# L-Phenylalanine Ammonia-lyase. I. Purification and Molecular Size of the Enzyme from Potato Tubers*

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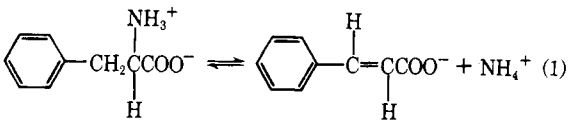
ABSTRACT: L-Phenylalanine ammonia-lyase (EC 4.3.1.5) has been extensively purified (316-fold) from extracts of acetone powders prepared from light-exposed slices of potato tubers. The extract contained two stable enzyme species, the minor species accounting for approximately 10% of the total activity. The major species was obtained free from the minor species and in a condition of near homogeneity as judged by Sephadex G-200 chromatography, sucrose density gradient centrifugation, and acrylamide gel electrophoresis. The sedimentation constant and the Stokes' radius for this species were estimated as 11.9 S and 68 Å by sucrose density gradient centrifugation and molecular sieve chromatography, respectively. These values lead to a provisional molecular weight of 330,000 and indicate that the enzyme is appreciably aspherical. The minor species of the enzyme may have twice this molecular weight.

L-Phenylalanine ammonia-lyase catalyzes the elimination of ammonium ions from L-phenylalanine to give *trans*-cinnamate (eq 1). In higher plants the



(1)

cinnamate thus formed is further metabolized to yield a great variety of phenylpropanoid compounds: lignin, flavonoids such as the anthocyanins, and conjugates of caffeic acid and *p*-coumaric acid with quinic acid, etc. The enzyme has been partially purified from several sources (Koukol and Conn, 1961; Minamikawa and Uritani, 1965; Young and Neish, 1966) and its distribution in the plant kingdom (Young *et al.*, 1966) and in an individual plant (Yoshida and Shimokoriyama, 1965) studied. It is reasonable to assume that the enzyme acts at a switching point in metabolism and diverts phenylalanine from the general pool of amino acids used in protein synthesis to the biosynthesis of phenylpropanoid compounds.

The study reported in this series of papers has been carried out with three aims in mind: (1) to obtain information at the molecular level that could be used to interpret the *in vivo* function of the enzyme, (2) to discover whether a variety of enzyme species are present in a single plant and whether the enzyme has been extensively modified in the course of evolution, and (3) to establish a mechanism for the enzyme's catalytic function.

The present paper is concerned with the size and shape of the species of enzyme present in potato tubers (Zucker, 1965). In paper II (Havir and Hanson, 1968) the main emphasis will be on the mechanism of the reaction. In paper III (Marsh *et al.*, 1968) the properties of the enzyme from maize will be described.

## Experimental Section

*Enzyme Assay and Definition of Enzyme Unit.* One unit (U) of enzyme is defined as the amount catalyzing the formation of cinnamic acid at an initial rate of 1 μmole/min at 30° under the standard assay conditions. Specific activities are expressed as milliunits per milligram of protein. The standard assay (*cf.* Zucker, 1965) was performed at the optimum pH with an amount of substrate that gave 96% of the saturation velocity. The reaction mixture (3.0 ml) contained L-phenylalanine (20 μmoles), pH 8.7 borate ($Na^+$) buffer (100 μmoles), and enzyme (3–15 mU). The blank cuvet contained buffer and enzyme. Addition of substrate initiated the reaction. Cinnamic acid formation was followed at 290 mμ; 1 μmole of cinnamic acid in 3 ml has an absorbancy of 3.0 (1-cm light path). Temperatures were measured with a 2-mm diameter cuvet thermometer (G. M. Manufacturing and Instrument Corp., New York, N. Y.) and a correction curve used to convert the observed rate into a standard rate at 30° (*e.g.*, the factor at 29.8° is 1.01). Protein was determined by the biuret method (Gornall *et al.*, 1949) or spectrophotometrically (Warburg and Christian, 1941). Both methods gave essentially the same values. The standard assay and all other initial rate measurements were carried out with a Beckman DU 2 spectrophotometer fitted with temperature-control plates and a deuterium lamp.

*Fractionation of Sephadex G-200.* About 6% of the spheres of water swollen Sephadex G-200, 40–120-μ diameter (Pharmacia, New York, N. Y.), have diameters

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less than 50 $\mu$. These spheres constitute a minute fraction of the gel volume and contribute little to the fractionating power of a column, but they seriously limit the flow rate. A fractionated Sephadex G-200 was prepared as follows (see Figure 1). Sieving the dry powder through a 140 mesh sieve (105 $\mu$) and then repeatedly through a nylon net with 70-$\mu$ openings gave fractions greater than 96-$\mu$, 50–96-$\mu$, and less than 50-$\mu$ diameter (8:46:43, w/w). The medium fraction still contained an appreciable number of aggregated small particles. A suspension of swollen medium-fraction gel (180 ml of settled volume) was then washed for 2 days at an upward-flow rate of 2.34 cm/min (8 ml/min) with distilled water in a 250 × 2 cm tube (*cf.* Whitaker and Strasdine, 1963; Hamilton, 1958). The product contained less than one sphere with a diameter of 50 $\mu$ or less per 100 particles.

