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The Metabolism of Aromatic Compounds in Higher Plants

VIII. ON THE REQUIREMENT OF HYDROXYNITRILE LYASE FOR FLAVIN*

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SUMMARY

The hydroxynitrile lyases of etiolated sorghum seedlings and ground almonds have been purified to homogeneity as seen in the analytical ultracentrifuge. The sorghum enzyme preferentially catalyzed the dissociation of *p*-hydroxymandelonitrile, whereas the almond enzyme exhibited its maximum rate on mandelonitrile; *p*-hydroxybenzaldehyde and benzaldehyde, respectively, together with HCN, were the products of the reaction. The almond enzyme was found to contain a flavin prosthetic group and the sorghum enzyme did not. Physical and kinetic characteristics of the two hydroxynitrile lyases were compared and were found to be quite different.

Hydroxynitrile lyase (mandelonitrile benzaldehyde-lyase, EC 4.1.2.10) was first observed in almond emulsin by Rosenthaler (1) in 1908. The enzyme was detected by its ability to catalyze the formation of optically active (+)-mandelonitrile from hydrogen cyanide and benzaldehyde. With use of an assay which measured the rotation of the optically active cyanohydrin synthesized, Albers (2) partially purified and characterized the enzyme. A similar enzyme which is only slightly active toward mandelonitrile but which asymmetrically dissociates racemic *p*-hydroxymandelonitrile to hydrogen cyanide and *p*-hydroxybenzaldehyde has been partially purified from *Sorghum vulgare* (3).

In 1963, Becker *et al.* (4) obtained a highly purified preparation of hydroxynitrile lyase from bitter almonds. Sedimentation analysis in the ultracentrifuge, immunological studies, electrophoretic observations, and, later, crystallization (5) were presented as evidence of homogeneity. The surprising observation was made that flavin adenine dinucleotide was required as a prosthetic group. This was the first demonstration of an enzyme which required a flavin cofactor for a reaction not involving oxidation-reduction. Another enzyme, glyoxylate carboligase, which requires FAD but which does not catalyze an oxidation-reduction reaction, was reported by Gupta and Vennesland (6, 7) in 1964.

These findings suggested that the sorghum hydroxynitrile lyase might possess a flavin cofactor which had been overlooked in the earlier work (3). The sorghum enzyme has therefore been further purified to a state of homogeneity, re-examined, and found to lack a flavin component. Almond hydroxynitrile lyase was also purified for a direct comparison of its properties with the sorghum enzyme.

EXPERIMENTAL PROCEDURE

Materials and Methods

The racemic cyanohydrins of *p*-hydroxybenzaldehyde, vanillin, and isovanillin were prepared according to the method of Ladenburg, Folkers, and Major (8). Seed of *Sorghum vulgare*, var. NK210, was purchased from Northrup King Company, Fresno, California. Bitter almonds (seed from bitter varieties of *Prunus amygdalus*) were the gift of the California Almond Growers' Association, Sacramento.

The assay for sorghum hydroxynitrile lyase was modified slightly from that previously employed (3). In this procedure the increase in light absorption which occurs at 285 mμ because of the formation of *p*-hydroxybenzaldehyde is measured. In the present work a buffer 0.022 M in Na_2HPO_4 and 0.056 M in citric acid, pH 5.5, was employed in place of 0.1 M acetate buffer, pH 5.4, and 1 unit of activity was defined as that amount of enzyme which catalyzed the dissociation of 1 μmole of substrate per min. A similar assay was developed for the almond hydroxynitrile lyase, in which mandelonitrile was used as substrate and the increase in light absorption due to the formation of benzaldehyde was measured at 249 mμ. When vanillin and isovanillin cyanohydrins served as substrates, the wave lengths employed were 308 mμ and 278 mμ, respectively.

All the sedimentation experiments described were carried out in a Spinco model E analytical ultracentrifuge with phase plate schlieren optics and either the standard 12-mm cells or matched 12-mm cells with a prismatic window. Molecular weight measurements were made by using the Archibald approach to sedimentation equilibrium method as modified by Ehrenberg (9). The electrophoretic measurements were carried out in a Spinco model H electrophoresis apparatus with a Tiselius cell.

Fluorometric analyses for flavin associated with the enzymes were carried out in an Aminco-Bowman spectrophotofluorometer. Protein was determined by the method of Lowry *et al.* (10).

