formed between two proteins, or a change in the state of aggregation were to occur, the volume fraction, or more especially the effective shape of the protein components, would be changed with a consequent deviation of the fluidity from that predicted on an additive basis (*cf.* Chap. V, ref. 3). The data in Table I and in Fig. 6 do not indicate such deviation, within the experimental error (*cf.* also ref. 2a). Nor do density determinations on beef albumin–globulin mixtures show any variation from strict additivity.[15] Even such a complicated mixture as serum yields a constant average partial specific volume, over the entire range, if one plots the density data of Chick and Lubrzynska.[16] A fair linear fluidity curve up to and including physiological concentrations of protein may also be obtained for this system.

Further discussion of this question, especially as to its bearing on the interesting effects observed in the ultracentrifuge by McFarlane[17] and by Pedersen[18] must await more accurate fluidity data, particularly on protein containing systems in which known compound formation and dissociation occur.

(15) Nugent and Towle, *J. Biol. Chem.*, **104**, 395 (1934).
(16) Chick and Lubrzynska, *Biochem. J.*, **8**, 59 (1914).
(17) McFarlane, *ibid.*, **29**, 407 (1935).
(18) Pedersen, *Compt. rend. trav. lab. Carlsberg*, **22**, 427 (1937).

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Summary

1. It has been found that the fluidity, or reciprocal of the relative viscosity, is linear with the protein concentration over a wide range of proteins and concentrations.

2. The fluidity provides a convenient interpolation formula for converting the fluidity or viscosity obtained at one concentration to that at any other.

3. It is shown that the fluidity is additive for mixtures of rabbit eu- and pseudoglobulin, and for horse albumin and globulin fractions.

4. The relation of additive fluidities to the presence of compounds in mixtures of proteins is discussed.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

Ascorbic Acid Oxidase from Summer Crook-neck Squash (C. pepo condensa)

BY P. L. LOVETT-JANISON AND J. M. NELSON

In 1928 Szent Györgyi[1] observed that certain plant tissues catalyzed the aerobic oxidation of ascorbic acid, and in 1930[2] he postulated the existence of a specific enzyme in cabbage leaves. Since then, other investigators[3,4,5,6,7] have reached the same conclusion working with different plant sources, such as apples, Hubbard squash, Drumstick, cauliflower, bananas, cabbage, carrots, cucumbers, potatoes and string beans.

Barron, DeMeio and Klemperer[8] were among the first to show that the aerobic oxidation of ascorbic acid was catalyzed by ionic copper, and in a later paper[9] they questioned the enzymic nature of ascorbic acid oxidase. Stotz, Harrer and King,[10] working upon this problem, were inclined to attribute the catalytic activity of squash juice to copper in combination with protein material, rather than to a specific oxidase. On the other hand, Spruyt and Vogelsang[11] working with fruit juices concluded that, "ascorbic acid oxidation and copper do not go hand in hand and that this oxidation is certainly not exclusively to be ascribed to the presence of copper."

From the above, it is obvious therefore that some confusion arises. It is the object of this paper to show that ascorbic acid oxidase is indeed a true enzyme, consisting of a copper-protein, similar in type to tyrosinase (polyphenol oxidase), which has been recognized as a true enzyme.

Purification of Enzyme from Summer Squash.—After experimenting with cucumber, cauliflower, summer squash, apples, parsnips, carrots, spinach, string beans, cabbage,

(1) A. Szent Györgyi, *Biochem. J.*, **22**, 1387 (1928).
(2) A. Szent Györgyi, *Science*, **72**, 125 (1930).
(3) S. S. Zilva, *Biochem. J.*, **28**, 663 (1934).
(4) H. Tauber, I. S. Kleiner and D. Mishkind, *J. Biol. Chem.*, **110**, 211 (1935).
(5) M. Srinivasan, *Biochem. J.*, **30**, 2077 (1936).
(6) F. G. Hopkins and E. J. Morgan, *ibid.*, **30**, 1446 (1936).
(7) W. Stone, *ibid.*, **31**, 508 (1937).
(8) E. S. G. Barron, R. H. DeMeio and F. Klemperer, *J. Biol. Chem.*, **112**, 625 (1935–1936).
(9) E. S. G. Barron, A. G. Barron and F. Klemperer, *ibid.*, **116**, 563 (1936).
(10) E. Stotz, C. J. Harrer and C. G. King, *ibid.*, **119**, 511 (1937).
(11) J. P. Spruyt and G. M. D. Vogelsang, *Arch. Néerland Physiol.*, **23**, 423 (1938).

oranges and green peppers, we have found the best source of ascorbic acid oxidase to be that from summer crook-neck squash (*C. pepo condensa*).