*Sephadex Analytical Column for Stokes' Radii Determinations.* Separations were performed in a 100 × 1.2 cm Chromaflex chromatography tube (Kontes Glass Co., Vineland, N. J.) with nylon net secured above the sintered-glass disk and a fine Teflon tubing leading directly from the small space below the disk. The column was poured as a thick suspension and buffer passed through the column for 2 days (3 ml/hr, 5-cm head). A section of a polypropylene, 5-ml disposable syringe covered at the lower end with nylon net was then secured in the top of the chromatography tube with the net in contact with the gel. Samples (*e.g.*, 0.4 ml) were layered onto this net after buffer had been removed. When the sample had been taken up a small volume of buffer was added and withdrawn. This ensured a sharp front and back to the sample. The development was 3 ml/hr with a head less than 20 cm. About six to eight runs could be obtained without repouring the column.

*Disc Electrophoresis on Polyacrylamide Gel.* A modification of the technique of Ornstein and Davis (1964; see directions supplied by Canalco, Bethesda, Md) was employed. A 4% (2% cross-linked) gel gave optimum separations. There appeared to be no advantage in using stacking gel. The sample was applied in a small volume, *e.g.*, 20 $\mu$l, 60 $\mu$g, and mixed with 10 $\mu$l of 25% sucrose. Enzyme was recovered after electrophoresis by subdividing the gel and leaving the final 2-mm slices at 0° overnight in 0.2 ml of 0.2 M borate ($Na^+$) buffer (pH 8.7). The recovery of activity was never more than 30%. The position of the protein bands was determined by staining gel from a parallel run.

## Results

*Enzyme Purification.* Freshly cut slices of potato tubers have no detectable phenylalanine ammonia-lyase activity. When the slices are exposed to light, the enzyme's activity develops until it reaches a maximum between 20 and 30 hr (Zucker, 1965). Slices left in darkness also develop such activity but to a much lower degree. The following purification (see Table I) uses acetone powders of light-exposed tuber slices as the starting material. An alternative method for extracting the enzyme has recently been devised (Zucker, 1968). The light-exposed slices are ground in 0.1 M borate ($Na^+$) buffer (pH 8.7) containing mercaptoethanol. We have purified the enzyme severalfold beginning with such extracts.

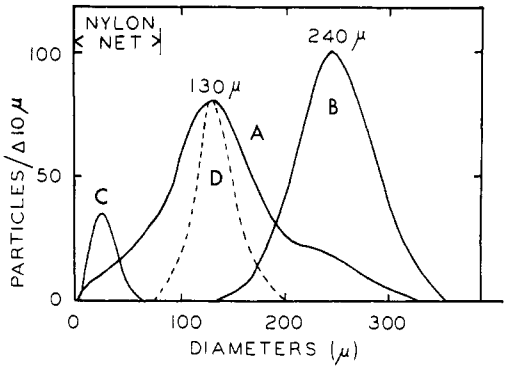


FIGURE 1: Size distribution of water swollen Sephadex G-200 spheres. (A) Unfractionated Sephadex G-200 (40–120 $\mu$); (B) after sieving and then removing fines by hydraulic fractionation; (C) fines removed by hydraulic fractionation from sieved material; (D) curve B redrawn for a most probable diameter of 130 $\mu$ instead of 240 $\mu$. Curves A and B show distributions for 1000 particles, C for 100 particles. The mesh size of the nylon net used for dry sieving and for supporting the columns of fractionated gel is indicated (70 $\mu$). Note that the large diameter shoulder of curve A corresponds to at least 40% of the volume of the unfractionated gel. Diameters were measured with a microscope.

All of the following operations were carried out at 0–4° unless otherwise indicated. The borate ($Na^+$) buffer employed had a pH of 8.7 (0.1 or 0.2 M). Centrifugations were performed at 2° in an A-2 Beta-Fuge (Lourdes Instrument Corp., Brooklyn, N. Y).: VRA Rotor; 1000*g*, 3000 rpm; 7100*g*, 8000 rpm. Calcium phosphate gel was prepared according to the method of Keilin and Hartree (1938).

*Light Exposure of Tuber Slices.* Slices 1 mm thick were cut from peeled potato tubers (*Solanum tuberosum* L., Kennebec), washed with tap water until the wash water was no longer cloudy, then washed once with distilled water. The washed slices were set out in white plastic trays (Columbian Co., Terre Haute, Ind.), neomycin sulfate solution (50 mg/l., 70 ml/ft$^2$) was added, and the trays were covered with transparent plastic screens. The slices were then exposed to fluorescent lights (500 ft-candles) at 21° for 22 hr. The slightly yellow slices were washed once with distilled water, shaken in a wire basket to remove water, weighed into plastic bags (450 g/bag), and cooled in ice. The amount of enzyme activity obtained from a given fresh weight of tissue decreased by about 30% during a 7-month period of potato storage. Potatoes stored at 4° for over 7 months were unsuitable for use as they blackened and became flaccid under the above conditions.

