L-Phenylalanine Ammonia-lyase (Maize and Potato). Evidence That the Enzyme Is Composed of Four Subunits†

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ABSTRACT: An electrophoretically homogeneous enzyme (EC 4.1.3.5) from the leaf-sheath tissue of maize shoots (*Zea mays* L.) gave only a single protein band upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol. The only significant band on treating highly purified enzyme from light-exposed slices of potato tubers (*Solanum tuberosum* L.) had a similar mobility. The radioactivity was associated with this band on sodium dodecyl sulfate gel electrophoresis of enzyme specifically labeled at the active site by sodium [$^3H_4$]borohydride reduction or by [^{14}C]nitromethane addition. Subunit molecular weights were estimated from relative mobility data. For both proteins the subunits, if not identical, differ in weight by <5%. The best estimate of the mean of the subunit molecular weights for potato and maize was 83,000 and of the difference, potato minus maize, was 1100 with 95% confidence limits of 3600 and −1400. Previous estimates of the molecular weights of the two enzymes and of the mean of these fell well within the range of 95% confidence calculated for a tetrameric molecule. The extent of labeling with [^{14}C]-nitromethane suggested that there are two active sites per fully active enzyme tetramer. The amino acid compositions of the enzymes are reported.

Phenylalanine ammonia-lyase (EC 4.1.3.5) catalyzes the elimination of $-NH_3^+$ and the (*pro*-3*S*)-hydrogen from L-phenylalanine to give *trans*-cinnamate. It is widely distributed in higher plants and also occurs in fungi. In higher plants it diverts L-phenylalanine and, in certain species, L-tyrosine to the biosynthesis of phenylpropanoid compounds. These include lignin, flavonoids, such conjugates of the hydroxycinnamic acids as chlorogenic acid, and certain alkaloids. The appearance of the enzyme's activity has been related to development, and to such external stimuli as light, wounding, and infection by plant pathogens. The enzyme resembles histidine ammonia-lyase in that both appear to possess the same novel prosthetic group which is thought to contain a dehydroalanine–imine system (Givot *et al.*, 1969; Hanson and Havir, 1969, 1970; also, Wickner, 1969; Hodgins, 1971). Both enzymes show the same overall stereochemistry for the elimination process (phenylalanine ammonia-lyase, Hanson *et al.*, 1971; Ife and Haslam, 1971; Ellis *et al.*, 1973; Strange *et al.*, 1972; Wightman *et al.*, 1972; hisitidine ammonia-lyase, Givot *et al.*, 1969; Rétey *et al.*, 1970). For a detailed review of many of the above topics, see Hanson and Havir (1972b; also, 1972a).

The enzymes examined in this study, from maize shoots and from light-exposed slices of potato tubers, differ in substrate specificity. The same active site of the enzyme from maize acts on both L-tyrosine and L-phenylalanine (Havir *et al.*, 1971; Reid *et al.*, 1972; Strange *et al.*, 1972), whereas the enzyme from potatoes is essentially inactive with L-tyrosine. Also, higher molecular weight forms of the potato enzyme, but not of the maize enzyme, have been found (Havir and Hanson, 1968a). These variations imply diversity in physiological function and there could, therefore, be major structural differences between the two enzymes. Conversely, common structural features could well be typical of the enzyme from higher plants. In searching for such resemblances we showed, by comparative methods, that the molecular weights of the maize enzyme and the major species of enzyme from the potato are of the order of 306,000 and 330,000, respectively (Havir and Hanson, 1968a; Marsh *et al.*, 1968). In this article we present evidence, summarized earlier (Havir and Hanson, 1972), that both are composed of four subunits of mol wt ~83,000.

