Ascorbate Oxidase: A New Method of Purification. Characterization of the Purified Enzyme*

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ABSTRACT: Ascorbate oxidase, prepared by a procedure which employs DEAE-cellulose chromatography and starch-column electrophoresis, has been obtained in high purity and in relatively high yield from either yellow or green summer squash. The enzyme had a specific activity of 3600 units per mg of protein. Average values for the weight-average and *z*-average molecular weights of the enzyme from sedimentation equilibrium experiments were found to be 140,000 and 147,000, respectively.

The larger value may be due to the presence of a small amount of a higher molecular weight species of the enzyme. The molecular weight from combined sedimentation and density data was calculated as 134,000. Selecting 140,000 as the best value from the molecular weight, the purified enzyme was found to contain 8 atoms of Cu per molecule. The absorption spectrum in the visible range showed a maximum at about 608 mμ, with an absorption coefficient at this wavelength of 1300/g-atom of Cu.

The preparation and properties of the copper-containing enzyme, ascorbate oxidase, which catalyzes the aerobic oxidation of L-ascorbic acid, have been studied by many investigators (see review by Stark and Dawson, 1963). One of the earlier purification procedures was developed by Powers *et al.* (1944). This method was later modified by Dunn and Dawson (1951) to obtain a still more highly purified enzyme. The properties of ascorbate oxidase so obtained were examined in detail by these authors and others and found to be considerably different from those observed using a less purified enzyme.

Although the method developed by Dunn and Dawson yields an enzyme of relatively high purity, their procedure has the disadvantages of involving many steps, a long time to accomplish the purification, and a low recovery of enzyme. Recently, the research investigations of the structure and function of ascorbate oxidase have required relatively large amounts of enzyme. It therefore seemed advisable to explore the possibility of utilizing new and potentially more productive techniques for the purification of ascorbate oxidase.

This report will describe a new purification procedure based on DEAE-cellulose column chromatography and starch column zone electrophoresis. This method of preparation yields an enzyme of significantly greater purity than is obtained by older methods. Some characteristics and properties of the purified enzyme will also be presented.

* From the Departments of Chemistry and Zoology, Columbia University, New York City. *Received January 4, 1965; revised March 22, 1965.* This investigation was supported in part by grants from the U.S. Public Health Service (A-3200 (C-2)) and the National Science Foundation (GB-37).

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Experimental Methods

Materials. The yellow crook-neck squash and the green zucchini squash (*Cucurbita pepo*) were used as sources of the enzyme. The substrate, ascorbic acid, was obtained from Merck & Co. and used without further purification. DEAE-cellulose was obtained from Eastman Organic Chemicals. The starch used in the zone electrophoresis was obtained from Fisher. Ammonium sulfate, Tris, acetone, and other chemicals of reagent grade were purchased from various manufacturers and used as received.

Enzyme Assay. The rate of the ascorbate oxidase–catalyzed ascorbic acid oxidation was measured manometrically in Warburg vessels in the following way. The standard reaction mixture for the measurement of specific activity consisted of an appropriate volume of the enzyme solution; 0.5 ml of 0.2 M citrate–0.4 M phosphate buffer, *p*H 5.5; 0.2 ml of a gelatin solution (5 mg/ml in distilled water); 0.1 ml of 0.1 M ascorbic acid; and a sufficient amount of distilled water to bring the final volume of the reaction mixture to 2.5 ml. The enzyme, buffer, and gelatin were placed in the main compartment of the reaction vessel; the substrate was placed in the side-arm and, after equilibration for 15 minutes, was added to the main compartment at time zero. The final volume of the reaction was always kept at 2.5 ml and the final *p*H of the reaction mixture was found to be 5.7.

The specific activity of the enzyme was expressed as units/mg as described by Lovett-Janison and Nelson (1940). The amount of protein was determined by using the Folin-Ciocalteau phenol reagent according to the method of Sutherland *et al.* (1949). The optical density of the resulting color was read in a Beckman Model DU spectrophotometer at 660 mμ. The concentration of the ascorbate oxidase protein was calculated from the

value of the optical density according to the following expression:

$$\text{concn protein} = (OD_{obsd})770 = \mu g/ml$$

The factor 770 was obtained by spectrophotometric measurements of a number of samples of the most purified ascorbate oxidase having known dry weights (Poillon, 1963).

A second factor, 800, determined from egg albumin and bovine serum albumin was used for the calculation of the protein content of those preparations which were not highly purified.

All copper analyses were carried out according to the procedure of Poillon and Dawson (1963b).

Sedimentation Experiments. Sedimentation velocity and equilibrium experiments were performed in a Spinco Model E analytical ultracentrifuge equipped with a phase plate as a schlieren diaphragm and also equipped with a rotor temperature indicator and control unit. The equilibrium experiments employed short columns of liquid (from 1 to 2 mm height) in a double-sector epoxy-resin cell with Kel-F oil placed below the solution column. The rotor was operated at 8225 rpm and the temperature was maintained at 24° until equilibrium was achieved throughout the cell. The photographic plates were read in a Gaetner two-dimensional microcomparator. The weight-average and *z*-average molecular weights were calculated from the equilibrium experiments according to the methods suggested by Van Holde and Baldwin (1958).