*Acetone Powder.* Tuber slices (450 g) were rinsed with cold acetone and homogenized (1 min, 55% line voltage) in an explosion proof Waring Blendor with acetone (520 ml) previously cooled to −20°. This work was carried out in a fume hood. The powder was collected by filtration and transferred into a second blendor

TABLE I: Purification of L-Phenylalanine Ammonia-lyase from Light-Exposed Slices of Potato Tuber.[a]

| Fractionation Step | Total Protein (mg) | Activity: Total (U = μmole/min) | Activity: Recov (%) | Activity: Sp Act. (mU/mg) | Purifcn/Step (-fold) |
|---|---|---|---|---|---|
| 1. Extraction and protamine treatment | 12,800 | 32.0 | 100 | 2.5 | |
| 2. $(NH_4)_2SO_4$ fractionation (27–40%)[a] | 1,870 | 19.7 | 62 | 10.5 | 4.2 |
| 3. Calcium phosphate gel treatment, pH 5.5; adsorption and elution | 575 | 14.4 | 45 | 25 | 2.4 |
| 4. $(NH_4)_2SO_4$ fractionation (27–40%) | 280 | 11.5 | 36 | 41 | 1.6 |
| 5. DEAE Sephadex column | 28 | 7.05 | 22 | 250 | 6.1 |
| 6. Sephadex G-200 column | 8.1 | 6.40 | 20 | 790 | 3.2 |
| | | | | Total purification | 316-fold |

[a] Tissue (2 kg fresh wt) gave 2.5 kg of light-exposed slices, which gave 138 g of sieved acetone powder. [b] The absorbancy ratio 280/260 mμ increased from 1.6 at step 2 to 1.7 at step 6.

vessel maintained at −20°. A second portion of tuber slices was similarly treated and the combined powders were homogenized (0.5 min) in cold acetone (520 ml). The powder was collected by filtration, rinsed with cold acetone (250 ml), and air dried. Starch grains were sieved from the powder with the aid of a 100 mesh sieve (149 μ). This reduced the weight by about 50%. The powder was stable for at least 1 year when stored at −20° in sealed jars.

STEP 1. EXTRACTION AND PROTAMINE TREATMENT. Acetone powder (138 g) was extracted (30 min) with a freshly made solution of reduced glutathione (600 mg) in 0.1 M borate buffer (2500 ml). The suspension was then strained through four layers of cheesecloth and 1 M acetate ($Na^+$) buffer (pH 5.0) (*ca.* 250 ml) was added to the stirred cloudy filtrate until the pH was 5.5 (Accutint 5.2–6.9 paper, Anachemia, Ltd., Montreal). Protamine sulfate solution (1 g plus 4 ml of 1 M acetate ($Na^+$) buffer (pH 5.0) diluted to 50 ml) was then added. After the mixture had been stirred (10 min), the precipitate was removed by centrifugation (15 min, 7100*g*). The clear supernatant solution (2178 ml; 5.9 mg of protein/ml) was not suitable for storage. Such solutions loose 30% or more of their activity in 5 hr at 0°.

STEP 2. FIRST AMMONIUM SULFATE FRACTIONATION (27–40%). To the above solution (2178 ml), $(NH_4)_2SO_4$ (415 g) was added with stirring (30 min) and stirring was continued for a further 30 min. The precipitate, removed by centrifugation (10 min, 7100*g*), was discarded. This operation was repeated with more $(NH_4)_2SO_4$ (200*g*) and the centrifuged precipitate was dissolved in the minimal volume of cold 0.2 M borate buffer. The solution (34.6 ml; 54 mg of protein/ml) was frozen and stored at −20°. The recovery of activity varied from 60 to 80% and the purification from 3.5- to 4.5-fold.

STEP 3. CALCIUM PHOSPHATE GEL: ADSORPTION AND ELUTION. The solution from step 2 was diluted with water (90 ml, 15 mg of protein/ml) and the pH of the stirred solution was adjusted to 5.5 (indicator paper) with 1 M acetic acid (*ca* 1.3 ml). Water-washed calcium phosphate gel (29 ml, 19 mg dry wt/ml) was then added, and stirring was continued for 15 min. The gel was collected by centrifugation (5 min, 1000*g*) and the supernatant was treated with a further volume of gel (98 ml) and centrifuged. In most preparations the supernatant solution from this second gel treatment had negligible activity. The two portions of gel were treated separately. In each case the gel was suspended in cold water (first gel, 50 ml; second gel, 100 ml), stirred occasionally (1 hr), and then centrifuged (1000 g). The supernatant solutions contained protein but little enzyme. A series of extractions was then performed with cold 0.2 M borate buffer (first gel, 50 ml; second gel, 100 ml/extraction). The fractions containing enzyme with a specific activity two- or fourfold higher than the starting material were combined (303 ml; 1.9 mg of protein/ml) and stored at −20°.