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TABLE I

Purification of hydroxynitrile lyase from S. vulgare, var. NK210

Step	Specific activity	Purification	Yield
	units/mg protein	*-fold*	%
I. Initial extraction	0.75	1	100
II. Acid-heat treatment	0.89	1.2	95
III. Ammonium sulfate precipitation	11.5	15.3	80
IV. Absorption on DEAE-cellulose	25.4	33.8	76
V. DEAE-cellulose column	162.0	215.0	9
VI. Sephadex G-75 column	188.0	251.0	3

Enzyme kinetic data were analyzed with the aid of an IBM 7040 computer to determine the kinetic parameters.

Purification of Sorghum Hydroxynitrile Lyase

Step I—Shoots (mesocotyl and epicotyl) of 5-day-old etiolated seedlings of *Sorghum vulgare* var. NK210 were frozen with liquid nitrogen, ground coarsely in a mortar, and then ground to a fine powder in a blender. The frozen plant material (1000 g) was then suspended in 2 liters of 0.01 M sodium phosphate buffer at pH 7.4. When the plant material had thawed, its temperature was maintained at 0–4° while it was stirred for 1 hour. This suspension was then filtered through two layers of cheesecloth, and the residue was washed with 1 liter of buffer.

Step II—The filtrate from Step I was adjusted to pH 5.4 with cold 1.0 M phosphoric acid and then heated to 60° during a period of 15 min while being swirled in an Erlenmeyer flask. The extract was maintained at 60° for an additional 10 min, and then was rapidly cooled to 4° and centrifuged for 40 min at 8000 × *g*. The precipitate was discarded. All subsequent steps were carried out at 0–4°.

Step III—The supernatant solution from Step II was adjusted to 45% saturation by the addition of solid $(NH_4)_2SO_4$, stirred for 30 min, and then centrifuged for 20 min at 30,000 × *g*. The precipitate was discarded and the supernatant fluid was adjusted to 75% saturation with additional $(NH_4)_2SO_4$, stirred for 30 min, and centrifuged as before. The precipitate from this step was taken up in approximately 300 ml of 0.01 M sodium acetate buffer, pH 5.4, and stirred for 30 min to redissolve the protein. The denatured protein which did not dissolve was removed by centrifugation for 20 min at 30,000 × *g*. The supernatant solution was then dialyzed against three changes of 4 liters each of 0.01 M acetate buffer, pH 5.4, for a total of 24 hours and again centrifuged for 20 min to remove a small amount of denatured protein which formed.

Step IV—The dialyzed enzyme preparation obtained in Step III was mixed with 5 g of diethylaminoethyl cellulose suspended in 50 ml of 0.01 M acetate buffer, pH 5.4. This mixture was stirred for 15 min and then filtered on a Buchner funnel while the DEAE-cellulose was kept moist. The DEAE-cellulose was then washed three times by suspension in 100 ml of 0.01 M acetate buffer and filtered. After the final rinse, the DEAE-cellulose with the adsorbed protein was suspended in 100 ml of 0.01 M acetate buffer containing 0.5 M NaCl to elute the protein. This mixture was stirred for 15 min and then filtered. The DEAE-cellulose was then resuspended in 100 ml of the elution buffer and again filtered. The two filtrates were combined and dialyzed against three changes of 4 liters of 0.01 M acetate buffer, pH 5.4, to remove the NaCl. The enzyme solution was then centrifuged to remove denatured protein, and at this stage could be stored at −15° for several months.

Step V—The thoroughly dialyzed enzyme from Step IV was applied to a DEAE-cellulose column, 1 × 30 cm, equilibrated with 0.01 M acetate buffer, pH 5.4. After washing with acetate buffer, the protein was eluted with a salt gradient which varied linearly from 0 to 0.5 M NaCl in 0.01 M acetate buffer, pH 5.4.

Step VI—Fractions from the column possessing the highest specific activity were combined and concentrated by dialysis against a saturated ammonium sulfate solution. This material was then placed on a Sephadex G-75 column, 1 × 30 cm, for a final fractionation. Acetate buffer, 0.01 M, pH 5.4, was used to develop the column. The results obtained in a typical purification are given in Table I.

Purification of Almond Hydroxynitrile Lyase

Step I—Ground almonds (200 g) were extracted with 1 liter of 0.01 M NH_4OH for 12 hours on a metabolic shaker at room temperature. This suspension was adjusted to pH 5.0 with 1.0 M H_3PO_4 and was centrifuged for 20 min at 35,000 × *g*; the residue was discarded.