Ebihara[12] has shown, in the cases of certain plants, that the enzyme is most abundant in the external layers. We have confirmed this in the case of summer crook-neck squash. Consequently, the rinds of squash were minced to a fine pulp, then subjected to hydraulic pressure in order to remove all juice. Determinations upon various crude juice preparations gave values ranging from 0.52 to 0.73 unit per mg. dry weight (for definition of unit see next section). This was followed by treatment with M barium acetate (20 cc. to 1 liter of juice), which removed a considerable amount of color and extraneous protein without appreciable loss of activity. After filtering, the juice was brownish-yellow in color. Excess barium ion was now removed as barium sulfate by the addition of 0.05 saturated ammonium sulfate solution and filtering. By the addition of ammonium sulfate to 0 6 saturated, the enzyme was precipitated. After filtering, the precipitate was washed with saturated ammonium sulfate solution until the filtrate was colorless. Addition of $M/15$ secondary sodium phosphate dissolved the precipitate which, after filtering, now contained about 85% of the original enzyme. The solution of the enzyme was then made 0.9 saturated with magnesium sulfate, which precipitated about two-thirds of the enzyme (Part I). After filtering the filtrate was now saturated with magnesium sulfate and the remaining third precipitated (Part II). After dialysis, these two parts, I and II, contained 70 and 144 units per mg. dry weight, respectively. Since on further treatment Part I failed to give preparations of low dry weight comparable with those obtained later from Part II, it was eventually discarded. The remaining steps in the purification apply, therefore, to Part II only.

Part II of the enzyme was now adsorbed to 10% alumina[12a] at *p*H 6.5, eluted with secondary phosphate and dialyzed in distilled water at *p*H 8.0. It was then fractionated with 0.1 saturated lead acetate and acetone (cooled with dry-ice) in the following manner. To the whole, lead acetate (14 drops per 100 cc. enzyme solution) was added, followed by the addition of 10% of the total volume of acetone and the precipitate centrifuged. The supernatant liquid was again treated with the same reagents (10 drops of lead acetate per 100 cc. of enzyme solution, followed by acetone up to 25% of the total volume), and the resulting precipitate again centrifuged. This procedure was repeated twice. The precipitates obtained were taken up in secondary phosphate, filtered and dialyzed in redistilled water at *p*H 8.0. Adsorption to 20% alumina followed by elution and dialysis yielded a fraction of 557 units per mg. dry weight. Further fractionations of this fraction with lead acetate and acetone, followed by treatments with alumina, yielded fractions of 600 to 631 units per mg. dry weight. Further treatments have, so far, failed to improve upon these values.

These purifications removed all the brownish-yellow pigment and it is interesting to note that while at dilutions of about 500 units per cc. the enzyme solution was colorless, at high concentrations of about 4000 units per cc. the solution was green and at intermediate dilutions the color was pale blue or greenish-blue.

Definition of Unit and Determination of Activity of Enzyme Preparations.—In these Laboratories one unit of ascorbic acid oxidase is that quantity which in the presence of its substrate, ascorbic acid, catalyzes a reaction involving an oxygen uptake of 10 c. mm. per minute.

The determinations were carried out in the Warburg respirometer at 25°. In the vessels were 2 cc. of 0.2 M phosphate–citrate buffer of *p*H 5.6, 1 cc. of gelatin (5 mg. per cc.), 1 cc. of *l*-ascorbic acid (2.5 mg. per cc.), the diluted enzyme solution and distilled water to make the total volume 8 cc. At all times one of the flasks contained no enzyme, being used as a control. Readings were usually taken every two minutes.