STEP 4. SECOND AMMONIUM SULFATE FRACTIONATION (27–40%). Enzyme from step 3 was treated as in step 1 (303 ml, 58 g of $(NH_4)_2SO_4$, then 28 g of $(NH_4)_2SO_4$). The solution of the second precipitate which usually contained all of the activity (7.8 ml; 36 mg of protein/ml) was frozen and stored at −20°.

STEP 5. CHROMATOGRAPHY ON DEAE SEPHADEX. A column 30 × 4 cm was prepared from DEAE Sephadex A-50 (Pharmacia, New York, N. Y.) which had previously been equilibrated (24 hr) with 0.05 M phosphate buffer (pH 6.8). Enzyme from step 4 (7.8 ml) was added to the column and the column was washed with the equilibrating buffer until a band of protein had been eluted. As much as 30% of the enzyme units added to the column was recovered in this band. The column was further eluted with phosphate buffer (pH 6.8) in a steadily increasing concentration (0.5 M buffer to 250 ml of 0.05 M buffer in a constant-volume mixing vessel). The enzyme appeared after 200 ml of effluent had been collected. Fractions with specific activities four- to fivefold greater than the starting

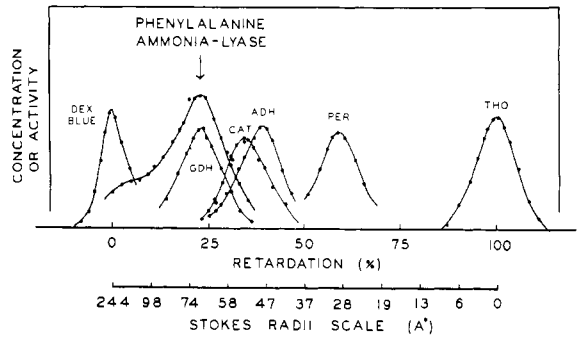


FIGURE 2: Molecular sieve chromatography of partially purified phenylalanine ammonia-lyase on the analytical Sephadex G-200 column. Buffer: sodium pyrophosphate–HCl, 0.1 M, pH 8.5. DEX BLUE, Dextran Blue (Pharmacia); PER, horseradish peroxidase; ADH, yeast alcohol dehydrogenase; CAT, beef liver catalase; GDH, beef liver glutamate dehydrogenase; THO, tritiated water. For further details, see Table II.

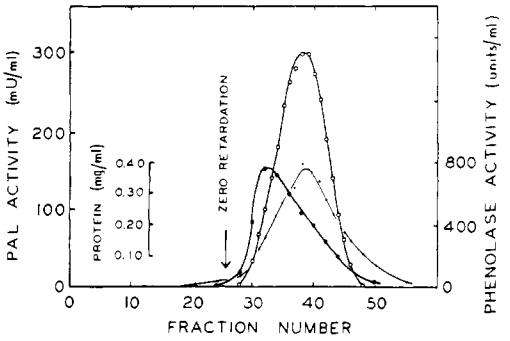


FIGURE 3: Rechromatography of purified phenylalanine ammonia-lyase (major species) on the analytical Sephadex G-200 column. (○) Ammonia-lyase activity; (·) protein concentration; (●) contaminating phenolase activity. Phenolase was assayed by following the oxidation of ascorbic acid spectrophotometrically (265 mμ) with chlorogenic acid as the enzyme substrate (Sisler and Evans, 1958; Patil and Zucker, 1965). The specific activities of the ammonia-lyase of fractions 32–41 (53% of total) lay within the region 730–790 mU/mg (*cf.* Table I). Total protein, 8 mg.

material were combined (95 ml, 0.29 mg of protein/ml). The protein was precipitated by $(NH_4)_2SO_4$ (33.5 g) as in step 2. The precipitate was collected by centrifugation and then dissolved in the minimal volume of cold, 0.1 M borate buffer (1.3 ml; 22 mg of protein/ml).

STEP 6. GEL FILTRATION WITH SEPHADEX G-200. A column 45 × 4 cm of fractionated Sephadex G-200 was prepared and the column was washed with two bed volumes of 0.1 M pyrophosphate ($Na^+$) buffer (pH 8.5). After enzyme from step 5 (1.3 ml) had been added, the column was developed (10 ml/hr) with the same buffer. Fractions with specific activities 2.5-fold or more greater than the specific activity of the starting material were combined (65 ml; 0.125 mg of protein/ml). This material contained very little of the larger molecular weight form of the enzyme found in the leading fractions of the column (see below). Protein was precipitated with $(NH_4)_2SO_4$ (29 g) as in step 2. The precipitate, collected by centrifugation, was dissolved in cold, 0.1 M borate buffer (1.0 ml; 8.1 mg of protein/ml) and stored at −20°. Some loss in total and specific activity was observed as a result of the precipitation and on occasion 30% of the enzyme units was lost. The activity of the frozen concentrated enzyme was essentially unchanged for at least 6 weeks, whereas the dilute enzyme from the column lost activity when stored at 4 or −20°. The leading fractions containing high molecular weight enzyme were stored at −20°.

