controversial. Recent statements by Pease[8] and Daniels[9] have shown that some investigators are far from satisfied with the present theories of quasi-unimolecular mechanisms. Reactions which throw light on this situation are still of great value, and the present series of experiments seems to have definite possibilities in this connection. The effect of pressure on rate has been studied at several temperatures, and more closely at 175°. The observed effects are shown graphically in Fig. 5 in which k is plotted against P_1. The fall off in rate has been found to begin appreciably above the expected value of about 40 mm.

The low pressure approach to a second order rate is demonstrated in Fig. 6. The reciprocal of reactant pressure has been plotted against time for several runs having initial pressures lower than 100 mm.

(8) Pease, *J. Chem. Phys.*, **7**, 749 (1939).
(9) Ref. 1, pp. 71, 72; ref. 5, p. 100.

The effect of foreign materials has been investigated quite thoroughly. An account of this effect will be published in the next paper of the present series.

Summary

1. Ethyl chlorocarbonate has been found to decompose homogeneously and by a unimolecular mechanism between 150 and 195° and over a pressure range from 10 to 700 mm. of mercury.

2. The specific reaction rate may be obtained from the equation

$$k = 5.5 \times 10^{-10}\, e^{-29,410/RT}$$

3. A slight effect has been observed in the comparison of runs in coated and uncoated vessels.

4. It has been found that at low pressures the rate constant falls appreciably and that the reaction approaches a second order rate.

BATON ROUGE, LA. RECEIVED AUGUST 3, 1939

[FROM THE DEPARTMENT OF PHYSIOLOGY AND BIOCHEMISTRY IN THE MEDICAL COLLEGE AND THE DEPARTMENT OF DAIRY INDUSTRY IN THE NEW YORK STATE COLLEGE OF AGRICULTURE, CORNELL UNIVERSITY]

Molecular Weight and Association of the Enzyme Urease

BY DAVID B. HAND

Introduction

The osmotic pressure of crystalline urease solutions corresponds to an average particle weight of 700,000[1] but the diffusion rate, when measured by activity, establishes the presence of active particles of 17,000 or less. Although much is known about the particle weights of proteins in general, there is yet no knowledge of the size of the smallest units which can exist with unaltered specific properties. In the case of the protein urease, it is possible to prove that smaller particles than those predominating in the solution possess enzyme activity.

Osmotic Pressure Measurements

An apparatus has been designed for measuring the osmotic pressure in a 1.0 ml. sample of solution. Even with these small volumes the expense and difficulty of obtaining the urease were considerable. The apparatus, which has proved convenient, is shown in Fig. 1. The collodion sack is mounted on the outer portion of a universal glass joint, thus making the filling of the sack very simple. Mineral oil is used as a manometer fluid and can be added through the side arm after the apparatus is set up. Equilibrium is reached rapidly since only 0.5 ml. of outer solution are needed. The details of the method are essentially those described by other workers.[2,3,4] Figure 2 gives the measurements of osmotic pressure which were made at pH 7.0 in 0.01 M citrate buffer and at pH 6.3 in 0.001 M phosphate. It is impossible to work nearer the isoelectric point of 5.0[5] or to increase the buffer concentration because of the lower solubility of urease under these conditions.[6] It was found that osmotic pressures were high in 0.001 M buffer due to unequal distribution of ions, but that this effect was repressed in 0.01 M buffer (see Fig. 3).

Mineral oil above both the inner and the outer solutions was allowed to rise in capillary tubes so that a value for osmotic pressure was obtained directly from the difference in the height of the oil in the two capillary tubes. The capillary tube for the outer solution is not illustrated in Fig. 1. The density of the oil was 0.842. In a typical experiment 32 lots of jack bean meal of 100 g. each were extracted with a total volume of 16 liters of 31.6% acetone by the method of Sumner and the urease allowed to crystallize. After centrifuging, washing and recrystallizing, the urease was dissolved in 4.0 ml. of water at 0°.

(1) Paper read before the American Chemical Society, New York City, April, 1935.