Sedimentation coefficients were determined from experiments in which the rotor was operated at 59,780 rpm. The observed values were corrected to standard conditions of water and 20°.

Correction for the effect of temperature on the viscosity of water was calculated from data in the "Handbook of Chemistry and Physics" (42nd edition). The viscosities of the solvents relative to water were determined in an Ostwald-type viscometer; densities of the solvents were measured in a 50-ml pycnometer.

A value of 0.73 for the partial specific volume of ascorbate oxidase was calculated from the amino acid analyses of this enzyme reported by Stark and Dawson (1962) and was used in the calculations of $s_{20,w}$ and the molecular weight.

Electrophoretic Mobility and Free Diffusion. The mobility and diffusion of the purified enzyme were studied at a single *p*H and protein concentration. These experiments were conducted in a Spinco Model H electrophoresis instrument and employed the 2-ml volume microcell. The diffusion coefficient was calculated from Rayleigh interference patterns as described by Schachman (1957). In the calculation of the mobility, the migration of the boundary was measured from schlieren patterns.

Results

Purification Procedure

Extraction of the Crude Enzyme. The juice from the minced peels of the summer squash was collected by press, treated with barium acetate, and precipitated with ammonium sulfate. Ammonium sulfate was added to the juice to give 60% saturation. The resulting precipitate was collected by filtration through Whatman No. 2 filter paper and stored in a deepfreeze at around −10° as the "starting material." This part of the procedure has been described in detail by Dunn and Dawson (1951).

Filtration of the Enzyme. A 500-g aliquot of the starting material was dissolved in 1 liter of distilled water. The solution was filtered through a funnel with the aid of Celite and under suction. A 5-g quantity of Celite was used for making a pad on the funnel and another quantity (5 g per 100 ml of solution) was mixed with the enzyme solution and filtered by passing through the pad of Celite under suction.

Precipitation with Acetone. Sodium chloride was added to the above filtrate to a final concentration of 1.3%; chilled acetone, in an amount equal to 0.9 volume of the filtrate, was added to the solution. The precipitation with acetone was effected at, or below, −5° with vigorous stirring. The precipitate was collected by filtration using suction and was then dissolved in ice-cold distilled water, using a volume of about 0.1 that of the original filtrate. The aqueous solution of the acetone precipitate was dialyzed against chilled running tap water for 4–5 hours and then filtered with suction through a thin Celite pad. The filtrate was dialyzed overnight against three changes of 1 liter of 0.01 M Tris buffer, *p*H 7.6.

DEAE-Cellulose Chromatography. PREPARATION OF COLUMN AND APPLICATION OF ENZYME. DEAE-cellulose was washed twice in 0.5 M NaOH (1 liter of solution per 20 g of cellulose), followed quickly by washing a few times with 1 liter of distilled water. The washed DEAE-cellulose was then suspended in 1 liter of 0.02 M Tris buffer, *p*H 7.6. If necessary, the *p*H of the cellulose mixture was adjusted to 7.6 by the addition of glacial acetic acid and the mixture was then stored in the cold (0–4°). Just before use, the buffer was decanted from the stock DEAE-cellulose solution and an appropriate volume of 0.01 M Tris buffer, *p*H 7.6, was added to the wet DEAE-cellulose (usually 50 volumes of buffer to 1 volume of DEAE-cellulose). The DEAE-cellulose was packed into the column under a constant air pressure of 25 cm and washed thoroughly with 0.01 M Tris, *p*H 7.6. The volume of the buffer used for the washing was about 50 times larger than the volume of the wet DEAE-cellulose. After washing the column, the enzyme solution was applied to the top of the column; the flow rate was about 100 ml/hour. The adsorbed enzyme was then eluted stepwise by Tris-NaCl solutions of higher ionic strengths, and samples of appropriate volume were collected by a fraction collector. After elution the enzyme fractions having the highest specific activities were pooled and dialyzed against 0.01 M Tris buffer.

FIRST CHROMATOGRAPHY. A column having a diameter of 3 cm was used for the first chromatography. DEAE-cellulose was packed in the column to a height of 15 cm, which was adequate to receive the entire crude enzyme solution prepared from 500 g of the starting

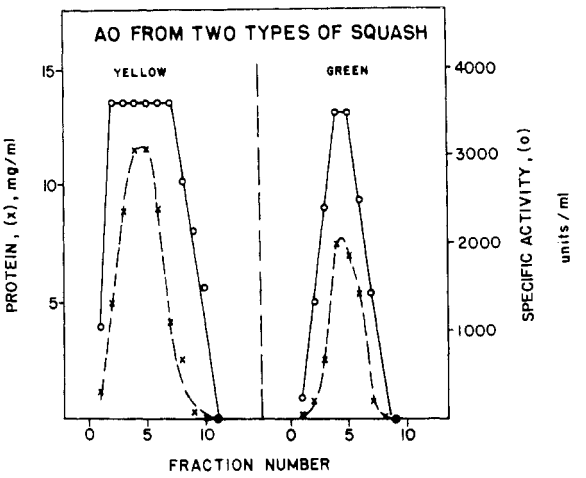

FIGURE 1: A comparison of the enzyme fractions prepared from yellow and green squash recovered after the second electrophoretic separation in 0.1 M veronal buffer, *p*H 8.6.