*Enzyme Stability*. The purified enzyme is not readily denatured. In general, there is little or no loss of activity at 25° from pH 7 to 10 over a period of several hours. Between pH 7 and 5.5, there is a slow loss of activity at 0° which is about 20% over several days. Below pH 5.5 the loss of activity occurs rapidly, as much as 50% in several hours. The enzyme retains 90% of its activity after heating for 5 min at 55° and pH 8.7. If the pH is lowered to 5.5, the loss of activity is at least 40% under the same conditions. The loss of activity at acid pH and on heating is irreversible.

*Homogeneity*. Partially purified phenylalanine ammonia-lyase may be resolved into two species differing markedly in size: a major species with a molecular weight of the order of 330,000, and a minor species with at least twice this molecular weight. This assertion is based on the following experiments.

Chromatography of partially purified preparations of the enzyme on the Sephadex G-200 analytical column in pyrophosphate or borate buffer showed a major peak of activity with a leading shoulder (Figure 2). When borate buffer was used the resolution was not as great and the shoulder was less noticeable (see next section). Sucrose density gradient centrifugation also showed a major peak with a small leading shoulder.

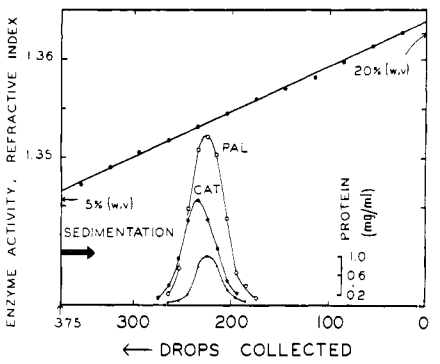


FIGURE 4: Sedimentation of purified phenylalanine ammonia-lyase (major species) in a linear sucrose gradient (see Martin and Ames, 1961). The gradient was established from 5 to 20% solutions of sucrose in 0.1 M sodium pyrophosphate–HCl buffer (pH 8.7) (w/v). Centrifugation was carried out with a Beckman Model L preparative ultracentrifuge and a SW-50L rotor at 39,000 rpm and 2° for 6 hr. Fractions of 10 drops each were collected after the run. The linearity of the gradient was checked by measurements of refractive index on 10-μl aliquots of every third fraction. Three tubes were run at one time. Tubes 1 and 2 contained phenylalanine ammonia-lyase (280 mU, sp act. 528 mU/mg) and tubes 1 and 3 catalase (0.03 mg, 750 U). The figure shows phenylalanine ammonia-lyase (PAL) and catalse (CAT) activities (assay: Beers and Sizer, 1952) for tube 1 (the parallel runs were closely similar) and protein concentrations for tube 2 (assay: Lowry *et al.*, 1951).

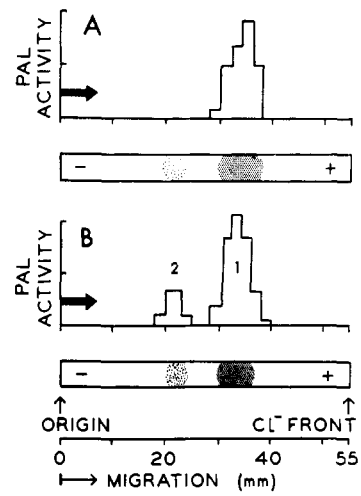


FIGURE 5: Disc electrophoresis of phenylalanine ammonia-lyase (PAL) on 4% (2% cross-linked) polyacrylamide gel. Separation takes place in glycine–Tris buffer at pH 8.9 (reported value, see Experimental Section). (A) Purified enzyme, major species only (as judged by G-200 chromatography and sucrose density gradient centrifugation). (B) Partially purified enzyme enriched in the minor species (peak 2). The lower portions in each section indicate the regions of protein staining.

When material purified as described above and corresponding to the major peak from the Sephadex column was rechromatographed, a single symmetrical peak of activity was observed (Figure 3). The activity peak and the protein peak coincided. The same material examined by sucrose density gradient centrifugation (Figure 4) showed a single symmetrical peak, and again the activity peak and the protein peak coincided. Rechromatography of material corresponding to the shoulder region from the Sephadex column (Figure 2) gave a peak of activity which corresponded in position to the original shoulder.

