from the primary and secondary halides gives addition and reduction products; with the *tert.*-butylmagnesium chloride only reduction occurred.

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THE ISOELECTRIC POINT OF CRYSTALLINE UREASE[1]

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Introduction

In connection with our study of the properties of crystalline urease[1a] we have been especially interested in finding the isoelectric point inasmuch as it has never been determined for an isolated enzyme. We thought that a knowledge of this value would help to classify urease among proteins, would aid in improving the method of purification and might eventually throw light on the mode of action of the enzyme on urea.

References to the isoelectric points of enzymes are not lacking in the literature and some of these references are contradictory. Some enzymes have been stated to function as ions over the P_H range at which they are active; other enzymes have been found to have isoelectric points coinciding with the P_H of their optimum activity and consequently they are thought to react best when not ionized. Thus, pepsin was found by Michaelis and Davidsohn[2] to have an isoelectric point at P_H 2.26. These authors state that pepsin must be positively charged to have a proteolytic effect. Northrop,[3] however, from a study of the distribution of pepsin and chloride and bromide ions between solid gelatin or coagulated egg albumin, concludes that pepsin is a monovalent ion from P_H 1 to 7. Michaelis[4] states that with invertase the uncharged particles are active, that with trypsin, erepsin, lipase and maltase the anions are the active part, while with pepsin the cation is active. Michaelis and Pechstein[5] have found the isoelectric point of liver catalase to be at P_H 5.37 and believe that the anions and uncharged particles are active, while the cations are not. Northrop[6] thinks it probable that the isoelec-

[1] The work reported in this paper was supported by a grant from the Heckscher Foundation for the Advancement of Research established by August Heckscher at Cornell University.

[1a] J. B. Sumner, *J. Biol. Chem.*, **69**, 435 (1926); **70**, 97 (1926); J. B. Sumner and D. B. Hand, *ibid.*, **76**, 149 (1928); *Naturwissenschaften*, **16**, 9, 145 (1928); J. B. Sumner and R. G. Holloway, *J. Biol. Chem.*, **79**, 489 (1928).

[2] L. Michaelis and H. Davidsohn, *Biochem. Z.*, **28**, 1 (1910).

[3] J. H. Northrop, *J. Gen. Physiol.*, **7**, 603 (1924–1925).

[4] L. Michaelis, *Biochem. Z.*, **60**, 91 (1914).

[5] L. Michaelis and H. Pechstein, *ibid.*, **53**, 320 (1913).

[6] J. H. Northrop, *J. Gen. Physiol.*, **6**, 337 (1923–1924).

tric point of trypsin is at P_H 10.2 and he finds trypsin to exist as a positive monovalent ion from P_H 10 to 2. On the other hand, Sherman, Thomas and Caldwell[7] have reported that highly purified malt amylase is isoelectric at P_H 4.3 to 4.5, which coincides with the P_H optimum for the action of this enzyme on starch.

Probably most of the enzyme preparations that have been employed to determine isoelectric points were far too impure to yield significant results. The presence of foreign proteins and other colloids must surely mask the ionic behavior of enzymes. It is of interest to note that in 1911 Pekelharing and Ringer[8] found that highly purified pepsin, which had a minimal solubility at P_H 3.3, showed no direction of migration under the influence of an electric current unless albumin, or albumose, were added, when the enzyme assumed the electrical properties of the added substance. The apparent purity of crystalline urease suggests that its isoelectric point may be determined accurately.

Electrophoresis Experiments.—The first method employed with crystalline urease was that of electrophoresis. We used the Sherman, Thomas and Caldwell[7] modification of the apparatus of Michaelis.[9]

The urease was prepared by methods already described and when obtained from meal low in enzyme activity was recrystallized. The media were phosphate, phthalate and acetate buffers, prepared from reagents which had been carefully purified. Cataphoresis experiments were run for periods varying from two to twenty-two hours, using a direct current of 110 volts. Various non-polarizable electrodes were tried in some cases and in others non-polarizable electrodes were not used. The amount of migration was tested by making determinations of the urease activity in all three chambers of the U-tube.

Our results showed that from P_H 7 to about 5.5 the urease migrated toward the anode, but in solutions more acid than this very little migration occurred. Since urease is a globulin, the lack of migration in the vicinity of what we later showed to be the isoelectric point is probably due to the presence of salts. This property of globulins of being dissolved without electric charge by salts has already been reported.[10] We would have repeated the migration experiments, using urease solutions containing minimal amounts of buffer, but at present we are unable to procure satisfactory jack bean meal.

Precipitation by Phthalate and Lead Ions.—In the electrophoresis experiments the addition of phthalate buffer to urease caused a precipitation at P_H 5 to 4. Since determination of the isoelectric point by migration had been unsatisfactory, we decided to find the P_H of maximum

[7] H. C. Sherman, A. W. Thomas and M. L. Caldwell, THIS JOURNAL, **46**, 1711 (1924).

[8] C. A. Pekelharing and W. E. Ringer, *Z. physiol. Chem.*, **75**, 282 (1911).