Step II—The supernatant solution from Step I was maintained at 4° and adjusted to 40% saturation with solid $(NH_4)_2SO_4$. The solution was centrifuged at 35,000 × *g* to remove the precipitate, and the resulting supernatant solution was adjusted to 60% saturation with $(NH_4)_2SO_4$. The precipitate was then collected by centrifugation, dissolved in 100 ml of buffer (pH 5.5) which was 0.022 M in Na_2HPO_4 and 0.056 M in citric acid, and dialyzed against three changes of 4 liters each of the phosphate-citrate buffer. The enzyme solution was then centrifuged at 35,000 × *g* to remove denatured protein.

Step III—DEAE-cellulose (5 g) was equilibrated with 50 ml of the phosphate-citrate buffer, pH 5.5, and added to the enzyme solution of Step II. The DEAE-cellulose with the adsorbed protein was rinsed twice with 100 ml of buffer. Elution of the protein was accomplished with two 100-ml portions of buffer, 0.5 M in NaCl. The NaCl eluate was then dialyzed against three changes of 4 liters each of the phosphate-citrate buffer and was centrifuged. The results of a typical preparation in which these three steps were followed are presented in Table II.

When examination showed that the protein obtained in Step III was not homogeneous, the dialyzed eluate from this step was placed on a DEAE-cellulose column previously equilibrated with phosphate-citrate buffer, pH 5.5. The enzyme was then eluted with a gradient of 0 to 0.5 M NaCl in the same buffer. This procedure gave a preparation which was homogeneous in the analytical ultracentrifuge and in the moving boundary electrophoresis apparatus. However, the specific activity of this sample was only 71.5 units per mg of protein, or less than the specific activity of the initial extract (98.4 units per mg of protein).

This result suggested that treatment on the DEAE-cellulose column removed a cofactor essential for maximum activity. The addition of FAD, thiamine pyrophosphate, FMN, or an ignited sample of the supernatant solution from Step III did not affect the activity of the column fraction. Addition of a boiled sample of the supernatant solution from Step III did increase the activity 1.8-fold, to yield a specific activity of 133 units per mg of protein. No further investigation into the nature of this stimulation has

been made. In the studies of the almond enzyme described under "Results," the homogeneous preparation with specific activity of 71.5 units per mg of protein is the one which has been employed.

RESULTS

Physical Properties of Purified Enzymes—A comparison of the physical properties of the sorghum and almond hydroxynitrile lyases (Table III) revealed interesting differences, the most obvious of which was their size. The sedimentation coefficients shown are the corrected values of $s_{20,w}$ obtained at pH 5.4 for the purified sorghum enzyme sample and at pH 5.5 for the almond enzyme. The sedimenting boundaries for both of the purified enzymes moved with a single symmetrical peak, as shown in Fig. 1. The average molecular weight calculated from data obtained at the meniscus during the experiments involving approach to sedimentation equilibrium did not decrease significantly with time during the course of the centrifuge run, indicating homogeneity of the preparation. Free boundary electrophoresis measurements in a Tiselius cell in buffers with pH in the range of 4.2 to 7.0 also revealed only a single migrating species in each enzyme preparation.

By way of comparison we cite the following data obtained by Becker *et al.* (4) for the almond hydroxynitrile lyase isolated from fresh almond press residues: molecular wieght, 80,000 g per mole; $s_{20,w}$ = 4.49 S; electrophoretic mobility, 5.78 × 10^{-5} cm^2 $volt^{-1}$ sec^{-1} at pH 8.53.

Prosthetic Group Analysis—Homogeneous preparations of sorghum hydroxynitrile lyase (Fraction VI) were examined for a flavin prosthetic group. The absorption spectrum of the enzyme (Fig. 2) lacked the characteristic maximum at 450 mμ due to flavin. A difference spectrum comparing the oxidized and reduced forms of the protein also showed no material absorbing at 450 mμ in the oxidized form.

Treatment with acid-ammonium sulfate by the method of Strittmatter (11) for the removal and detection of flavins yielded only negative results. Also, there was no decrease in the specific activity of the enzyme as a result of precipitation with ammonium sulfate at acid pH, showing that neither dissociation nor inactivation occurred. Moreover, no increase in specific activity resulted from incubation of the acid-treated enzyme with FAD or FMN for intervals of 5 to 60 min.