Materials and Methods

General. L-[U-^{14}C]Phenylalanine, 472 Ci/mol, L-[U-^{14}C]-tyrosine, 396 Ci/mol, and sodium [$^3H_4$]borohydride, 200 Ci/g, were purchased from New England Nuclear, and [^{14}C]-nitromethane, 1.81 Ci/mol, was purchased from Mallinckrodt (custom synthesis, purified by gas chromatography). Sodium dodecyl sulfate was purchased from Pierce Chemical Co., and guanidine hydrochloride was purchased from Heico. The reference proteins ovalbumin, bovine serum albumin, and α-chymotrypsinogen A (beef pancreas) were from Sigma and paramyosin was prepared from clams (Johnson *et al.*, 1959). Sephadex G-200 from Pharamacia was hydraulically fractionated (Havir and Hanson, 1968a). Agarose 1.5m and agarose 15m were purchased from Bio-Rad Laboratories as agarose Bio-Gel A-1.5m and agarose Bio-Gel A-15m, both 200–400 mesh.

A Gilford 2400 recording spectrophotometer was used for spectrophotometric assays, absorption spectra measurements, and, in conjunction with a linear transport attachment, for scanning stained polyacrylamide gels for protein bands. Radioactivity was determined in a 720 Nuclear-Chicago scintillation counter.

Enzyme Units, Specific Activity, and Spectrophotometric Assay. One unit (U) of phenylalanine ammonia-lyase activity catalyzes the formation of *trans*-cinnamate from L-phenylalanine at an initial rate of 1 μmol/min at 30° under the standard assay conditions. This spectrophotometric assay employs near saturating substrate concentrations and the optimum pH of 8.7 (Havir and Hanson, 1970). The analogous assay

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for tyrosine ammonia-lyase activity has been described (Havir *et al.*, 1971). Specific activities of purified enzyme (in milliunits per milligram of protein) are expressed in terms of "Folin protein" using bovine serum albumin as a standard (Lowry *et al.*, 1951). The conversion factors for purified maize enzyme are: protein from spectrophotometer readings at 280 and 260 nm (Warburg and Christian, 1941) to "Folin protein," 1.10; "Folin protein" to "analysis protein" (protein determined from the amino acid analyzer recoveries plus the tryptophan estimation), 1.03.

Radioactivity Assay for Enzyme Activity in Polyacrylamide Gels. In order to determine the distribution of activities, gels were cut by subdivision into 2.5-mm segments. Each segment was cut lengthwise into two equal portions of semicircular cross section. One of these was covered with a solution (100 μl) of L-[U-^{14}C]phenylalanine (0.167 μCi) in 0.2 M borate (Na^+) buffer, pH 8.7, and the other with an equal volume of L-[U-^{14}C]tyrosine (0.167 μCi) in 0.1 M phosphate (K^+) buffer, pH 7.5. After 18 hr at 22°, the [U-^{14}C]cinnamate or *p*-[U-^{14}C]coumarate formed was separated from the substrate amino acid by thin layer chromatography on ChromAR Sheet-500 (Mallinckrodt) and the distribution of radioactivity determined by scintillation counting (Havir *et al.*, 1971). Before applying the sample (10 or 25 μl) to the ChromAR sheet the point of application was first spotted with sufficient cinnamate or *p*-coumarate to yield a fluorescence quenching spot after chromatography.

Enzyme Purification. Enzyme was purified from the leaf-sheath tissue of outdoor grown maize (*Zea mays* L.; tall allele of dwarf-1 mutant, Phinney, 1956) essentially as in the large scale procedure described previously (Reid *et al.*, 1972). The final stage employed a large (50 × 8.5 cm) agarose 1.5m column (200–400 mesh). The enzyme from light-exposed potato tubers (*Solanum tuberosum* L., Kennebec) was also purified to the corresponding agarose column stage as previously described (Havir and Hanson, 1970). Kennebec potatoes have ceased to be readily available in Connecticut, but Katahdin potatoes, used in recent preparations, gave similar amounts of enzyme. Our procedure did not give satisfactory results with the variety Norchip.

Some high specific activity fractions of maize and potato enzyme from the agarose 1.5m column were further purified by chromatography on an agarose 15m column (45 × 4 cm; 200–400 mesh; void volume, 230 ml; internal volume, 380 ml; 35–50 mg of protein in 3.5 ml applied; flow rate, 15 ml/hr). The peak volume for the maize enzyme, 414 ml, corresponded to a 48% retardation.