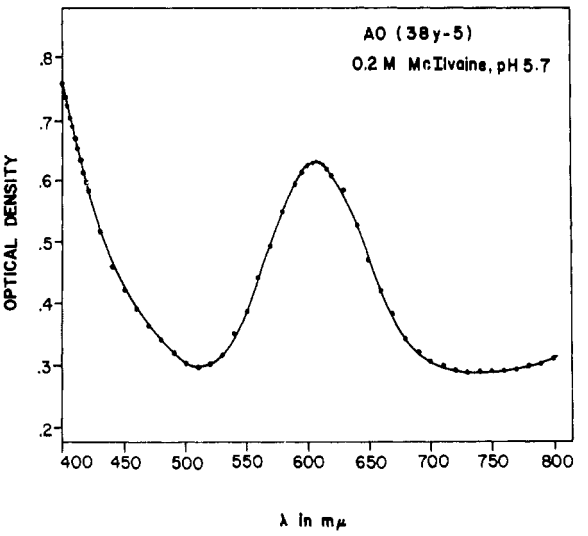

FIGURE 2: The visible spectrum of ascorbate oxidase in 0.1 M McIlvaine buffer, *p*H 5.7, showing a single maximum at about 608 mμ.

material. The first elution employed 100 ml of 0.07 M Tris-NaCl (0.01 M Tris–0.06 M NaCl), *p*H 7.6. The elution of the enzyme could easily be followed as a blue-green band of color moving down the column.

The first eluate having a yellow color was discarded (usually the first 20 ml). The following eluate having either a yellow-green, green, or dark green color was collected (usually the next 80 ml) in 6-ml fractions. For complete elution of enzyme still adsorbed on the cellulose, another 100 ml of 0.1 M Tris-NaCl (0.01 M Tris–0.09 M NaCl), *p*H 7.6, was used. Only the green- or blue-colored fractions were collected. In the case of enzyme from the yellow squash, the first 50–70 ml of the 0.1 M eluate was collected, while in the case of the enzyme from the green squash the second elution involving the 0.1 M Tris–NaCl was not necessary.

The green- or yellow-green-colored fractions eluted with the 0.07 M–0.1 M Tris-NaCl solutions were pooled and dialyzed twice against a 2-liter volume of 0.01 M Tris buffer, *p*H 7.6, overnight with stirring.

SECOND CHROMATOGRAPHY. A column having a diameter of 1 cm was used for the second chromatography, and was packed with DEAE-cellulose to a height of 15 cm. The procedure of making the column, applying the sample, and eluting the enzyme was essentially the same as in the first chromatography. However, the volume of the solvent employed in this step was smaller. All of the enzyme was eluted by the first 15 ml of 0.07 M Tris-NaCl, *p*H 7.6, and collected by means of the fraction collector in 3-ml aliquots. The fractions were assayed, and those having the highest specific activities were pooled and dialyzed as described in the preceding step.

THIRD CHROMATOGRAPHY. The column used in this step was identical with that used in the second chromatography. The procedures were the same as in the second chromatography except that, before eluting the enzyme, 15 ml of 0.04 M Tris-NaCl (0.01 M Tris–0.03 M NaCl), *p*H 7.6, were used for washing inert protein from the column after the application of the enzyme solution. The enzyme was then eluted with 15 ml of the 0.07 M Tris-NaCl solution. Selected fractions were then dialyzed against a 2-liter volume of 0.01 M Tris buffer, *p*H 7.6, overnight with stirring. If necessary, 0.1 M Tris-NaCl could be used for eluting any residual enzyme strongly adsorbed on the DEAE-cellulose after elution with the 0.07 M Tris-NaCl. However, this fraction often had a lower specific activity which indicated the presence of a greater amount of inactive or contaminating protein, and usually was not combined with the other enzyme fractions.

Starch-Column Zone Electrophoresis. The three-times-chromatographed sample was further purified by means of zone electrophoresis on a vertical starch column connected to balanced electrode vessels as described by Kunkel (1954). The starch was washed thoroughly with 1 liter of distilled water 10 times, equilibrated with about 5 volumes of veronal-HCl buffer (μ = 0.1, *p*H 8.6), and then packed (via a slurry) in a column having a diameter of 3 cm. The column was initially packed to a height of about 8 cm and the enzyme was then carefully applied so as to produce a narrow uniform band. The column was then immediately packed with another 7 cm of starch. The electrophoresis was performed in the veronal-HCl buffer at 16 ma and 600 v for 24 hours. At the end of this time the veronal-HCl buffer preparation was fractionated simply by allowing the buffer remaining in the top of the column to flow through the starch. Generally, 2-ml fractions were collected. The fractions showing the highest specific activity for ascorbate oxidase, when pooled and subjected to a second electrophoresis for 24 hours under the same conditions as used previously, showed no further improvement in specific activity. All of the procedures were carried out in the cold room (4°).

TABLE I: Recovery of Enzyme during Purification.