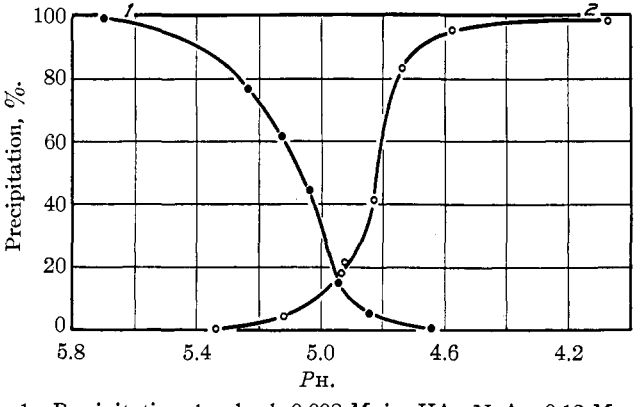
[9] L. Michaelis, *Biochem. Z.*, **16**, 81 (1909).

[10] H. Chick, *Biochem. J.*, **7**, 318 (1913).

precipitation by phthalate buffer, expecting that this P_H could be taken as the isoelectric point.

Experiments were conducted by adding 25 cc. of 0.04 *M* phthalate buffer, made up at different P_H values, to 25 cc. of crystalline urease solution, containing 2500 to 3500 units of urease, mixing and allowing to stand in the ice chest for one hour. The material was then centrifuged until the supernatant liquid was water-clear. The centrifuging was continued for intervals of ten minutes with cooling in the ice chest before centrifuging again. In some cases it was necessary to centrifuge for a total of fifty minutes. The supernatant liquid was poured off as completely as possible, and in those cases where either very little, or nearly all of the urease had been precipitated, the centrifuge tube was freed of the last drop of supernatant fluid by a filter paper.

Both supernatant liquid and precipitate were analyzed separately for urease activity. The precipitate was dissolved in dilute neutral phosphate buffer before analyzing. The P_H of the supernatant liquid was determined by the quinhydrone electrode. In these experiments very little of the urease was destroyed when the reaction was near P_H 5, but at P_H 4.07 the amount destroyed in one case was 21%. After determination of urease activity and P_H, the remainder of the material was analyzed for total nitrogen by the macro-Kjeldahl method, using a refined technique suitable for small amounts of material.



1, Precipitation by lead 0.008 *M* in HAc–NaAc 0.12 *M*; 2, precipitation by phthalate, 0.02 *M*.

Fig. 1.

The analyses demonstrated that precipitation of urease by phthalate is parallel to the precipitation of the protein and that no significant separation of urease from protein takes place. A curve was constructed, showing the percentage of total urease precipitated with change of P_H. We had expected to find a point of maximum precipitation but there was none, as will be seen from Fig. 1. The curve shows that urease begins to precipitate at P_H 5.3 and is almost all precipitated at P_H 4.6.

Acetate buffer (Walpole's[11] 0.2 *M*) was substituted for the phthalate

[11] W. M. Clark, "The Determination of Hydrogen Ions," The Williams and Wilkins Co., Baltimore, Maryland, 1927.

buffer in order to study the precipitation of urease in more acid solutions but we were surprised to find the urease to be completely soluble in acetate buffer from P_H 3.8 to neutrality. We concluded that the precipitation by phthalate is not a P_H effect solely, but is caused by phthalate ions which unite with positively charged urease.

On account of the results with phthalate we decided to investigate the precipitation of urease with positively charged ions. For this purpose we employed lead acetate, since we had previously observed that lead acetate precipitates urease without much destruction and the insoluble precipitate actively decomposes urea.

The precipitation was carried out by adding to 5 cc. of phosphate-free urease solution, containing about 500 units, 10 cc. of 0.2 *M* acetate buffer, followed by 4 drops of 25.5% lead acetate. The lead acetate was Kahlbaum's and had been recrystallized three times. The material was mixed and allowed to stand for an hour in the ice chest; then it was centrifuged and the urease activity determined as was done with the phthalate precipitation. A few experiments were run using larger amounts of enzyme, and analyses for total nitrogen showed that the urease accompanied the protein in its precipitation. Treatment with lead was found to have very little destructive action on urease in the region of P_H 5, but in more acid media some destruction occurred. However, even when the addition of lead acetate did not inactivate the urease, centrifuging down the lead precipitate did cause partial inactivation. The way in which the lead precipitate was redissolved apparently made a difference. Here the addition of gum arabic had a favorable effect. In no case in our experiments was more than 25% of the urease inactivated.