In preparing electrophoretically homogeneous maize enzyme for amino acid analysis, fractions from the agarose 1.5m column were chromatographed on the analytical column (95 × 1.2 cm) of fractionated Sephadex G-200 (see Havir and Hanson, 1968a). Fractions having the highest specific activity (300–350 mU/mg) from a total of eight columns (4.5 mg of protein in 0.4 ml applied per column) were combined (52 ml, 0.149 mg of protein/ml) and the protein was precipitated with ammonium sulfate (22 g). The precipitate was collected by centrifugation and dissolved in the minimum amount of 0.1 M borate (Na^+) buffer, pH 8.7, to give 4.9 mg of protein in 0.6 ml; sp act. 350 mU/mg. The poor recovery is attributable to the precipitation step. Material of equal purity and in greater yield has since been obtained more readily with the above agarose 15m column. (The calculated pore size was 400 Å compared to 250 Å for the fractionated Sephadex G-200.) Repeated attempts to increase the specific activity of the maize enzyme above the value 400 mU/mg observed in some fractionations, *e.g.*, by interposing a chromatographic DEAE-Sephadex step between the agarose and Sephadex fractionations, were unsuccessful.

The small sample of highly purified potato enzyme used for amino acid analysis was a portion of the batch whose preparation has been described (Havir and Hanson, 1970).

Labeling the Active Site with [^{14}C]Nitromethane and Sodium [^{3}H]Borohydride. In a typical experiment $^{14}CH_3NO_2$ (1 μCi, 0.55 μmol in 25 μl of ethanol) was added to maize enzyme (1.7 mg, sp act. 345 mU/mg) in 12 ml of 0.1 M borate (Na^+) buffer, pH 8.7, at 30°. Samples were withdrawn at 2, 4, 6, 8, 10, and 15 min. Each portion (2 ml) was added to a cuvet containing 0.2 ml of 0.1 M L-phenylalanine and assayed for enzyme activity. The phenylalanine completely protected the active site against further nitromethane attack. The 15-min sample corresponded to 60% inactivation. Each sample was dialyzed against at least three 2-l. portions of 0.02 M borate buffer and assayed for "Folin" protein and incorporated radioactivity. In several other experiments the time of nitromethane treatment was considerably lengthened to obtain a range of incorporation values including values for complete inactivation.

A similar procedure was employed with samples of potato enzyme (*e.g.*, 4.2 mg; sp act. 186 mU/mg) but, to achieve comparable rates of inactivation, higher concentrations of the reagent were necessary (10 μCi, 5.5 μmol in 25 μl of ethanol was added). Preparations of both the major species (mol wt 330,000) and the higher molecular weight species were examined. From the observed rate of inactivation with $^{14}CH_3NO_2$ and the results of detailed kinetic studies with unlabeled compound (to be reported elsewhere), the concentration and therefore the specific radioactivity of the reagent were calculated. The value agreed with that determined by Mallinckrodt.

The active site of the potato enzyme was specifically labeled with ^{3}H by first treating the enzyme in the presence of cinnamate with unlabeled sodium borohydride and then, after removing the cinnamate, reducing the enzyme with the minimal amount of sodium [$^3H_4$]borohydride necessary for 90% inactivation (see section on Preparation of Type I Tritiated Enzyme; Hanson and Havir, 1970). The maize enzyme was converted to type I tritiated enzyme with the minimal amount of the reagent necessary to cause 90% inactivation (Havir *et al.*, 1971). (By type I tritiated enzyme it is implied that [3-^{3}H]alanine was the only significant radioactive product, other than tritiated water, obtained after hydrolysis with 6 N HCl at 110° for 18 hr.)