	Source[a]	Specific Activity (units/mg)	Protein Content (mg/ml)	Volume (ml)	Total Activity (units)	Yield (%)
Starting extract (500 g in 1 liter water)	Y	32.3	23.0	1250	930,000	100
	G	26.0	21.3	1250	700,000	100
Filtrate	Y	56.1	15.8	1050	930,000	100
	G	34.0	18.1	900	554,000	79
Dialysis after acetone ppt.	Y	280	4.8	700	940,000	100
	G	150	4.4	830	548,000	78
1st DEAE	Y	1300	2.83	126	464,000	50
	G	840	4.2	74	261,000	37
2nd DEAE	Y	2100	9.7	22	447,000	48
	G	1700	6.75	13	150,000	21.5
3rd DEAE	Y	2200	18	6.6	262,000	28
	G	2100	8.04	5.6	94,500	13.5
1st electrophoresis	Y	3600	30	2	216,000	23
	G	3500	11.7	2	81,900	12
2nd electrophoresis	Y	3600	27	2	194,400	21
	G	3500	10.5	2	73,500	10.5

[a] Y refers to preparation VII from yellow squash. G refers to preparation II from green squash.

The specific activity and protein content of the purified enzyme, plotted against fraction number after electrophoresis on the starch column, are shown in Figure 1. The specific activity and the recovery of enzyme at each step in the purification are summarized in Table I. Preparations having a specific activity of about 3500 units per mg of protein showed no further change upon further electrophoresis.

For clarification, it should be pointed out that all of the values listed in Table I having reference to the DEAE-cellulose columns and the electrophoresis are based on the selection of fractions having the highest specific activities. It should also be noted that the data given are for two specific preparations. The specific activity and recovery values shown for these two preparations are, with one exception, close to the average values of several different preparations. The exception is the unusually high specific activity (2100) and recovery (48%) noted for the Y enzyme after elution from the second DEAE-cellulose column. The G values at this stage are more characteristic. The yellow enzyme was chromatographed a third time (as usual) in order to concentrate the sample in preparation for electrophoresis and to see if further purification could be achieved by another chromatographic step. When the third chromatography treatment on DEAE-cellulose is omitted, the electrophoretic separation is usually less effective in producing an enzyme of maximal specific activity, color, and copper content.

Exploratory sedimentation velocity experiments using fractions of enzyme having specific activities in the range of 2000 units/mg, as obtained from the DEAE-cellulose columns, showed only a very small amount of contaminating protein (observed as a distinct boundary). In other words, such preparations usually showed only a slight curvature of the base line behind the enzyme boundary. This point will be elaborated in more detail in the Discussion.

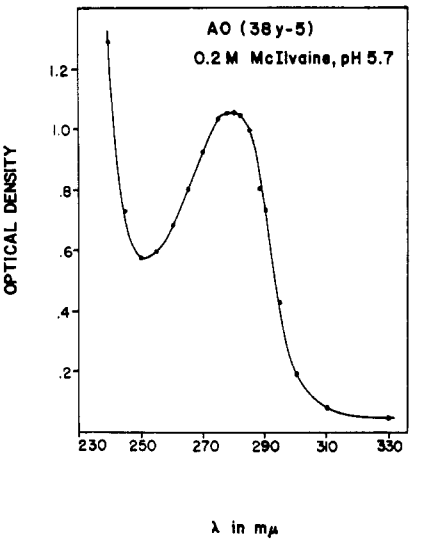

FIGURE 3: The ultraviolet spectrum of ascorbate oxidase in 0.1 M McIlvaine buffer, *p*H 5.7, showing a maximum at 280 mμ.

It has often been demonstrated, in these laboratories, that the activity of an ascorbate oxidase preparation is critically dependent on its copper content, and the activity per μg of copper increases with each stage in the purification until a maximum value is reached.

TABLE II: Cu Content of Purified Enzyme from Yellow and Green Squash.

	Source	
	Yellow	Green
Specific activity (units/mg protein)		
Freshly prepared	3600	3500
After 3 months storage (4°)	3200	2600
Protein (mg/ml)	3.5	2.4
Cu content (μg/ml)	11.4	8.2
(%)	0.33	0.35
(mole Cu/mole enzyme[a])	8	8
Activity (units/μg Cu)	1100	1030

[a] Assuming the molecular weight of the ascorbate oxidase protein as 140,000.

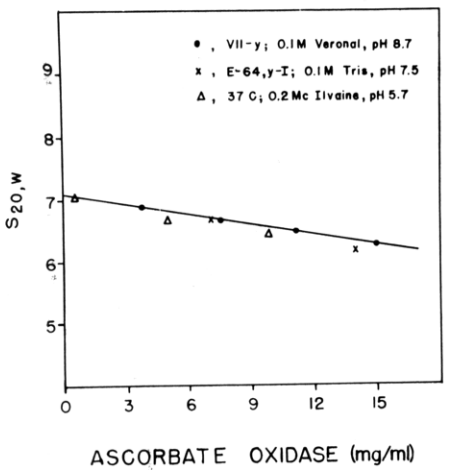

FIGURE 4: A graph of s versus c using three different preparations of purified ascorbate oxidase. All values are corrected to standard conditions of water and 20°; however, the $s^0_{20,w}$ extrapolation used only the values obtained from preparation VII-Y.