A curve was constructed showing the percentage of total urease precipitated with changing P_H. As will be seen from Table I, urease begins to precipitate under these conditions at P_H 4.7 and is almost completely precipitated at P_H 5.6. The curves of precipitation of urease by phthalate and by lead clearly show the amphoteric nature of crystalline urease. On the acid side of P_H 5 the colloidal particles bear a positive charge and are precipitated by negative ions such as phthalate. On the alkaline side of P_H 5 the particles are negative and are precipitated by the lead ions. The two curves intersect at P_H 4.95 and this value we thought might be taken as the isoelectric point. However, further experiments with lead precipitation have shown that the concentration of lead ions and of acetate buffer have a decided effect. Increasing the amount of lead acetate added was found to push the precipitation more toward the acid side, while increasing the concentration of the acetate buffer dissolved the lead–urease precipitate, or prevented its appearance. Hence it is evident that the accuracy of the isoelectric point established by these two curves can be questioned. The curves do give the isoelectric point approximately and they illustrate the precipitation of urease by certain electrolytes without destruction.

Mercuric and cupric ions precipitate urease over a much wider P_H range than do lead ions and unlike lead cause complete inactivation if

present in sufficient concentration. Here a portion of the urease can be reactivated by buffered hydrogen sulfide, but all of the urease cannot be reactivated.

Solubility Relationships.—As a result of further experiments we are able to summarize the solubility relationships of urease very simply. Urease is completely insoluble on the alkaline side of the isoelectric point in excess of lead acetate and on the acid side in excess of phthalate buffer. Urease is soluble in excess of phosphate buffer, acetate buffer and carbonic acid–bicarbonate buffer at all P_H values, but is partly precipitated by these reagents over a narrow P_H zone, provided a minimal amount of buffer is used. This range lies between P_H 4.8 and 5.3. On adding more of the buffer the precipitate immediately redissolves. Hence it is necessary to distinguish between the precipitating action of certain ions, such as lead and phthalate, and that of certain buffers which in small amount are able to precipitate urease by P_H effect. However, these buffers have a solvent action and if present in sufficient quantity are able to dissolve urease at any P_H. These findings confirm our previous statement that urease is a globulin.

Determination of Minimal Solubility.—Having an understanding of the solubility relationships of urease, it proved a simple matter to determine the isoelectric point by finding the minimal solubility in greatly diluted acetate buffer.

For this purpose 1-cc. portions of urease solution, containing about 150 units of enzyme, were pipetted into pyrex test-tubes and to each tube was added 2 drops of 0.2 *M* acetate buffer of various P_H values. It was readily apparent that the heaviest precipitation occurred in tubes which were at P_H 5.0 and 5.1, as was afterwards shown by testing with the quinhydrone electrode. Repetition of the experiment gave the same result. In a few instances the urease precipitate was centrifuged off and analyzed for its activity. The urease was found to be about 50% precipitated at P_H 5.0 to 5.1. Below P_H 4.8 and above P_H 5.3 no precipitate was produced. We were able also to precipitate urease by bubbling carbon dioxide through its solution. As nearly as could be found maximum precipitation by carbonic acid occurred at P_H 5.0 to 5.1.

TABLE I

PRECIPITATION OF UREASE BY ACETATE BUFFER

Experiment 1		Experiment 2	
P_H	Precipitate	P_H	Precipitate
4.82	None	4.80	Faint
4.91	Moderate	4.88	Moderate
5.00	Heavy	4.98	Heavy
5.10	Heavy	5.10	Heavy
5.20	Moderate	5.18	Faint
5.30	Very faint	5.30	None

Accordingly we report the isoelectric point of crystalline urease as determined by the P_H of minimum solubility to be at P_H 5.0 to 5.1. The greatest possibility of error is dependent upon the purity of the urease

employed. Our material is admitted not to be as pure as that which might have been prepared by several recrystallizations, starting with crystals obtained from jack bean meal of highest activity.

The isoelectric point is thus shown to be far to the acid side of the P_H of optimum activity. We have previously found this for crystalline urease to be at P_H 7.0. Urease is active on both sides of the isoelectric point with no sudden change in activity in passing from one side to the other. Indeed, the enzyme is still active in solutions as acid as P_H 3.8, although it is being steadily destroyed at this acidity.

Summary

1. Crystalline urease undergoes electrophoresis toward the anode in solutions of buffer varying from neutrality to about P_H 5.5. In buffer more acid than 5.5 no migration could be confirmed.

2. Urease begins to be precipitated by potassium acid phthalate at P_H 5.3 and the precipitation is almost complete at P_H 4.6.

3. Urease begins to be precipitated by buffered lead acetate at P_H 4.7 and the precipitation is almost complete at P_H 5.6. The regions for precipitation by phthalate and lead overlap at P_H 4.95, where 20% of the urease is precipitated by either ion. Urease is precipitated by mercuric and cupric ions over a much wider range than for lead ions.

4. Dilute electrolytes have three effects on the solubility of urease: a precipitation by certain ions such as phthalate and lead, over a certain range of P_H; a solvent effect on urease; a precipitating action where the dilute salts bring the solution to the P_H of the isoelectric point of urease.

5. The isoelectric point of urease as determined by the point of minimum solubility in dilute acetate buffer is at P_H 5.0 to 5.1. This point is far to the acid side of the P_H of optimum activity for urease. Urease is active on both sides of its isoelectric point.

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