(2) Adair, *Proc. Roy. Soc.* (London), **A109**, 292 (1925); **A120**, 573 (1928); **A126**, 16 (1929).
(3) Krogh and Nakazawa, *Biochem. Z.*, **188**, 241 (1927).
(4) Weber and Stöver, *ibid.*, **259**, 269 (1933).
(5) Sumner and Hand, THIS JOURNAL, **51**, 1255 (1929).
(6) When the concentration of citrate is increased, the urease crystallizes in octahedra which are better defined and larger than those obtained with 32% acetone.

The clear solution contained 77.3 mg. of urease per cc. To 1.0 ml. of the urease solution was added 0.1 ml. of 0.1 *M* citrate buffer, *p*H 7.0. The osmotic pressure of this solution at 3° was found to be 4.1 cm. of oil.

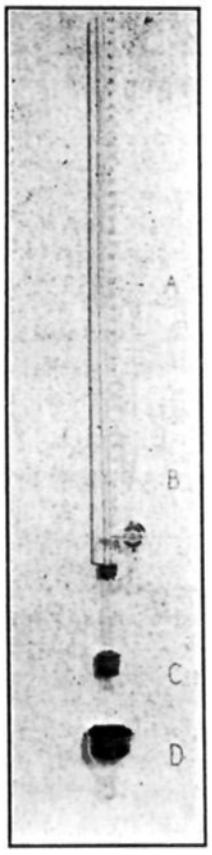

Fig. 1.—Osmometer for measuring osmotic pressure of protein solutions: A, scale and manometer tube; B, side tube for adding mineral oil; C, collodion sack mounted on interchangeable joint; D, outer vessel.

The curve for the variation of osmotic pressure with concentration of urease is not a straight line, so the calculation of particle weight has to be made by extrapolation. Particle weights are calculated from the osmotic pressure at the various concentrations and these values are extrapolated to zero concentration. When this is done the particle weight is found to be 700,000. This is a minimum value for the average particle weight depending on how completely the unequal distribution of ions was suppressed. The apparatus and method have been checked with hemoglobin which is known to be homogeneous with particle weight of 68,000.

Diffusion Measurements

The rate of diffusion of urease through a porous glass membrane was determined by the method of Northrop and Anson[7] as simplified by Dawson.[8] The urease was crystallized once and dissolved in 0.1 molar glycine, *p*H 6.0, to protect against destruction in the outer solution due to extreme dilution. The concentrations in the inner and outer solutions were determined by allowing aliquot portions to act on urea in phosphate buffer and then aerating off the ammonia into standard acid.[9] The rates of diffusion of oxyhemoglobin and hydrochloric acid were also determined with the same cell, for comparison with the urease and calculation of the cell constant. The diffusion of hemoglobin was measured colorimetrically by comparison of the outer solution with the original inner solution. Tables I, II, and III give the experimental data together with the calculated values of diffusion rate. For convenient calculation of diffusion coefficient the rate of diffusion was expressed in cubic centimeters of inner solution diffusing per hour.[7] The diffusion rate of the proteins decreased during the first two or three days, but then remained constant. The final value found for hemoglobin is in agreement with those of other workers using cells either with sintered glass membranes[7,10] or without membranes.[11,12]

No error is introduced by the fact that the diffusion rates of urease and hemoglobin decrease during the first few days in the diffusion cell since calculation of particle weight is made by comparison of the two proteins. Both