Adams and Nelson[13] have shown in the case of tyrosinase preparations that the addition of gelatin furnishes protection to the enzyme against inactivation. Similarly with ascorbic acid oxidase we have found that the addition of 3.0 mg. of gelatin helps by stabilization of the enzyme when in a highly purified state.

Determination of Copper.—The method used for the determination was that devised by Warburg[14] and is based upon the oxidation of cysteine by copper in the presence of sodium pyrophosphate, which inhibits the action of any iron or manganese which may be present.

The operation was carried out in a Warburg respirometer at *p*H 7.6. A typical run is shown in Table I.

TABLE I

Flask	1	2	3	4	5	6
0.2 M pyrophosphate, *p*H 7.75, cc.	2	2	2	2	2	2
(1) Copper soln. 0.1 γ/0.1 cc., cc.	0.1	0.2	...	...	...	...
(2) 0.2 M hydrochloric acid, cc.	.1	.1	0.1	0.1	0.1	0.1
(3) Enzyme solution, cc.	.1	.1	...	.1	.2	.3
(4) Cysteine hydrochloride 6 mg./0.2 cc., cc.	.2	.2	.2	.2	.2	.2
Water, cc.	.1	...	.3	.2	.1	...

The reagents (1), (2), (3), (4) were placed in the side arm and added to the buffer solution and water, contained in the reaction flask, when the temperature was constant at 25°. Flask 3 contained no enzyme, and served as a control, by being a blank on all the reagents used. The total volume in each flask was 2.6 cc., and the *p*H after mixing was 7.6. Readings were taken at five-minute intervals. It is obvious that when the manometer readings were converted to c. mm. of oxygen, then subtraction of the value for flask 4 from those of flasks 1 and 2 gave the values of the uptakes due to 0.1 γ and 0.2 γ of copper, while subtraction of the value for flask 4 from flasks 5 and 6 gave the values of the uptakes due to 0.1 cc. and 0.2 cc. of enzyme solution. In such a way two determinations of copper and enzyme uptakes were obtained in each run. In all cases at least two runs were performed. Dividing the uptake due to 0.1 cc. of enzyme solution by that due to 0.1 cc. of copper solution gave the concentration of copper in the enzyme solution. Knowing also the activity of enzyme in units per cc. enabled one to obtain the activity of enzyme in units of enzyme per γ of copper. The results of copper estimations carried out on different enzyme prepa-

(12) T. Ebihara, *J. Biochem. (Japan)*, **28**, 415 (1938).

(12a) Suspension 15 g. of alumina per liter.

(13) M. H. Adams and J. M. Nelson, THIS JOURNAL, **60**, 2472 (1938).

(14) O. Warburg, *Biochem. Z.*, **187**, 255 (1927).

rations have been found to give a constant value of 432 ± 30 units per γ of copper.

Table II shows that the percentage of copper increased with rise of enzyme activity until it reached the value of 0.15%. In spite of further attempts at purification, this was the highest purity reached to date. The figures given are those obtained only after a more or less constant value for the activity of the enzyme (in units per γ Cu) had been reached. This proportionality between the copper contents and enzyme activities (column 2) is, we think, strong evidence for the conclusion that the active group of ascorbic acid oxidase contains copper.

TABLE II

Prepn.	Concn. enzyme in sample (units/cc.)	Activity, units/γ Cu	% Cu	Activity, units/mg.
1	1412	488	0.029	142
2	161	400	.040	144
3	1287	390	.047	183
4	2248	480	.060	286
5	496	400	.10	379
6	730	415	.12	480
7	3480	431	.12	512
8	530	452	.12	525
9	786	405	.14	586
10	5640	429	.15	600
11	531	406	.15	612
12	516	490	.15	631
		432 ± 30		

Further purification failed to alter this value for copper, which represented about 5% of the total copper present in the original squash juice. It was only by successive stages of purification that the copper which did not belong to the ascorbic acid oxidase was gradually eliminated, together with other impurities.