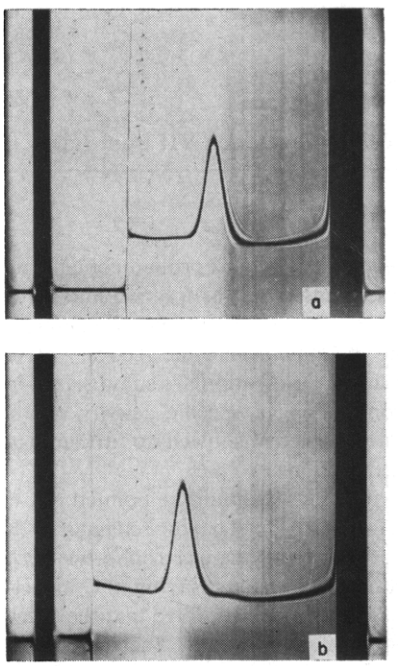

FIGURE 5: Schlieren patterns of ascorbate oxidase 38 Y II-3 and ascorbate oxidase VII-Y. (a) Ascorbate oxidase 38 Y II-3, 7.9 mg/ml in 0.1 M veronal buffer, *p*H 8.7; 48 min after reaching 59,780 rpm; bar angle 70°; T = 19°. (b) Ascorbate oxidase VII-Y, 11 mg/ml in 0.1 M veronal buffer, *p*H 8.7; 40 min. after reaching 59,780 rpm; bar angle 70°; T = 25°C.

Preparations having a specific activity of 2000–2500 units/mg of protein usually show an activity of 750–850 units/μg of copper. The copper content of such preparations has been found to correspond to 6 atoms of copper per mole of enzyme (Dawson, 1950; Dunn and Dawson, 1951; Magee and Dawson, 1962).

Properties of the Most Purified Ascorbate Oxidase Preparation

Spectrum and Copper Content. The visible and ultraviolet spectra of the most purified ascorbate oxidase preparation are shown in Figures 2 and 3. In the visible range of the spectrum it is seen to exhibit a maximum at 606–611 mμ. In the ultraviolet region the maximum occurs at 280 mμ. The absorption coefficient at 606–611 mμ was 1300/g-atom Cu.

The analyses of the amount of copper in the most purified preparations were performed on four different fractions of ascorbate oxidase: two from the yellow squash and two from the green squash, each having a specific activity of 3600 and 3500 units/mg of protein, respectively. As shown in Table II, it was found that the average copper content was 0.34%, which would be equal to 8 atoms of copper per mole of enzyme when the molecular weight of the enzyme is taken as 140,000, and the activity per μg of copper was found to be about 1000 units. It is to be noted that these preparations have significantly higher values of specific activity,

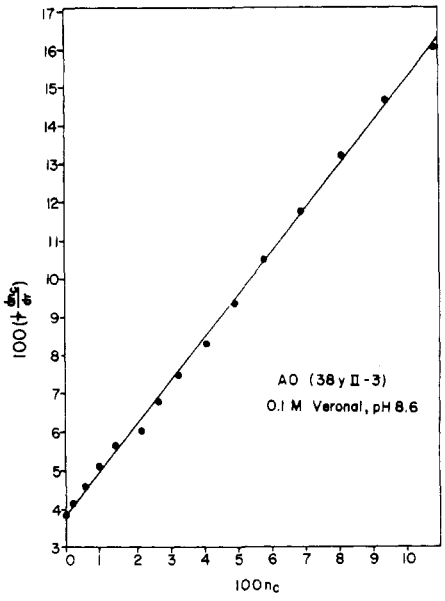

FIGURE 6: A graph of $(1/r)(dn_c/dr)$ versus n_c at equilibrium; operating speed, 8225 rpm, $T = 20°$; M_z calculated from the slope of the line between extremes.

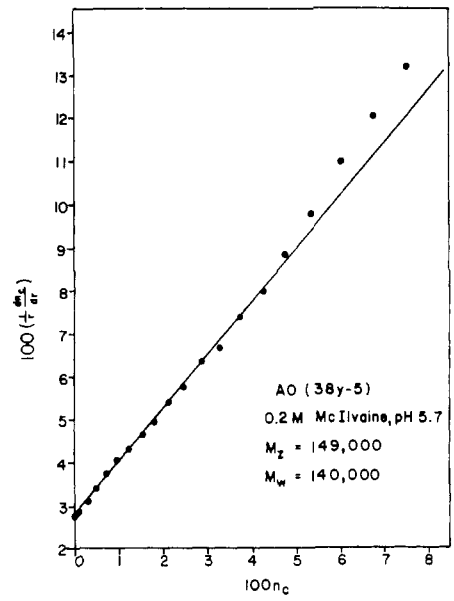

FIGURE 7: M_z calculated from the slope of the line drawn ignoring the upward curvature of the points obtained from near the bottom of the cell. Conditions as in Figure 6.

copper content, and the activity per μg of copper than earlier preparations. As indicated in Table II the Cu-containing ascorbate oxidase is relatively stable on storage in the cold.

The Cu is tightly bound to the apoenzyme. It can, however, be removed by reduction with KCN followed by dialysis against 0.02 M citrate-phosphate buffer, at a *p*H of below 4. When a dilute sample of the enzyme was used in this experiment, the copper-free enzyme became very unstable and was irreversibly denatured in a short time. However, in more concentrated samples and at neutral *p*H, preliminary experiments have shown that the apoenzyme is sufficiently stable to be partially converted to active enzyme by the addition of copper ions in the presence of ascorbic acid. Further and more recent studies have revealed that under proper conditions almost all of the catalytic activity and blue color are regained upon treatment of the apoenzyme with cuprous ion (Penton and Dawson, 1965).