Kearney, in her studies on succinic dehydrogenase (12), found that flavin material associated with this protein could be released only after proteolytic digestion of the enzyme. Application of this method, as modified by Cerletti, Strom, and Giordano (13), did not release flavin from a homogeneous (Fraction VI) preparation of sorghum hydroxynitrile lyase.

The homogeneous almond enzyme was also examined for the presence of flavin. As shown in Fig. 2, this preparation contained material absorbing at 380 mμ and 450 mμ, which is characteristic of flavin. The absorption maxima at these wavelengths could be eliminated completely by treatment of the enzyme with sodium hydrosulfite. Acid hydrolysis (14) and quantitative determination of the flavin released gave a value of 1.11 moles of flavin as FAD per molecular weight of 82,000 for the enzyme.

In contrast to the sorghum enzyme, treatment of the almond enzyme with acidified $(NH_4)_2SO_4$ (11) did result in a decrease of 54% in the specific activity, and indicated that flavin was required. However, all efforts to reactivate the enzyme with FAD or FMN met with failure at our hands.

Substrate Specificities—In the present study the same reaction was used to study both the sorghum and almond enzymes, namely, the dissociation of an aromatic cyanohydrin to yield the corresponding aldehyde and HCN. As shown in Table IV, the two

TABLE II

Purification of hydroxynitrile lyase from bitter almonds

Step	Specific activity	Purification	Yield
	units/mg protein	*-fold*	%
I. Initial extraction	98.4	1.0	100
II. Ammonium sulfate precipitation	433.0	4.4	70
III. Absorption on DEAE-cellulose	1015.0	10.3	47
IV. DEAE-cellulose column	71.5	0.73[a]	

[a] The decrease in purification apparently results from loss of an unknown cofactor as described in the text.

TABLE III

Properties of sorghum and almond hydroxynitrile lyases

Properties	Sorghum lyase	Almond lyase
Sedimentation coefficient	6.90 S	4.45 S
Molecular weight	180,000 g/mole	82,000 g/mole
Electrophoretic mobility[a]	4.0 × 10^{-5} cm^2 $volt^{-1}$ sec^{-1}	1.52 × 10^{-5} cm^2 $volt^{-1}$ sec^{-1}
Prosthetic groups	None found	1.1 mole FAD/mole protein
Specific activity[b]	188 units/mg protein (*p*-hydroxymandelonitrile)	71.5 units/mg protein (mandelonitrile)
pH optimum	5–6	5–6
K_m	7.0 × 10^{-4} M	2.9 × 10^{-4} M
V_{max}	1680 μmoles/min	787 μmoles/min
pK	4.5	5.0

[a] The electrophoretic mobilities cited are for the sorghum enzyme in 0.1 M sodium acetate buffer, pH 5.4, and for the almond enzyme in 0.01 M barbiturate buffer, pH 3, containing 0.1 M NaCl, and are therefore not directly comparable. The mobilities were calculated from movement in the ascending limb of the Tiselius cell.

[b] The specific activity was measured with the use of 2 × 10^{-4} M racemic *p*-hydroxymandelonitrile and mandelonitrile, respectively, for the sorghum and almond enzymes in buffer, pH 5.5, 0.022 M in Na_2HPO_4 and 0.056 M in citric acid.