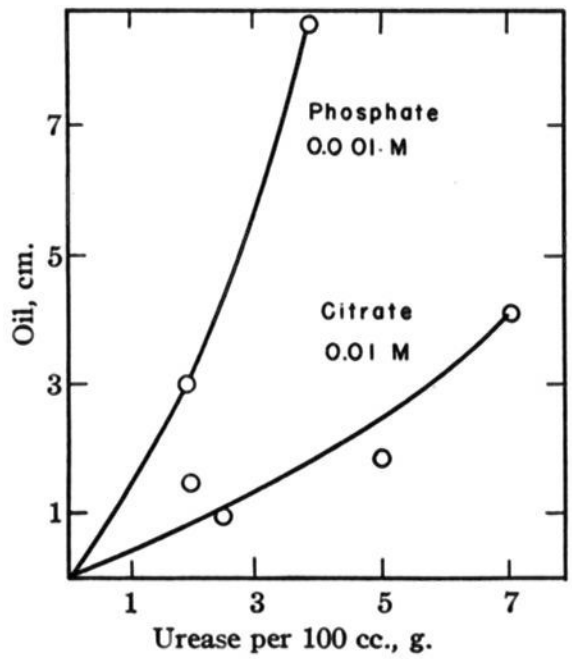

Fig. 2.—Osmotic pressures of urease solutions at 3°.

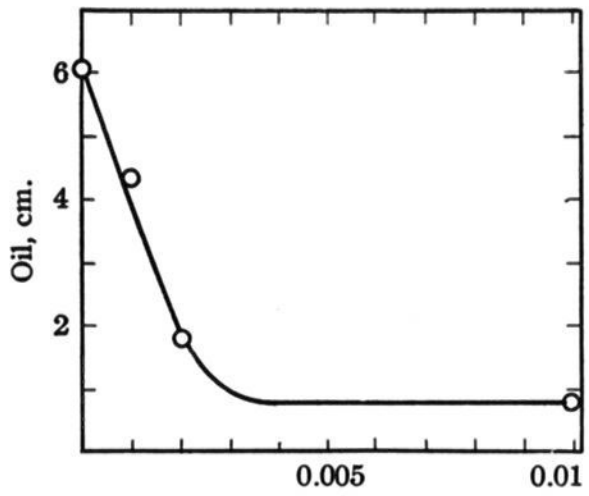

Fig. 3.—Effect of buffer concentration on the osmotic pressure of urease.

TABLE I

DIFFUSION OF UREASE

0°, 0.10 g. per 100 cc. in 0.1 molar glycine; cell no. 1, inner volume 21.0 cc., outer volume 20.4 cc., amount of urease in inner solution at start, 2850 units.

Run	Duration (in hr.)	Amt. diffusing per run (in urease units)	Amt. left in inner soln. at middle of run (in urease units)	Diffusion rate (in cc. inner soln. per hr.)
0	....	*ca.* 7	2848	.....
1	20.5	38.4	2826	(0.0139)
2	20.0	36.7	2789	(.0138)
3	8.0	13.8	2754	(.0132)
4	15.0	21.0	2737	.0107
5	23.25	31.0	2711	.0103
6	9.25	12.8	2689	.0108
7	15.0	18.5	2674	.0097
8	26.0	*ca.* 35	....	.....
				.0104

(7) Northrop and Anson, *J. Gen. Physiol.*, **12**, 543 (1928–1929).
(8) Dawson, THIS JOURNAL, **55**, 432 (1933).
(9) Sumner and Hand, *J. Biol. Chem.*, **76**, 149 (1928).
(10) Zeile, *Biochem. Z.*, **258**, 347 (1933).
(11) Tiselius and Gross, *Kolloid-Z.*, **66**, 11 (1934).
(12) Svedberg and Nichols, cited by Nichols, *Coll. Symp. Monograph*, **6**, 287 (1928).

TABLE II

DIFFUSION OF HEMOGLOBIN

0°, 2.93 g. per 100 cc. in $M/15$ phosphate, pH 7.0, cell no. 1, inner volume 21.0 cc., outer volume 20.4 cc., amount of hemoglobin in inner solution at start, 615 mg.

Run	Duration (in hr.)	Amt. diffusing per run (in mg.)	Amt. left in inner soln. at middle of run (in mg.)	Diffusion rate (in cc. inner soln. per hr.)
1	20.0	4.88	613	(0.00836)
2	23.0	4.86	608	(.00728)
3	23.25	4.43	604	.00661
4	22.5	4.06	599	.00632
5	23.25	4.39	595	.00664
				.00650

TABLE III

DIFFUSION OF HYDROCHLORIC ACID

0°, 0.979 normal, cell no. 1, inner volume 21.0 cc., outer volume 20.4; HCl in inner solution at start equivalent to 2900 cc. $Ba(OH)_2$; N, 0.0071.