Polyacrylamide Gel Electrophoresis. Gels (5 × 100 mm) were prepared in the proportions and following the directions given by Gabriel (1971), *e.g.*, 7.5% gel = "7.5% acrylamide, 0.18% Bis." Unless otherwise indicated, the standard discontinuous buffer system was employed with a Tris-glycine electrode buffer, pH 8.3, and Tris-chloride, pH 8.9, in the gel. A continuous sytem, phosphate (Na^+) buffer, pH 7.2, as used for sodium dodecyl sulfate gel electrophoresis (see below), but without the sodium dodecyl sulfate, was also employed. Persulfate was not removed by prior electrophoresis and stacking gels or sample gels were not employed. Enzyme together with tracking dye was applied in buffered glycerol. Gels were stained with Coomassie Brilliant Blue and scanned at 575 nm.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The method of Weber and Osborn (1969) was followed closely. A gel (5 × 100 mm) formed from 7.5% acrylamide and 0.18% Bis (7.5% gel) (Gabriel, 1971) gave the best resolu-

tion in the molecular weight range investigated and was routinely used. The samples (5–40 μg of protein) were equilibrated with sodium dodecyl sulfate and mercaptoethanol under the proscribed conditions and then applied to the gel along with tracking dye and glycerol without prior dialysis. Chymotrypsinogen was added to some samples after separate equilibration to serve as an internal standard. Gels were stained and scanned as above.

Distribution of Radioactivity in Gels. After electrophoresis the stained gels were scanned and then cut by subdivision into 2.0- or 2.5-mm segments. Each segment was placed in a scintillation vial and covered with 30% H_2O_2 (0.25 ml). The tightly capped vials were maintained at 50–55° for 18 hr and then cooled to room temperature and the depolymerized gel was dissolved in toluene–ethanol scintillation fluid (20 ml) prior to counting for radioactivity.

Amino Acid Analyses. Duplicate hydrolyses of maize enzyme were performed with constant boiling HCl (Pierce Chemical Co.) on equal samples at 110° for 24, 48, and 72 hr in evacuated and sealed tubes. After hydrolysis, the HCl was removed in a stream of N_2 at 40°, then water was added and removed twice similarly. The dried samples were stored at −20° in the presence of silica gel and KOH. Each sample was analyzed at Brookhaven National Laboratory on a two-column ion exchange chromatography system. The yields of each amino acid (range, 25–204 nmol) were computed automatically from the data output (Hirs, 1967a). Total recovered amino acids from both columns (mean ± standard error of mean) for the 48- and 72-hr hydrolyses were 1.662 ± 0.001 μmol (N = 4). Recoveries indicated that 95 μg of protein was applied per column. Two additional samples were oxidized with performic acid (Hirs, 1967b), hydrolyzed for 24 hr, and analyzed as part of the same series.

A provisional estimate of the tryptophan content was made by the method of Edelhoch (1967). The ratio $r = A_{280}/A_{288}$ was 1.43 for the enzyme in 6 M guanidine hydrochloride and 0.2 M phosphate (K^+) buffer, pH 6.5. It is necessary to take into account the effect of an unidentified chromophoric group whose presence is evidenced by an extended shoulder of low intensity in the 310–350-nm region of the spectrum both in guanidine hydrochloride and in buffer alone. If N_{Trp}, N_{Tyr}, and N_{Cys} are the moles of amino acid per mol wt 83,000 subunit and x is the contribution of the chromophoric group ($x = \epsilon_{280} - \epsilon_{288}r$ per subunit), then $N_{Trp} = [N_{Tyr}(1280 - 385r) + N_{Cys}(120 - 72r) + x]/(4815r - 5690)$. On substituting from Table II, column 4 for N_{Tyr} and N_{Cys}, $N_{Trp} = 9$ if $x = 0$. Inspection of the absorption curve suggested that the correction term $x/(4815r - 5690)$ is probably $< \pm 1$.

The value $E^{1\%}_{280} = 8.9$ was calculated for the protein sample hydrolyzed using the analyzer recoveries plus the tryptophan estimation. A small correction to A_{280} was made according to the procedure of Warburg and Christian (1941).

Amino acid analyses of potato enzyme were performed on 24- and 48-hr hydrolysis samples in the laboratory of Dr. F. M. Richards, Yale University, also using a two-column ion-exchange system.

Results

Polyacrylamide Gel Electrophoresis (Maize Enzyme). The final stages of purification of phenylalanine ammonia-lyase from maize leaf-sheath tissue were monitored by polyacrylamide gel electrophoresis. The fractions of highest specific activity from the agarose 1.5m purification step showed one major and several minor protein bands on electrophoresis. Upon rechromatography on a Sephadex G-200 or agarose 15m column, fractions were obtained that were electrophoretically homogeneous in discontinuous systems with Tris-glycine buffer, pH 8.9, on 5 and 7.5% gels (Figure 1), and also at pH 7.2 in a continuous phosphate buffer system on 7% gel.