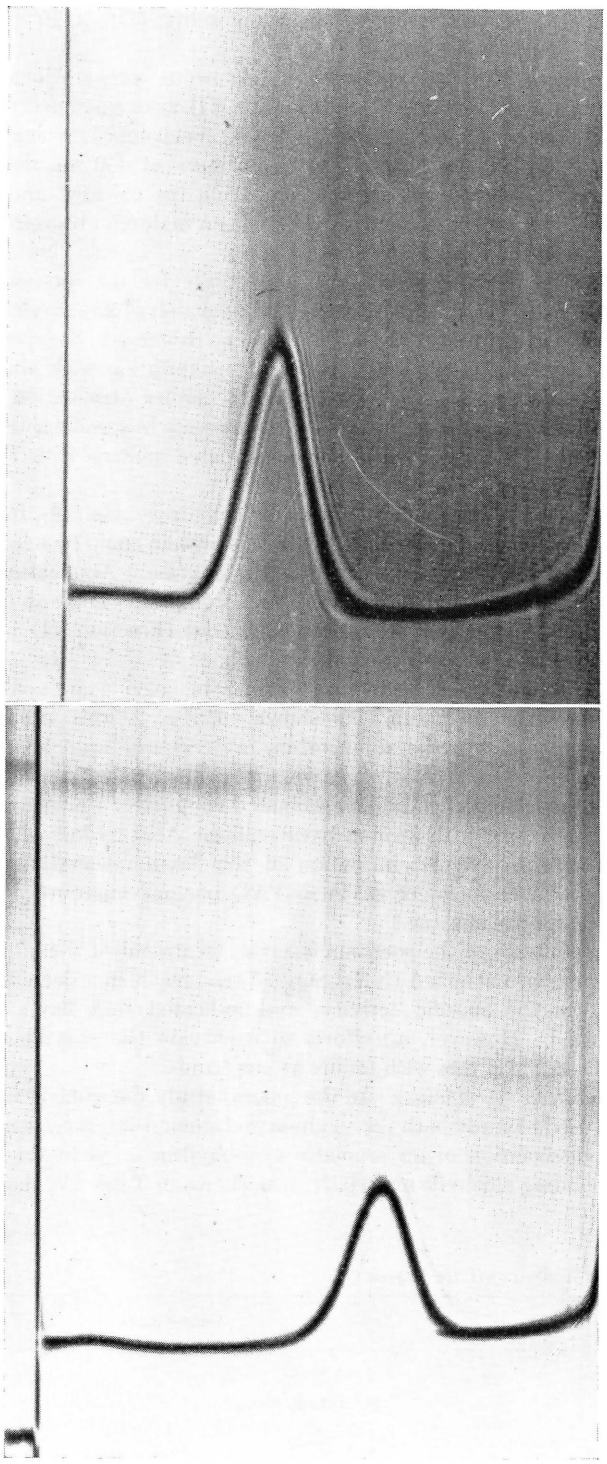

FIG. 1. Sedimentation boundaries of purified hydroxynitrile lyases from almond (*top*) and sorghum (*bottom*). Sedimentation was run at 59,780 rpm in phosphate-citrate buffer at pH 5.4 and pH 5.5 for the sorghum and almond enzymes, respectively. Photographs were taken after sedimentation for 52 min at 5°. Sedimentation is from *left* to *right*. Note that the light absorption due to the flavin moiety associated with the almond enzyme migrates with the boundary.

enzymes varied in their action toward four cyanohydrins examined as substrates. The values for K_m and V_{max} were computed by analysis with least squares plots of v against v/S. As reported previously (3), only one of the two isomers of the racemic mixtures used as substrates was acted upon by the enzymes, and no attempt was made to determine which isomer served as substrate.

In their work Becker *et al.* (4) used the assay devised by Albers (2), which is based on the polarimetric analysis of the optically active mandelonitrile formed from benzaldehyde and

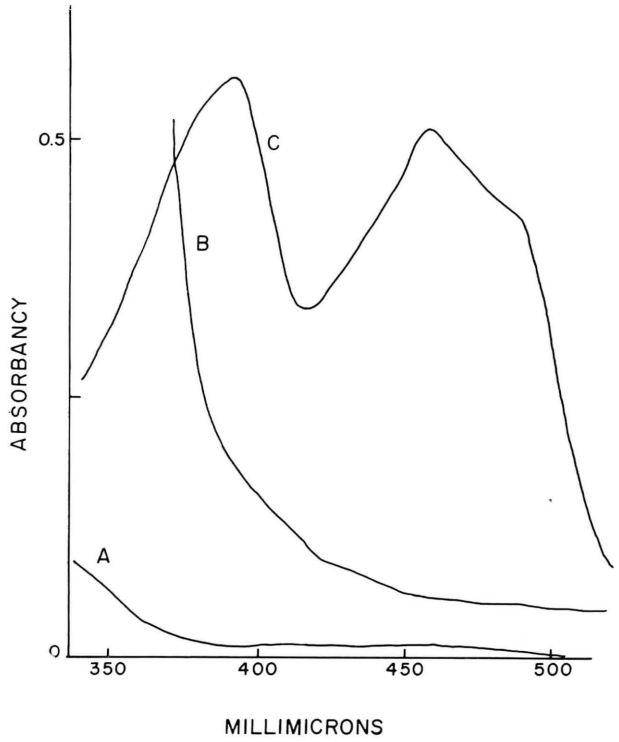

FIG. 2. Absorption spectra of purified hydroxynitrile lyases. *Curve A*, sorghum enzyme; *Curve B*, oxidized almond enzyme; *Curve C*, reduced almond enzyme. The protein concentration in all cases was 10 mg per ml.

