iso-electric points of proteins of the same group. The iso-electric points of albumins were found to range in general from *P*H 4 to *P*H 5, those of globulins from *P*H 5 to *P*H 5.5, and those of prolamins from *P*H 6 to *P*H 6.5.

Proteins requiring the greater quantity of ammonium sulfate for their precipitation from saline solutions have lower iso-electric points than proteins which require less ammonium sulfate for their precipitation. Thus the β-globulins have lower iso-electric points than the α-globulins.

Albumins were found to be more soluble, in the buffers used, on the alkaline side of their iso-electric points, while globulins, on the other hand, showed greater solubility on the acid side.

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