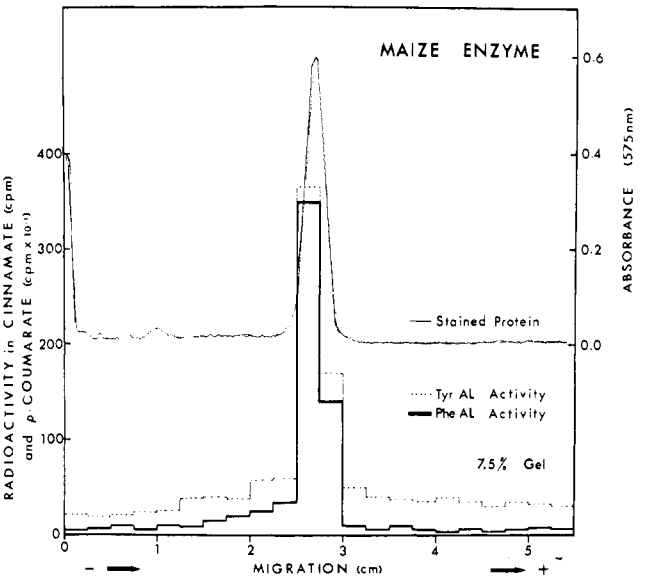

FIGURE 1: Polyacrylamide gel electrophoresis of purified maize enzyme. Electrophoretic separations of equal samples of purified enzyme (sp act. 280 mU/mg; from Sephadex G-200 chromatography) were carried out in parallel runs on 7.5% gels. Immediately afterward one gel was stained (scale corrected for expansion) and the other subdivided. Half-segments were assayed by the radioactivity assay. Abbreviations used in this figure are: Phe AL, phenylalanine ammonia-lyase; Tyr AL, tyrosine ammonia-lyase.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (Maize Enzyme). The results of sodium dodecyl sulfate gel electrophoresis on 7.5% gel support the view that the above enzyme preparation consisted of a single protein. The purified enzyme gave a single sharp staining band (Figure 2A) which corresponded in mobility to a polypeptide chain of about mol wt 82,500 (see below). Sodium dodecyl sulfate gel electrophoresis of crude enzyme fractions showed that two proteins present in similar barely detectable amounts and having approximately this molecular weight would appear as a doublet if their molecular weights differed by about 4000. We have been unable to observe such doublets and conclude that the subunits of the enzyme, if not identical, differ in molecular weight by less than 5%.

The failure to observe other peaks or shoulders on the main peak when the stained gel was scanned makes it unlikely that significant amounts of contaminant proteins were present or that the enzyme had been damaged by proteolytic enzymes or by cleavage of a labile linkage. A minor protein component, whether a contaminant or derived from cleavage, would have been detected if it differed in molecular weight from the undamaged subunit by 6000 (*e.g.*, see Figure 2C, peaks d, e, and f).

Active-Site Labeled Enzyme. Both the maize and potato enzymes may be labeled at the active site by reduction of the prosthetic group with sodium [$^3H_4$]borohydride and by the addition of [^{14}C]nitromethane to the prosthetic group (Hanson and Havir, 1969, 1970; Havir *et al.*, 1971). When maize enzyme of relatively high specific activity was labeled with ^3H or ^{14}C and examined by polyacrylamide gel electrophoresis,