TABLE IV

Substrate specificities of sorghum and almond hydroxynitrile lyases

Substrate	Almond enzyme		Sorghum enzyme	
	V_{max}[a]	K_m	V_{max}[a]	K_m
		M		M
dl-Mandelonitrile	47.0	2.9×10^{-4}	0.7	7.9×10^{-4}
dl-*p*-Hydroxymandelonitrile	1.8	2.0×10^{-3}	100.0	7.0×10^{-4}
dl-Vanillin cyanohydrin	6.3	4.3×10^{-3}	13.7	7.3×10^{-4}
dl-Isovanillin cyanohydrin	0.5	1.1×10^{-3}	0.1	1.2×10^{-3}

[a] One unit of relative activity represents 16.8 μmoles of cyanohydrin dissociated per min per mg of protein in buffer, pH 5.5, 0.022 M in Na_2HPO_4 and 0.056 M in citric acid. For each assay 0.3 μg of protein was used in a volume of 1 ml.

HCN. In order to dissolve the benzaldehyde, an assay mixture containing 0.01 M sodium acetate buffer, pH 5.4, in 50% ethanol was employed. We have examined the ability of the sorghum and almond enzymes purified in our laboratory to catalyze the dissociation of their preferred cyanohydrin substrates in 0.01 M acetate buffer, pH 5.4, containing 50% of ethanol. Even at the higher protein concentrations employed by Becker *et al.* (4) no cleavage occurred, and the enzyme was inactive at protein concentrations known to be active in the absence of ethanol. There was every indication that the enzymes were inactivated by the high alcohol concentration.

Inhibitors—The enzymatic activity of sorghum hydroxynitrile lyase was not affected by any of the common enzyme inhibitors. There was no evidence that any metal ion was required by the enzyme. On the other hand, the almond hydroxynitrile lyase was inhibited by iodoacetamide and *N*-ethylmaleimide. The inhibition with iodoacetamide was noncompetitive, and a K_i value of 1.49×10^{-3} M was computed as described by Dixon and Webb (15). The almond enzyme, in contrast to the sorghum enzyme, therefore apparently does require free sulfhydryl groups for activity.

Activity Characteristics—The pH values for optimum activity of both the almond and the sorghum enzymes were between 5.0 and 6.6 with all the substrates examined. The pH-activity curves at lower pH values coincide mainly with a major alteration

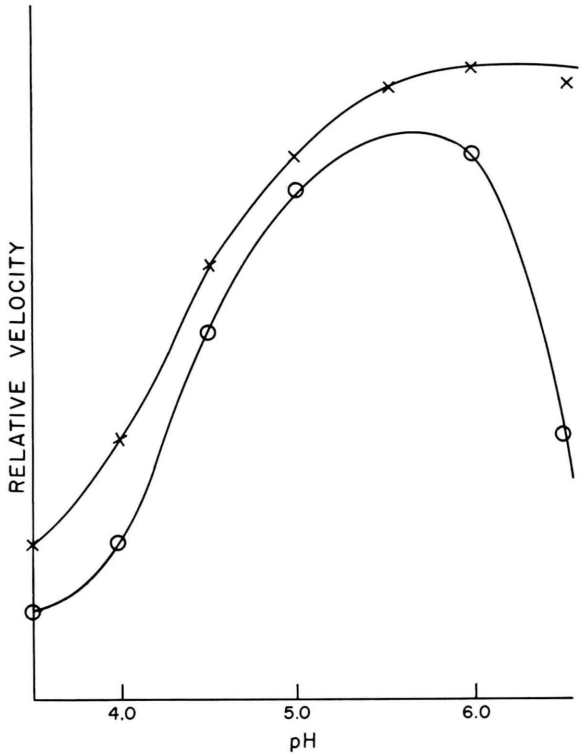

FIG. 3. Enzyme activity and stability curves for sorghum hydroxynitrile lyase. The activity curve (○——○) was obtained by assaying the enzyme at the pH indicated. The stability curve (×——×) was obtained by incubating the enzyme for 5 min at the pH indicated and then assaying at pH 5.5. In these experiments, the buffer employed was that described as citrate-phosphate buffer in Reference 16. In all experiments 0.3 μg of protein and 2×10^{-4} M *p*-hydroxymandelonitrile in a total volume of 1 ml were employed.