The two species appear to be resolvable by polyacrylamide gel electrophoresis. On electrophoresis the purified major species used in the Sephadex and and density gradient experiments showed a single region of phenylalanine ammonia-lyase activity and this region corresponded to the major protein band (Figure 5A). A trace of slower moving protein was present. Electrophoresis of material from the shoulder region of the G-200 column showed two bands of activity and two protein bands (Figure 5B). The larger species thus migrated more slowly. Such a result is in keeping with the molecular sieve function of the polyacrylamide gel. Similar differences in migration rate were observed when partially purified enzyme was subjected to sucrose density gradient centrifugation and material corresponding to the peak and shoulder regions examined by electrophoresis.

Two conclusions follow from the above experiments. (1) Some 80% of the protein of the best preparations of the major component is probably phenylalanine ammonia-lyase. (2) The two species of enzyme are stable and not in dynamic equilibrium under normal conditions of manipulation.

The two species are also stable to treatment by moderate concentrations of urea. When a partially purified preparation of the enzyme was left at 22° for 3 hr in 5 M urea and the solution then diluted and assayed, a 13% loss of activity was observed, but sucrose density centrifugation of this enzyme under the conditions employed previously, *i.e.*, without adding urea to the sucrose–pyrophosphate buffer solutions, showed that the large species was still present in essentially the same proportion as in the original preparation.

The possibility was considered that the minor species represented a complex of ribonucleic acid with the small species as the 280/260 mμ ratio was 1.4, compared with 1.7 for the major species. Overnight treatment of a partially purified sample of the enzyme with crystalline bovine pancreatic ribonuclease (Sigma) did not alter the position or the size of the shoulder observed on sucrose density gradient centrifugation and it is thus very unlikely that nucleic acid is associated with either species.

Phenolase (EC 1.10.3.1) activity was associated with the enzyme throughout its purification. The highest specific activity of the phenolase associated with the purified major component was about 18% of that reported for a purified potato phenolase fraction by Patil and Zucker (1965). The possibility was therefore considered that some species of phenolase molecule was permanently bound to the phenylalanine ammonia-lyase, *i.e.*, a multienzyme complex was being purified rather than a single species. On sucrose density gradient centrifugation of the purified major species the phenolase activity was skewed toward the lower molecular weight side of the ammonia-lyase peak and on Sephadex G-200 chromatography (Figure 3) toward the higher side. On polyacrylamide gel electrophoresis phenolase was found in all fractions up to 5 cm from the origin. We conclude, therefore, that the phenolase is a contaminant and that the two activities are, in principle, completely resolvable without changing the properties of the ammonia-lyase. Jolley and Mason (1965) have shown that mushroom phenolase has a marked tendency to undergo self-association and dissociation and it seems probable that the difficulty in removing phenolase from the ammonia-lyase is the result of such phenomena.

The purified major species was also examined for other enzyme activities which might conceivably be associated with phenylalanine metabolism *in vivo*. Neither quinate dehydrogenase (Gamborg, 1966) nor phenylalanine-2-ketoglutarate aminotransferase (Gamborg and Wetter, 1963) could be detected in samples of the purified major species containing 20 mU of phenylalanine ammonia-lyase.

*Molecular Size and Shape.* SEDIMENTATION CONSTANT DETERMINATIONS. From sedimentation runs in sucrose density gradients (Figure 4) the mean $s^{0.725}_{20,w}$ value and standard error of the mean for the major species of ammonia-lyase was 11.93 ± 0.11 S (*N*, the number of observations, = 6). Beef liver catalase was employed as a standard ($s^{0.725}_{20,w}$ = 11.30 S; Martin and Ames, 1961; Sumner and Gralen, 1938). When the true partial specific volume of the protein is known it will be possible to calculate a true $s_{20,w}$ value (Martin and

TABLE II: Stokes' Radius Determinations by Molecular Sieve Chromatography on Fractionated Sephadex G-200.[a]

| Proteins | Retardation (%) 1 | 2 | 3 | 4 | 5 | 6 | 7 | Mean $a/r$ (%) | Ref[b] Stokes' Radii ($a$) (Å) | Calcd Pore Size ($r$) (Å) | Calcd Stokes' Radii ($a$) (Å) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Pyrophosphate buffer, 0.1 M, pH 8.5, mean void volume 39.2 ml, mean internal volume 79.4 ml | | | | | | | | | | | |
| Phenylalanine ammonia-lyase | 23.7 | 20.2 | | 22.1 | 23.1 | | | 28.8 | | | 70 |
| Glutamate dehydrogenase | (22.4 | 22.0 | 22.4 | 22.1 | 22.9) | | | (28.9) | | | (71) |
| Catalase | 36.4 | 33.4 | 36.4 | 31.8 | 35.0 | | | 21.6 | 52.1 | 241 | |
| Alcohol dehydrogenase | 41.7 | 37.9 | | 37.7 | 41.2 | | | 19.4 | 45.5 | 236 | |
| Peroxidase | | 57.9 | 60.2 | 57.2 | 60.0 | | | 11.8 | 30.3 | 257 | |
| Estimated pore radius ($r$): 244 ± 4 Å ($N$ = 13) | | | | | | | | | | | |
| Borate buffer, 0.05 M, pH 8.5, mean void volume 37.9 ml, mean internal volume 77.1 ml | | | | | | | | | | | |
| Phenylalanine ammonia-lyase | 11.8 | 15.0 | 13.1 | 13.7 | 15.0 | | | 36.0 | | | 66 |
| Catalase | 22.1 | 22.2 | | | | 22.4 | 22.4 | 28.8 | 52.1 | 181 | |
| Alcohol dehydrogenase | 29.8 | 27.2 | | 26.8 | 32.0 | | 30.9 | 24.4 | 45.5 | 186 | |
| Bovine serum albumin | | | | | | 38.0 | | 20.0 | 34.7 | 180 | |
| Peroxidase | | | (60.6 | 58.8 | 58.8)[c] | | | (11.6) | 30.3 | | (21) |
| Estimated pore radius ($r$): 182 ± 2 Å ($N$ = 10) | | | | | | | | | | | |