On comparing the activity of copper in units per γ with those of Stotz, Harrer and King,[10] who used solutions of copper only, copper–gelatin, and copper–albumin mixtures, and the juices of cauliflower and squash (somewhat purified), we find that our purified enzyme is 1100 times more active than copper alone, 13,000 times more active than the copper–albumin mixture, 4100 times more active than the copper–gelatin mixture, and 3800 and 1700 times, respectively, more active than the juices taken from cauliflower and squash and used by these investigators. This very great activity or increased specificity shown by our purified enzyme constitutes, we consider, a strong argument in favor of our purified squash juice containing a true enzyme. This is in line with the copper content of other enzymes such as tyrosinase (polyphenol oxidase).

Dry Weight Determinations.—For details of the method by which these determinations were performed, refer to the work of Lutz and Nelson[15] in 1934.

Determination of Manganese.—Theorell,[16] working upon dihydroxymaleic acid oxidase, has offered evidence to show that manganese is the activator for this enzyme. Since the configurations of dihydroxymaleic and ascorbic acids show similarity, it was thought at one time in this Laboratory that manganese might play some part as activator for ascorbic acid oxidase. With this end in view, the direct microdetermination of manganese, as outlined by Wiese and Johnson,[17] was carried out upon samples of ascorbic acid oxidase from squash, in which the samples varied from crude juice to purified preparations. It was found that the quantity of manganese in the samples was very small and bore no relation to enzyme activity. With each stage of purification, the value for manganese slowly diminished, even when the enzyme concentration was increased. From this work we must conclude that manganese seems to play no part in the catalytic function of ascorbic acid oxidase. This seems to be in agreement with the work of Barron, DeMeio and Klemperer,[8] who found that of the metals, manganese, iron, copper, nickel, cobalt and calcium, copper was the only metal to catalyze the oxidation of ascorbic acid.

Determination of Peroxidase.—Several investigators[5,18,19] have shown that ascorbic acid oxidase preparations contain peroxidase. We also have found this to be the case in crude or partially purified samples.

The method used by us for the determination of peroxidase was that of Balls and Hale,[20] a method which we used on samples of ascorbic acid oxidase varying from crude juice to our purest preparations. Determinations conducted through the various stages of purification indicated that considerable amounts of peroxidase were being removed as the purification proceeded. In the filtered crude squash juice the concentration of peroxidase was found to be 0.0022 unit of peroxidase per unit of ascorbic acid oxidase, while in the purest preparation (Prepn. 10 (see table) containing 600 units of ascorbic acid oxidase per mg. dry weight) this ratio was 0.0000078. This indicated that 99.65% of the peroxidase had been removed during purification, and for all practical purposes it may be considered as a peroxidase-free preparation. This should be of interest to other workers in this field, because of the importance that has been attributed to peroxidase in the respiration of plants by investigators such as Szent Györgyi,[21] St. Huszák,[18] and Robězieks.[22]

Behavior of Ascorbic Acid Oxidase toward Catechol, *p*-Cresol and Hydroquinone.—Since

(15) G. J. Lutz and J. M. Nelson, *J. Biol. Chem.*, **107**, 169 (1934).
(16) H. Theorell and B. Swedin, *Naturwissenschaften*, **27**, 95 (1939).
(17) A. C. Wiese and B. C. Johnson, *J. Biol. Chem.*, **127**, 203 (1939).
(18) St. Huszák, *Z. physiol. Chem.*, **247**, 239 (1937).
(19) T. Ebihara, *J. Biochem. (Japan)*, **29**, 217 (1939).
(20) A. K. Balls and W. S. Hale, *J. Assoc. Off. Agr. Chem.*, **16**, 445 (1933).
(21) A. Szent-Györgyi, *Bull. soc. chim. biol.*, **20**, 846 (1938).
(22) I. Robězieks, *Z. physiol. Chem.*, **255**, 255 (1938).

we have shown that ascorbic acid oxidase is a copper–protein enzyme similar to tyrosinase (polyphenol oxidase), we thought it of interest to try its effect upon the above substrates, although some investigators[4,5] already have shown that the enzyme does not catalyze the oxidation of phenols. Using one of our preparations of highest purity (600 units per mg.) we found that it was about 520 times more active toward ascorbic acid than toward catechol and about 335 times more active toward its own substrate than toward hydroquinone. No activity toward *p*-cresol was obtained when an amount of ascorbic acid oxidase as high as 846 units was used. These activities are extremely small and to all intents and purposes it may be concluded that the enzyme does not catalyze the oxidation of these substances except at high concentrations.