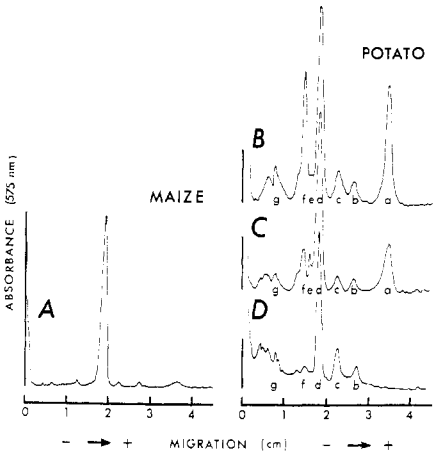

FIGURE 2: Protein bands after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The purified maize gave only one subunit band and the potato enzyme, after rechromatography on agarose 15m, gave only one significant band (peak d, curve D), the protein having a similar mobility to that of the maize enzyme subunit: (A) same preparation as in Figure 1; (B and C) fractions 78 and 82, respectively, from the separation shown as an inset to Figure 4A (sp act., *ca.* 250 mU/mg); (D) peak fraction from the rechromatography of fractions 76–78 of the same separation (sp act. 350 mU/mg). Molecular weights of unidentified proteins: a, 46,000; b, 61,500; c, 71,000; e, 90,000; f, 96,000.

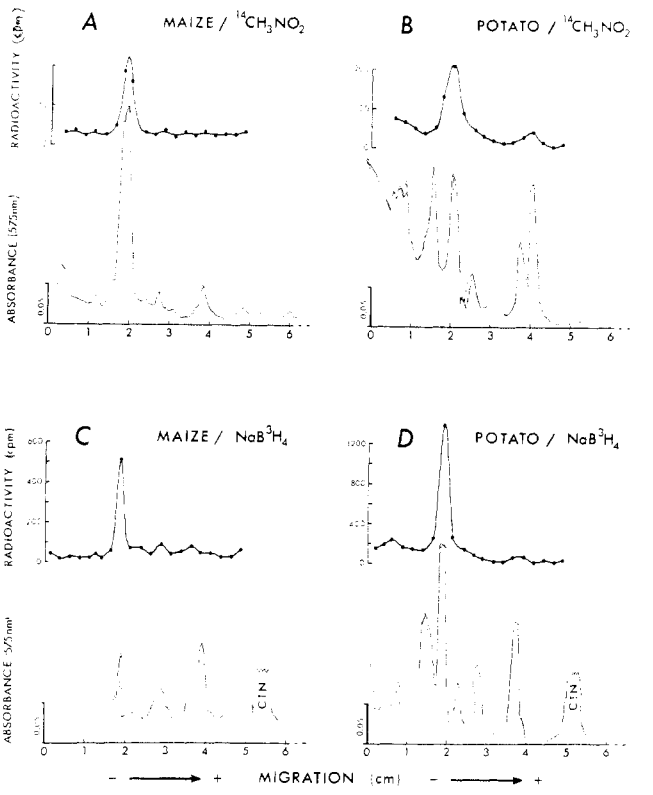

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of partially purified maize and potato enzymes specifically labeled at the active site. The stained gels were first scanned and then subdivided and the distribution of radioactivity was determined. Specific activities prior to labeling: (A) 330, (B) 440, (C) 67, (D) 380 mU/mg.

the radioactivity was associated with a protein of the same mobility as the active enzyme. Upon sodium dodecyl sulfate gel electrophoresis (Figure 3) the only radioactivity was associated with the same band observed for purified unlabeled enzyme. The corresponding results for the partially purified potato enzyme and the stoichiometry of labeling for both enzymes are discussed below.

Molecular Forms and Subunits of the Potato Enzyme. From the point of view of this study, the higher molecular weight forms of the potato enzyme are an unfortunate complication. We showed previously (Havir and Hanson, 1968a) that the forms were not in dynamic equilibrium and, as they differed markedly in size, they were *not* isozymes in any broad or strict sense of the term. On Sephadex G-200 or agarose 15m chromatography of enzyme purified from buffer extracts of fresh tissue the same high molecular weight shoulder (Figure 4A, inset) was observed as that encountered when the purification started with acetone powders (Havir and Hanson, 1968a). Fractions from the peak region (mol wt 330,000) gave a single peak of enzyme activity on polyacrylamide gel electrophoresis (Figure 4A). After rechromatography of these fractions on the agarose 15m column, a sample giving only one major peak on sodium dodecyl sulfate gel electrophoresis was obtained (Figure 2D). (Traces B and C of Figure 2 correspond to fractions 78 and 82 of Figure 4A, inset.) The relative mobility of the peak corresponded to a mol wt of ~83,500 (see below). The only labeled band from ^{14}C-labeled potato enzyme, whether from the mol wt 330,000 form or from the higher molecular weight forms, had this same mobility (Figure 3). We conclude that the major species of the potato enzyme, like the maize enzyme, is composed of a single size of subunit.