or destruction of the purified enzyme itself. This was shown by incubating aliquots of the sorghum enzyme for 5 min at the different pH values indicated and then assaying for activity after a short incubation at pH 5.5. The resulting enzyme stability curve is shown in Fig. 3. Note that the decrease in activity at high pH values is apparently the result of a readily reversible change affecting the active center of the enzyme but allowing complete recovery of enzymatic activity. It is interesting to note that, although enzymatic activity is lost under these rather mild conditions of exposure to acid pH, treatment with acid in the presence of ammonium sulfate at pH 1 to 2 in the attempts to remove flavin did not prevent almost complete recovery of activity.

The pK values for the enzyme-substrate complex formed by the two enzymes with their preferred substrates were obtained by plotting log V_{max} as a function of pH (17), and are given in Table III.

DISCUSSION

The experiments described in this study show that sorghum hydroxynitrile lyase does not possess a flavin type of prosthetic group. On the other hand, and in agreement with the results of Becker *et al.* (4), the almond hydroxynitrile lyase was found to contain 1 mole of FAD per mole of protein. While Becker *et al.* also established that the almond enzyme requires FAD for enzymatic activity, the results obtained in our laboratory do not permit this conclusion. Treatment with acid-ammonium sulfate did result in a loss of activity, but this activity could not be restored with FAD or FMN.

Why two enzymes as closely related as are the sorghum and almond hydroxynitrile lyases should differ in their requirement for a flavin cofactor is an interesting question. Since the sorghum protein is devoid of flavin, it may be concluded that the enzyme-catalyzed cyanohydrin dissociation does not have an absolute requirement for a flavin cofactor. This conclusion would be in harmony with the fact that the reaction catalyzed does not involve oxidation-reduction and therefore would not necessarily require a cofactor known to participate in oxidation-reduction reactions. The FAD present in almond hydroxynitrile lyase does not appear to undergo a change during the enzyme reaction. There is no change in the absorption at 457 mμ, and the fluorescence remains constant in the presence of substrate. The reaction proceeds at the same rate under either anaerobic or aerobic conditions. Gupta and Vennesland (6, 7) have also reported that the glyoxylate carboligase flavoprotein which is assayed under anaerobic conditions is not bleached in the presence of substrate.

These observations suggest that the role played by the flavin in the almond hydroxynitrile lyase is of a secondary nature. Indeed, Gupta and Vennesland (6, 7), in considering a role for the FAD found in glyoxylate carboligase, suggested that the flavin may maintain a protein conformation necessary for activity. Support for such a function for FAD is found in the case of the bacterial luciferase of *Photobacterium fischeri* (18), in which the importance of the flavin prosthetic group appears to be primarily structural. An increased amount of energy applied to the enzyme system can elicit a response even in the absence of the flavin formerly thought to be required.

In view of the size of the two enzyme molecules (sorghum hydroxynitrile lyase, 180,000; almond hydroxynitrile lyase,

80,000) it can also be suggested that the role of the FAD in the smaller (almond) enzyme is to stabilize the active site. In the larger (sorghum) protein, that role could be performed by the remainder of the protein. Experiments by Becker *et al.* (4) indicate that only the oxidized form of the prosthetic group plays a role in the enzymatic activity of almond hydroxynitrile lyase. Pfeil[1] has also suggested that FAD in the almond enzyme exerts its influence in determining specificity at the active site of the enzyme.

There are several possible explanations for the observed differences in the FAD requirement between our preparation of almond hydroxynitrile lyase and that of Becker *et al.* (4). The starting materials (freshly ground almonds and fresh press residues) employed in the two investigations were unavoidably different. However, it seems unlikely that a difference in the requirement for an essential cofactor would be dependent on varietal differences or on the manner of preparation of the plant material. The assay methods employed were not identical in these two investigations. The possibility therefore exists that the different assays led to the isolation of different enzymes. Finally, strong protein-protein interactions among cellular components could permit the simultaneous isolation of several tightly bound proteins, only one of which contains the associated flavin group. The methods of purification in this case could affect the composition of the final homogeneous preparation.

[1] E. Pfeil, personal communication.

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