Physical Properties. The sedimentation coefficient of the purified enzyme obtained from yellow squash was found to show some dependence on concentration. Figure 4 shows the $s_{20,w}$ values obtained from experiments using several different preparations of the enzyme. It can be seen from the figure that all of the values fall on approximately the same line. Further, these data indicate little or no *p*H dependence of the sedimentation rate of the enzyme through the *p*H range of 5.7 to 8.7. Extrapolation of the $s_{20,w}$ values obtained from these experiments gave a sedimentation coefficient for ascorbate oxidase at infinite dilution of 7.08 S.

Single sedimentation velocity experiments performed on two samples of the enzyme obtained from the green squash gave $s_{20,w}$ values of 6.43 S at a concentration of 6.3 mg/ml and of 6.84 S at 2.05 mg/ml. Such values indicate a general similarity of molecular properties of the enzyme obtained from the two sources.

In the majority of preparations examined a single boundary was observed in the sedimentation velocity experiments (Figure 5a). Occasionally, however, a small amount of a faster sedimenting component was observed in addition to the major component (Figure 5b). The observed sedimentation coefficient of the faster component was found to be 9–10 S.

In one preparation the identification of the 9 S component as a second molecular species of the enzyme was accomplished by an experiment which utilized a fixed partition separation cell. After a period of centrifugation this second molecular species of the enzyme was preferentially concentrated in the bottom compartment. The rotor was gradually decelerated and the solution recovered separately from the top and bottom sections. Assay of these solutions showed that the specific activity of each sample was the same, 3200 units, and the same as the original enzyme solution before centrifugation. Had the leading component been nonenzymatic, the specific activity of the enzyme from the lower compartment of the centrifuge cell would have been significantly less than that of the enzyme recovered from the upper compartment.

Molecular weights of ascorbate oxidase were calculated directly from sedimentation equilibrium experiments. Preparation 38 Y II-3 in 0.1 M veronal, *p*H 8.8, was calculated to have a *z*-average molecular weight (M_z) of 144,000. A plot of $(1/r)(dn_c/dr)$ versus n_c gave good linearity (Figure 6), indicating homogeneity of the sample. Unfortunately, supporting data for calcu-

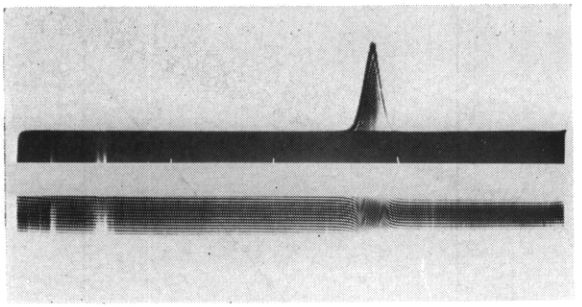

FIGURE 8: Ascorbate oxidase 38Y-5, 4 mg/ml in 0.2 M McIlvaine buffer, *p*H 5.78, after free diffusion for 583 min at $T = 3.5°$.

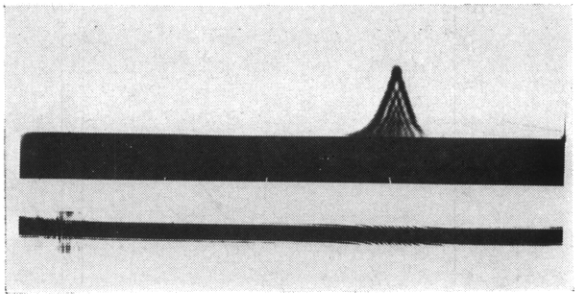

FIGURE 9: Ascorbate oxidase 38Y-5, 2 mg/ml in 0.2 M McIlvaine buffer, *p*H 5.78, after 6 hours migration at 4 ma, $T = 3.5°$.

lating a weight-average molecular weight (M_w) was not available for this same sample. A plot of $(1/r) \cdot (dn_c/dr)$ versus n_c of a second (and perhaps more typical) sample showed a significant upward curvature (Figure 7), indicating heterogeneity of the sample even though only a single boundary had been observed in both sedimentation velocity and electrophoresis experiments. The M_z value calculated from this sample was 149,000, while the M_w value was found to be 140,000. Further experimentation will be necessary in order to demonstrate conclusively whether the observed heterogeneity represents an aggregated species of the enzyme or possibly an impurity in the preparation.

A value for the diffusion coefficient of the ascorbate oxidase was obtained on two preparations using two types of experiments. A $D_{20,w}$ of 4.89×10^{-7} cm²/sec was determined from an analysis of boundary spreading as observed in the Spinco Model E analytical ultracentrifuge employing a synthetic boundary cell and the schlieren optical system. A free diffusion experiment conducted in a Spinco Model H electrophoresis apparatus employed preparation 38 Y-5 (Figure 8). Calculation of the apparent diffusion coefficient from the Rayleigh interference patterns gave a $D_{20,w}$ value of 4.95×10^{-7} cm²/sec. While the two values are in close agreement, the value obtained from the free diffusion experiment was used for calculating the molecular weight of 134,000 from combined s and D measurements, which is in reasonable agreement with the values obtained from direct measurements. If $\bar{v} = 0.75$ is used in this calculation a molecular weight of 138,000 is obtained, still somewhat lower than the 146,000 reported by Dunn and Dawson.