Higher molecular weight forms of the maize enzyme have not been observed by us in crude tissue extracts, purified preparations stored at −20° for 1 year, or ^3H- and ^{14}C-labeled enzyme stored for 1 year. In contrast, the high molecular weight shoulder has been reported upon chromatography of fresh extracts of potato tissue (Sacher *et al.*, 1972) and we have observed that the higher molecular weight species accumulate on storage of samples in which the only significant species initially was the mol wt 330,000 enzyme. This process can be observed in terms of enzyme activity (Figure 4B) and the radioactivity of labeled enzyme (Figure 4C).

Subunit Molecular Weights. The molecular weights of the enzyme subunits were determined by comparing their mean relative mobilities upon sodium dodecyl sulfate gel electrophoresis with the mean relative mobilities of a series of reference proteins (Figure 5). The standard errors in y about the fitted regression lines were so similar that there were no grounds for preferring one method for calculating relative mobilities over the other—method B gave values that were lower by about 500. The best estimates for the molecular weights indicated above and in the figure are the rounded means of the results of both methods of calculation.

The difference between the mean relative mobilities of the maize and potato subunits is small compared to the scatter of the individual observations. There is a two in three chance that an equal or greater difference would be observed if the two proteins were identical. The best estimate of the molecular weight difference (potato minus maize) is 1100. There is a 19 in 20 chance that the difference lies between the limits 3600 and −1400 (inset, Figure 5). These conclusions are insensitive to the actual molecular weight values calculated from the regression lines.

As the confidence limits of the separate estimates of the subunit molecular weight overlap considerably, it is useful