[a] Calculation: Retardation (%) = $100\,(V_e - V_0)/V_i$, where $V_0$ is the void volume, $V_i$ is the internal volume, $V_e$ is the volume of effluent collected while the protein passes from the top to the bottom of the column. Retardation values in each case were converted into $a/r$ values by means of a theoretical curve (Ackers, 1964). For the reference proteins these were further converted into $r$ values. The estimated values of $r$ are means of these, *i.e.*, each observation is given equal weight. The over-all estimates of $r$ together with the mean $a/r$ values for the ammonia-lyase (28.8 ± 1.0% and 36.0 ± 1.6%) led to the calculated Stokes' radii given in the test. [b] Stokes' radii for the reference proteins, expressed in angstrom units, were calculated from the relationship $a_{20,w} = 213.6/(D_{20,w} \times 10^7)$: glutamate dehydrogenase (EC 1.4.1.2) from beef liver (Boehringer Mannheim Corp., New York, N. Y.); assay: Olson and Anfinsen (1952), catalase (EC 1.11.1.6) from beef liver (Worthington Biochem. Corp., Freehold, N. J.); $D_{20}$: Sumner and Gralen (1938); assay: Beers and Sizer (1952); alcohol dehydrogenase (EC 1.1.1.1) from yeast (Sigma Chem. Co., St. Louis, Mo.); $D_{20,w}$: Hayes and Velick (1954); assay: Racker (1955): bovine serum albumin (Sigma); $D_{20,w}$: Creeth (1952); peroxidase from horseradish (EC 1.11.1.7), grade D (Worthington); $D_{20,w}$: Cecil and Ogston (1951); assay as Worthington catalog. [c] The behavior of peroxidase in the borate system was so out of line that these results were not used in calculating $r$.

Ames, 1961), but the correction may well be negligible compared with the observational error.

Any systematic bias in these determinations is probably small. There appeared to be no interaction between catalase and the ammonia-lyase as the distances of migration and the shapes of the peaks were identical in the control tubes run simultaneously and containing the separate enzymes. Also the rate of sedimentation of the ammonia-lyase is not a function of its natural contaminants as the same results were obtained when less purified samples of the ammonia-lyase were used (except that a shoulder of activity corresponding to the minor component was observed). As the reference enzyme and the ammonia-lyase sediment at approximately equal rates the effects of nonlinearity in the sucrose gradient or of error in estimating the meniscus position should be very small.

Enzyme prepared by extracting light-exposed tissue slices with buffer and purified to a specific activity of 45 mU/mg has also been examined by sucrose density centrifugation: $s^{0.725}_{20,w} = 12.17 \pm 0.11$ S ($N = 2$). This value does not differ significantly from that for the enzyme prepared from acetone powders. The probability is roughly three out of four that two such samples drawn from the same population would differ in their means by at least the observed amount (6 DF, $t = 0.48$).

The sedimentation constant for the minor species of ammonia-lyase, as judged from the position of the shoulder in runs with partially purified enzyme, is 16 or 17 S.

STOKES' RADIUS DETERMINATIONS. The Stokes' radius $a$ of a protein is the radius of a sphere that has the same diffusion constant, $D$, as the protein under the conditions specified. In Table II the determinations of $a$ for the major species of ammonia-lyase are recorded. A Sephadex G-200 column and a series of proteins with known $D_{20,w}$ (and therefore $a_{20,w}$) values were employed (Ackers, 1964). Giving each observation equal weight, $a$ determined in pyrophosphate buffer was 70.3 ± 1.8 Å, and in borate buffer 65.5 ± 1.5 Å. The difference between the calculated values of $a$ for the two buffer systems is clearly significant and requires to be explained. One qualitative result, however, stands out: in both buffers $a$ for the ammonia-lyase is appreciably greater than that for catalase (52 Å), although the sedimentation constants for the two proteins are almost identical.