The electrophoretic mobility of the enzyme was measured at a single *p*H near the isoelectric point of the enzyme. As seen in Figure 9, the enzyme migrated as a single symmetrical boundary having a mobility of -1.5×10^{-5} cm²/v-sec. Although the reproduction of the fringe pattern is poor, close inspection of the original film revealed no distortion of the fringes or trace of contaminating protein of different mobility characteristics.

Discussion

Ascorbate oxidase obtained from yellow squash by the foregoing procedure may be characterized as having a molecular weight of 134,000–140,000, an $s^0_{20,w}$ of 7.08×10^{-13}, D of approximately 4.95×10^{-7} cm²/sec, and a prosthetic copper content of 8 atoms of copper per molecule of enzyme. The specific activity of such a purified enzyme averages 3300 units/mg of protein. In respect to physical parameters, these findings are in substantial agreement with the earlier results of Dunn and Dawson (1951). However, in respect to specific activity and activity per μg of copper, the new preparation is significantly more active and has a higher copper content than preparations previously described. The increased activity and copper content values obtained after starch-column zone electrophoresis show that large amounts of inactive and copper-free protein have been removed. This observation is of particular interest because of the following facts.

It will be recalled that Dawson (1950) reported a purified enzyme which had a specific activity of about 2000 units. This enzyme was shown to contain 6 atoms of copper per molecule and to have no detectible protein contaminants (Dunn and Dawson, 1951). In the present investigation it was observed that the enzyme obtained from the third DEAE-cellulose chromatographic column having a specific activity of about 2000 units also showed only a trace of detectible protein contaminant by ultracentrifugal analysis (see Figure 10). The lack of ultracentrifugal heterogeneity in these preparations is obviously explainable in terms of a contaminant which, by chance, has the same sedimenting properties as the enzyme under the particular conditions that were employed. It has recently been found that under certain conditions the copper-free and inactive apoenzyme of ascorbate oxidase is not distinguishable from the native enzyme in the ultracentrifuge (E. E. Clark, W. N. Poillon, and C. R. Dawson, paper in preparation). Furthermore it is known that by adding copper, in the proper form, to the apoenzyme essentially full activity can be recovered (Penton and Dawson, 1965). Although these observations suggest the possibility that the ultracentrifugally indistinguishable contaminant(s) may be a modified form(s) of the enzyme there is not sufficient data available at this time to substantiate such a view. Work is not in progress in these laboratories to explore the possibility that the contaminant may have a

structural and functional relationship to the enzyme.

More exhaustive studies of this enzyme will be required to produce a definitive value of the molecular weight. It is not inconceivable that 134,000 (from the *s* and *D* data) represents a more reliable value, the equilibrium values reported being the result of higher molecular weight aggregates. That the higher molecular weight material represents ascorbate oxidase protein rather than an impurity in the sample is supported by the constancy of the specific activity and copper content of all the purified samples examined. This contention is also supported by the failure to effect a differential sedimentation of the ascorbate oxidase activity when a sample containing a 9 S component was centrifuged in a fixed partition cell.

The discrepancy between the molecular weight value of ascorbate oxidase customarily cited (150,000) and the lower value reported herein (134,000–140,000) has several contributing factors: namely, the use of an enzyme of increased purity for the analyses; direct measurements of the molecular weight from equilibrium methods; and finally, information on the amino acid content of the enzyme as reported by Stark and Dawson (1962). From their values on the amino acid residues present in the enzyme, one can calculate a partial specific volume of 0.73 for ascorbate oxidase. Using this value rather than the 0.75 value assumed by Dunn and Dawson leads to a significantly lower molecular weight for the enzyme. The existing data would thus favor 134,000–140,000 as the molecular weight range of this enzyme.

The finding that the prosthetic Cu is present in amounts of 8, rather than 6, atoms of Cu per molecule of enzyme is of singular significance. Interest in the catalytic role of the Cu moiety has been heightened by the findings that as high as 85% of the prosthetic Cu is exchangeable, and that the exchange occurs only when the enzyme is in the functional state (Magee and Dawson, 1962); that the so-called "reaction inactivation" of ascorbate oxidase is probably caused by H_2O_2 formed by a slow, copper-involved but nonenzymatic secondary reaction (Tokuyama and Dawson, 1962); and most recently, the observation that only 6 of the 8 Cu atoms are enzymatically active (Poillon and Dawson, 1963a,b), Such results not only lend credence to the existence of mixed valency states of the prosthetic Cu but might also indicate specialized roles for each of the two types of Cu, one leading to the formation of H_2O, the other to H_2O_2 as end products of the oxidation. On the other hand, the oxidation of ascorbic acid could involve the transfer of electrons between the two types of Cu with H_2O_2 being formed as an incidental product of the primary reaction. These speculations underscore the need for definitive data on the molecular states of the enzyme (as any attempt to elucidate the catalytic mechanism of the enzyme must focus on the role of the prosthetic Cu).