Summary

1. A method has been described for the preparation of a highly purified ascorbic acid oxidase having an activity of 600 to 630 units per mg. dry weight.

2. At high concentrations the enzyme was green, at lower concentrations blue or bluish-green.

3. The ascorbic acid oxidase was found to be a copper-protein compound, containing 0.15% of copper.

4. The purest enzyme preparations obtained had an activity of 432 units per γ of copper.

5. Manganese seems to play no part as activator for this enzyme.

6. The purest enzyme preparation was found to have lost 99.65% of the peroxidase accompanying the enzyme in the crude juice and may be considered practically free of this enzyme.

7. Ascorbic acid oxidase has no action toward *p*-cresol and only shows a slight action toward catechol and hydroquinone at comparatively high concentrations.

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[CONTRIBUTION FROM THE DIVISION OF INDUSTRIAL AND CELLULOSE CHEMISTRY, MCGILL UNIVERSITY]

Studies on Lignin and Related Compounds. XLVII. The Synthesis of Xylosides Related to Lignin Plant Constituents

BY J. H. FISHER, W. LINCOLN HAWKINS AND HAROLD HIBBERT

The question of whether lignin is in chemical combination with a carbohydrate constituent in the plant has been a matter of speculation for many years. Some workers[1] believe that lignin is present as an incrustant in the cell wall and is not in chemical union with any other product. Evidence[1] from a study of X-ray patterns of pure cotton cellulose and cellulose from wood appears to indicate the absence of a chemical union. However, the possibility of the existence of a lignin-hemicellulose complex as suggested by Harris, Sherrard and Mitchell[2] and later supported by Norman and Shrikhande[3] is not excluded.

Recent studies on the enzymatic degradation of wood by Ploetz[4] provide further evidence for the presence of a lignin carbohydrate complex. Bailey[5] in work dealing with lignin extracted by the use of butyl alcohol obtains evidence indicating a chemical combination of part of the lignin with cellulose in certain woods. Norman[6] and also Hägglund[7] have presented comprehensive reviews concerning the relationship between lignin and cellulose in the cell wall.

Possible Mode of Combination.—The two principal carbohydrate constituents of the cell wall, cellulose and xylan, may be combined with the lignin through ether, ester or glycosidic bonds, that of the last-named occurring most frequently in natural products. To obtain further information on the nature of the lignin union in wood, a number of glycosides have been synthesized from products presumably closely related to lignin.

Although the structure of "protolignin" is unknown, recent studies on the ethanolysis of wood[8] and the hydrogenation of certain isolated lignins[9]

(1) Freudenberg, *J. Chem. Education*, **9**, 1171 (1932).
(2) Harris, Sherrard and Mitchell, THIS JOURNAL, **56**, 889 (1934).
(3) Norman and Shrikhande, *Biochem. J.*, **29**, 2259 (1935).
(4) Ploetz, *Ber.*, **73**, 57, 61, 74 (1940).
(5) Bailey, *Paper Trade J.*, **110**, No. 1, 29 (1940).
(6) Norman, "Biochemistry of Cellulose, Polyuronides, Lignin," Clarendon Press, Oxford, 1937, pp. 59–62.
(7) Hägglund, "Holzchemie," Akademische Verlagsgesellschaft, Leipzig, 1939, pp. 206–212.
(8) Cramer, Hunter and Hibbert, THIS JOURNAL, **61**, 509 (1939); Hunter, Cramer and Hibbert, *ibid.*, **61**, 516 (1939).
(9) Harris, D'Ianni and Adkins, *ibid.*, **60**, 1467 (1938).