Run	Duration (in hr.)	Amt. diffusing per run (in cc. $Ba(OH)_2$)	Amt. left in inner soln. at middle of run (in cc. $Ba(OH)_2$)	Diffusion rate (in cc. inner soln. per hr.)
1	1.0	41.8	2880	0.305
2	1.0	39.6	2860	.291
3	0.5	19.8	2830	.294
4	.517	20.1	2810	.290
5	.5	19.7	2790	.297
				.295

proteins behaved in the same way and the same value is obtained when either the initial or final rates of diffusion are compared. The decrease in diffusion rate was found not to be due to denaturing of the proteins because there was no measurable loss of native protein in the inner cell except that accounted for by diffusion. The decrease in diffusion rate was also shown not to be due to a progressive clogging of the pores during the experiment. No clogging of the pores occurred during the diffusion experiments. An independent measure of the clogging of the pores can be obtained from the rate of flow of solution through the membrane under a given hydrostatic pressure. During the flow of protein solution, but not during the diffusion, the pores become clogged so that the rate of flow for a given pressure becomes progressively slower. Even after the pores have been so completely clogged that the flow of liquid is practically prevented, the rate of diffusion through the cell is unchanged. Zeile[10] has reported a decrease in the rate of diffusion of hemoglobin during the course of the experiment and he has attributed this to a change in concentration gradient.

Calculations of diffusion coefficients are based on a value of 1.655×10^{-5} cm.^{2} per sec. for the diffusion coefficient of molar hydrochloric acid at 0°.[10,13,14,15] The diffusion coefficient of urease, when measured by activity, is 5.83×10^{-7} cm.^{2} per sec. and that of hemoglobin is 3.67×10^{-7} cm.^{2} per sec.

The diffusion rate of urease activity is thus 59% faster than that of hemoglobin under the same conditions. Particle weights of proteins calculated from diffusion by the Einstein equation[16] are larger than the true weights if the particles are hydrated or non-spherical. Polson has proposed a method for obtaining the shape of protein particles from viscosity data and has found, for all the proteins investigated, that the diffusion coefficients are about 0.7 as great as they would be for spherical particles and also that the hydration factor is approximately the same for all the proteins.[17] This relationship might reasonably be expected to hold for crystalline proteins which yield solutions of similar and comparatively low viscosity.[18] Insofar as these relationships hold, a very satisfactory value for the particle weight of a given protein can be obtained by comparison of the diffusion rate with that of some known protein such as hemoglobin. Particle weights are inversely proportional to the third power of the diffusion coefficients. The particle weight so obtained is equal to the weight of the diffusing particle only if the solution is homogeneous with respect to particle size. Otherwise the calculated value is somewhat larger than the smallest, most rapidly diffusing particle. Thus by comparison with hemoglobin it can be calculated that a maximum value for the weight of the diffusing particle of urease is 17,000.

The experiments described in this paper to determine the particle weight of urease[1] are not in disagreement with the later measurements of sedimentation and diffusion velocity[19] by Sumner, Gralén and Eriksson-Quensel in Svedberg's laboratory. It was shown by these workers that urease solutions (twice crystallized) could be prepared which consisted of two to five components. In all the solutions the component with particle weight 483,000 predominated. In the most homogeneous solutions all but 15% of the protein consisted of this component. For the urease solution containing five components of different particle weights, the relative amounts of the components and the activity of this preparation were not reported. No experiments were made to determine whether the enzyme activity was restricted to a single particle size.