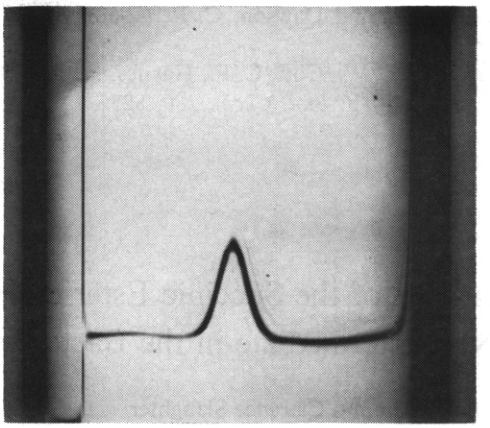

FIGURE 10: Ascorbate oxidase recovered from a third chromatography on a DEAE-cellulose column. Specific activity, 2050 units per mg protein; 4.9 mg/ml in Tris-NaCl (μ = 0.06; *p*H 7.5); 48 min. after reaching 59,780 rpm; bar angle 60°; T = 24°. Note slower sedimenting material.

Acknowledgments

The authors are indebted to Dr. W. N. Poillon for performing the Cu analyses, to Mr. S. Lewis for technical assistance with the purification of the enzyme, and to Mr. R. Hyde and the Marine Biological Laboratory, Woods Hole, Mass., for assistance and generous provision of facilities used in the physical studies.

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Synthesis of the Succinic Ester of Homoserine, a New Intermediate in the Bacterial Biosynthesis of Methionine*

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ABSTRACT: Procedures are described for the synthesis of *O*-succinyl-L-homoserine, a new intermediate in the bacterial biosynthesis of methionine; and of the racemic ester and *N*-succinyl-DL-homoserine lactone. The titration behavior, infrared spectra, and optical rotatory dispersion curves are reported, as well as analytical procedures for the determination of these compounds. In base, *O*-succinylhomoserine was rapidly converted to *N*-succinylhomoserine; the reaction prevailed over that with hydroxylamine. The reverse nitrogen-to-oxygen acyl transfer was not observed; in anhydrous acid conditions *N*-succinylhomoserine was converted to *N*-succinylhomoserine lactone. At physiological *p*H the ester was only slowly decomposed, partly by hydrolysis and partly by acyl transfer. The rates at which several different *O*-acylamino acids underwent oxygen-to-nitrogen transfer were compared as a function of *p*H and temperature. For *O*-succinylhomoserine, *O*-acetylserine, and *O*-acetylthreonine the rates were in the ratio of 1:25:200, between *p*H 7.5 and 9.5.

The first organic sulfur compound formed in the microbial biosynthesis of methionine is cysteine (Schlossman and Lynen, 1957). Sulfur must then be transferred from the 3- to a 4-carbon chain to yield homocysteine. This process, termed transsulfuration, had long been postulated from indirect evidence (Flavin, 1963) to occur by condensation of homoserine and cysteine to the thioether, cystathionine, and cleavage of the latter by β-elimination to yield homocysteine, pyruvate, and ammonia. The enzymatic synthesis of cystathionine from homoserine and cysteine was first reported 3 years ago, with extracts of a mutant strain of *Escherichia coli* (Rowbury, 1962). This synthesis appeared to involve the formation of an intermediate containing the elements of succinate and homoserine. More recently the intermediate has been positively identified as *O*-succinyl-L-homoserine, by studies of the enzymatic reactions undergone by synthetic samples of the succinic ester and amide of homoserine (Flavin *et al.*, 1964). The pyridoxal-P enzyme-catalyzing reaction (1) has been purified 1000-fold from a *Salmonella* mutant,[1] and the reaction product has been crystallized and shown to be L-cystathionine (Kaplan and Flavin, 1965). Reaction (1), a γ-replacement, is the only known example of its kind (Flavin and Slaughter, 1964). This paper reports the procedures for synthesizing the succinic ester and amide of homoserine (Flavin *et al.*, 1964), and some of the physical and chemical properties of these compounds.

O-succinyl-L-homoserine + L-cysteine $\longrightarrow$ L-cystathionine + succinic acid (1)

Experimental Procedure

Synthesis of O-Succinyl-DL-homoserine. To 25 g of DL-homoserine (210 mmoles) dissolved in 800 ml of water saturated with $NaHCO_3$ at 0° was added 38 ml (300 mmoles) of carbobenzoxy chloride. The latter was dispersed to a stable emulsion by agitating the mixture for 2 minutes at top speed in a Waring Blendor. The mixture was then placed in a 4-liter flask on a reciprocal shaker for 2 hours at 25°. After filtration of the mixture through Celite, the *p*H of the filtrate was lowered from 8.2 to 1.5 with 60 ml of 11 N HCl, and the solution, including some reaction product which separated as an oil, was quickly frozen in an ethanol–dry ice bath. The solution was thawed after overnight frozen storage, and 15.1 g of crystalline *N*-carbobenzoxy-DL-homoserine (Flavin and Slaughter, 1960) was recovered by filtration, mp 82–84°. The filtrate was lyophilized to a dry residue in which most of the remaining product had undergone ring closure to the lactone (Flavin and Slaughter, 1960), as measured by hydroxylamine assays (*vide infra*). The residue was suspended in 50 ml of water at 25° and stirred for 3 hours

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[1] M. M. Kaplan and M. Flavin, unpublished results.