As the enzyme shows the same catalytic activity in borate and pyrophosphate buffer (Havir and Hanson, 1968), and in the absence of other information, it may be assumed that the two values of $a$ are estimates of the same physical parameter, *i.e.*, that the size and shape of the molecule is not appreciably affected by the buffer. The retardation of the ammonia-lyase did not appear to be significantly related to the purity of the preparation or the amount of protein added to the column. The possibility must be considered, therefore, that Ackers' pore diffusion model does not apply accurately at high values of $a$. It is significant that Rogers *et al.* (1965), using pyrophosphate buffer and a Sephadex G-200 column calibrated according to the method of Ackers, estimated the radius of this enzyme to be 61Å, whereas in the present study the value 71 Å was obtained. The only obvious difference between the two studies was in the Sephadex used. Rogers *et al.* (1965) report $r = 169 \pm 5$ Å whereas we calculated $r = 244 \pm 4$ Å, thus the lower pore size is correlated with the lower estimate of $a$. Similarly, in the present study, the borate column with the lower $r$ leads to a lower value of $a$ for the ammonia-lyase. As Rogers *et al.* were led to calculate a molecular weight for glutamate dehydrogenase of 270,000, which is considerably less than the established monomer value of 400,000 (Colman and Frieden, 1966) the low values for $r$, which lead to much poorer resolution in the region of interest, may also lead to an underestimation of Stokes' radii for proteins migrating in that region. As this topic requires further investigation we shall adopt an average value of 68 Å as a provisional estimate.

MOLECULAR WEIGHT AND FRICTIONAL RATIO. From the above values of $s$ and $a$ a provisional molecular weight may be calculated using beef liver catalase as a reference ($M = 244{,}000$: Sumner and Gralen, 1938; Samejima and Shibata, 1961), and ignoring differences in the partial specific volumes of the reference protein and the ammonia-lyase. The equation $M_1/M_2 = (a/a_2)(S_1/S_2)$ leads to the estimate $M = 330{,}000$ for an average value of $a$ and $M = 340{,}000$ for the value of $a$ found in the pyrophosphate buffer experiments.

A value for the frictional ratio, $f/f_0 = a/a_0$, may also be calculated where $a_0$ is the radius of an unhydrated sphere with the same molecular weight as the enzyme. If the partial specific volume is 0.725, then $f/f_0$ is of the order 1.4–1.5 compared with 1.25 for catalase (Sumner and Gralen, 1938). If it is assumed that the partial specific volume and the degree of hydration of the molecule are not unusual, then the qualitative conclusion may be drawn that the ammonia-lyase is appreciably aspherical.

## Discussion

The above results establish the presence of two species of phenylalanine ammonia-lyase in extracts of light-exposed potato tuber tissue. The same major species is obtained both by extraction of acetone powders made from the slices and by direct extraction with buffer of the slices. The best preparations of the major species, molecular weight of *ca.* 330,000, had a specific activity of 790 mU/mg and the methods of separation employed suggest that the contaminating proteins associated with the enzyme do not amount to more than 20% of the total protein. The specific activity of the purified enzyme is considerably higher than the specific activities reported for preparations from other sources (55 mU/mg or less; Koukol and Conn, 1961; Young and Neish, 1966; Minamikawa and Uritani, 1965), but is lower than the specific activities reported for purified enzymes catalyzing analogous reactions: *threo*-β-methyl-L-aspartate ammonia-lyase, 280 U/mg at 25° (Hsiang and Bright, 1967); aspartate ammonia-lyase, 35 U/mg (Williams and Lartigue, 1967); histidine ammonia-lyase, 35 U/mg (Williams and Hiroms, 1967); and arginosuccinate arginine-lyase, 15 U/mg at 38° (Havir *et al.*, 1965).

It is unclear exactly how much of the activity of the enzyme in the initial extracts is attributable to the minor species. The activity of these extracts is too low and too unstable for examination, but for enzyme purified through step 3, 10% of the activity was that of the minor species. Although its size cannot be estimated with accuracy it is sufficiently large and stable that the molecule could be formed by a chemical linkage between two molecules of the smaller species. Indeed the reduction in activity observed when potato tuber slices are exposed to light for prolonged periods (Zucker, 1967) could be the result of such coupling. Minamikawa and Uritani (1965) reported that sweet potato contains two species of phenylalanine ammonia-lyase resolvable by DEAE-cellulose chromatography, but it is not known whether these species differ in size.

A protein with a molecular weight of 330,000 is undoubtedly composed of subunits. In considering the possible regulatory functions of the enzyme it is important to know whether the purified major species is a mixture of isozymes. Evidence that this is not the case will be discussed by Havir and Hanson (1968) in part II.

If a single protein is under examination, then the observed high frictional ratio may give an indication of the enzyme's subunit structure. Proteins formed from more than two protomers are likely to be constructed in the form of isologous rings (Hanson, 1966) and it is conceivable that the high frictional ratio for the enzyme arises because the enzyme is doughnut shaped.

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