Diffusion experiments with other enzymes have not indicated any dissociation or association of the particles. The diffusion rate for the activity is the same as the diffusion rate for protein material with pepsin[20] and chymotrypsin.[21] The results reported for catalase are uncertain. Stern's experiments[22] on the diffusion of catalase by activity measurements indicate a particle weight somewhere between 8500 and 500,000 with an average of 68,900, while Zeile finds a destruction of the catalase during diffusion.[10] By physical measurements the catalase particle is found to have a weight of 250,000.[23,24,26] In the case of horse antibody, Kabat[25] has reported that many lots could be prepared which were inhomogeneous in

(13) Öholm, *Z. physik. Chem.*, **50**, 309 (1904–1905).
(14) McBain and Liu, THIS JOURNAL, **53**, 59–74 (1931).
(15) Anson and Northrop, *J. Gen. Physiol.*, **20**, 575 (1936–1937).
(16) Einstein, *Z. Elektrochem.*, **14**, 235 (1908).
(17) Polson, *Nature*, **137**, 740 (1936).
(18) Hand, *J. Gen. Physiol.*, **18**, 847 (1934–1935).
(19) Sumner, Gralén and Eriksson-Quensel, *J. Biol. Chem.*, **125**, 37 (1938).
(20) Northrop, *J. Gen. Physiol.*, **13**, 739 (1929–1930).
(21) Kunitz and Northrop, *ibid.*, **18**, 433 (1934–1935).
(22) Stern, *Z. physiol. Chem.*, **217**, 237 (1933).
(23) Stern and Wyckoff, *Science*, **87**, 18 (1938).
(24) Sumner and Gralén, *J. Biol. Chem.*, **125**, 33 (1938).
(25) Kabat, *J. Exp. Med.*, **69**, 103–118 (1938).
(26) Agner, *Biochem. J.*, **32**, 1702 (1938).

amounts up to 65% of the total protein present. He found that the "degraded components" possessed antibody activity. Frampton and Saum have found that the particle weight of tobacco mosaic virus protein as determined from diffusion and viscosity is decreased in 6 molar urea solution to 100,000 without any decrease in biological activity.[27]

Discussion

Although it has been shown by the diffusion rate of urease that particles of a weight as low as 17,000 can carry the activity, it has not been proved that urease exists in this dissociated form at the time that it exerts its catalytic action. The particles may associate again under the conditions in which the activity is measured. Similarly the associated fractions, as separated in the ultracentrifuge, might dissociate in the test solution. There is no evidence to show that dissociation or association has any effect on the activity of urease. Incidentally, urease activity is determined in a solution which is very dilute with respect to protein and which contains 1.5% urea. Both of these conditions are known to be favorable to the dissociation of proteins.

However, in the case of enzymes other than urease, the conditions for measurement of activity might not be so favorable to complete dissociation. It is conceivable that the state of aggregation might have a considerable influence on the activity per gram. This may account for the enzyme preparations which have been reported to be more active than the same enzymes in crystalline preparations. Furthermore, a slow dissociation and association might well give rise to one or more forms of the same enzyme similar to the lyo- and desmo-enzymes of Willstätter.

The weight of the smallest particle of active enzyme is perhaps of more interest than the weight of the associated particles. Theoretically the aggregate dissociates by breaking very weak bonds under such influences as the presence of urea or a shift in *p*H. However, a final unit is reached which cannot be further dissociated except by breaking strong bonds, as for example by peptic or acid hydrolysis. Any further breakdown of this basic unit is accompanied by loss of enzyme activity. Although the term molecular weight has been justifiably applied to the aggregate, it probably would be less confusing to chemists if molecular weight referred only to the smallest particle still possessing enzyme activity, and the term associated molecular weight were used for the aggregates. For example, the molecular weight of acetic acid is 60, although it is known to associate. In this sense the molecular weight of urease is no greater than 17,000, and the average associated molecular weight is 700,000.

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Summary

1. From the osmotic pressure the average particle weight of crystalline urease solutions is found to be 700,000.

2. From the rate of diffusion, particles with a weight as low as 17,000 are shown to possess urease activity.

3. There is no evidence to show in which form urease exists at the time that it exerts its catalytic action.

(27) Frampton and Saum, *Science*, **89**, 84 (1939).

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