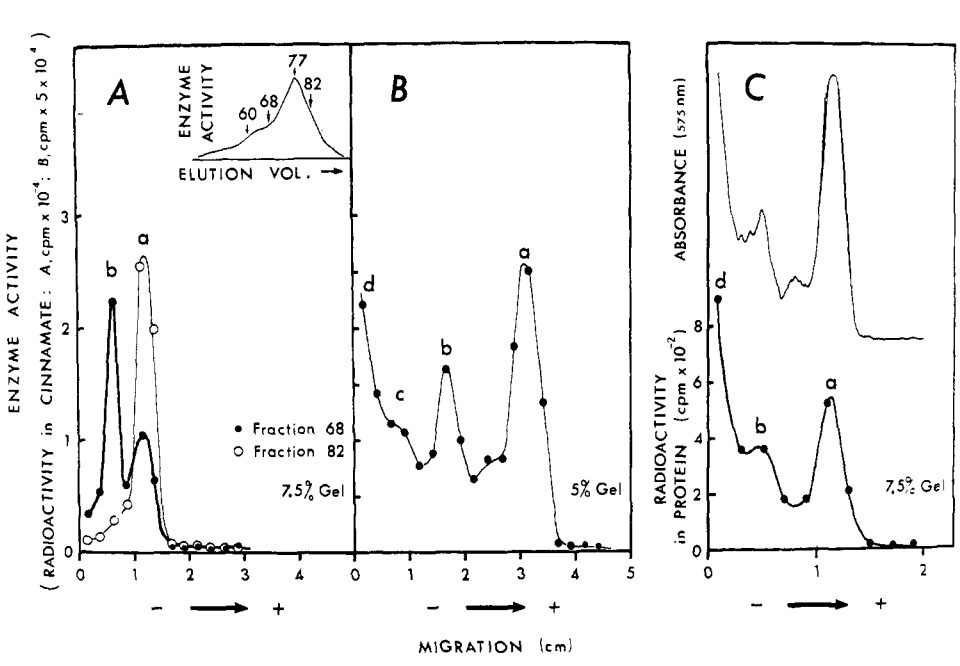

FIGURE 4: Demonstration by polyacrylamide gel electrophoresis that the potato enzyme can exist in high molecular weight forms. The major species of the potato enzyme is characterized by a single peak (peak a). Peak b enzyme could be a dimer of the major species. (A) Inset shows distribution of enzyme activity in fractions (3 ml) from the chromatography of potato enzyme on an agarose 15m column (4 × 45 cm). The enzyme chromatographed (sp act. 190 mU/mg) had been purified to the agarose 1.5m stage and then stored for 2 years at −20°. Fraction 82 on electrophoresis gave only a single protein band corresponding to peak a enzyme activity (see also Figure 2B and C). (B and C) Accumulation of higher molecular weight forms on storage of enzyme and tritiated enzyme for 2 years at −20°. (For panel C, see also Figure 3D.)

(next section) to pool the relative mobility data. The means of the subunit molecular weights calculated for the two enzymes and its 95% confidence limits are: best estimate, 83,000; lower bound, 79,500; upper bound, 87,000. This assumes that the molecular weights of the standards were correct. The value for the mean subunit molecular weight is less by 100 if the appropriate mol wt of ovalbumin is 43,000 and less by 700 if that of bovine serum albumin is 66,000. Variations in dodecyl sulfate binding should compensate for large proteins (see Dunker and Rueckert, 1969) and it is, therefore, unlikely that the subunits of phenylalanine ammonia-lyase are atypical in the number of dodecyl sulfate molecules they bind. We conclude that the true 95% confidence limits about the 83,000 value are well within the ±10% "accuracy" said to be realizable (Weber and Osborn, 1969). A ±10% accuracy corresponds to roughly twice the above range of confidence, *i.e.*, 75,000–91,000.

Subunit Composition. The above estimate of the mean of the subunit molecular weights of the two enzymes with the broadened confidence limits 75,000–91,000 may be expressed as a skewed probability distribution curve (curve 1, Figure 6). This yields estimates of the possible mean molecular weight of the oligomeric enzyme represented by the curves ×2, ×3, etc. The direct estimate of this mean by sucrose density gradient centrifugation and molecular sieve chromatography (Havir and Hanson, 1968a; Marsh *et al.*, 1968) is indicated by the heavy arrow which falls well within the probability range for four subunits. It is pertinent that almost all enzymes studied have been found to have even numbers of subunits (Klotz *et al.*, 1970; Darnall and Klotz, 1972).

Number of Active Sites per Tetramer. When enzyme from maize and potato was treated with [^{14}C]nitromethane and samples were withdrawn at various intervals, the loss of enzyme activity was found to be proportional to the amount of ^{14}C labeling (for analogous proportionality in ^{3}H labeling, see Hanson and Havir, 1970). The results of such experiments, expressed as nanomoles of active sites (=nanomoles of ^{14}C incorporated) per unit of enzyme activity lost, are listed in Table I, column 1. To calculate the number of active sites

TABLE I: Stoichiometry of [^{14}C]Nitromethane Incorporation into Phenylalanine Ammonia-lyase.

Source	Active Sites:[a] Enzyme Act. (nmol/U)	k_{cat},[b] pH 8.7, 30° (sec^{-1})	Highest Obsd Sp Act.[c] (mU/mg)	App No. of Active Sites/Enzyme Tetramer[d] (mol/mol)
Maize (N = 5)	12[e]	1.4	400	1.6[e]
Potato (N = 2)	14[f]	1.2	440	2.1[g]

[a] It is assumed that for each molecule of [^{14}C]nitromethane incorporated one active site is destroyed. [b] k_{cat} = turnover number per active site. Calculated from column 1. In the standard assay both enzymes are ~95% saturated with respect to L-phenylalanine. [c] Fractions from agarose 15m columns, this investigation. [d] From columns 1 and 3. [e] Range from N = 5 experiments: 9.9–13.9 nmol/U giving 1.3–1.8 mol of active site/mol. [f] Two experiments: 14.0 and 14.6 nmol/U. A value of 15 nmol/U was obtained for a sample containing largely higher molecular weight forms of the enzyme. [g] If the highest specific activity observed previously (Havir and Hanson, 1968a) is used (790 mU/mg, instead of 440 mU/mg), the value is 3.7 mol of active site/mol.

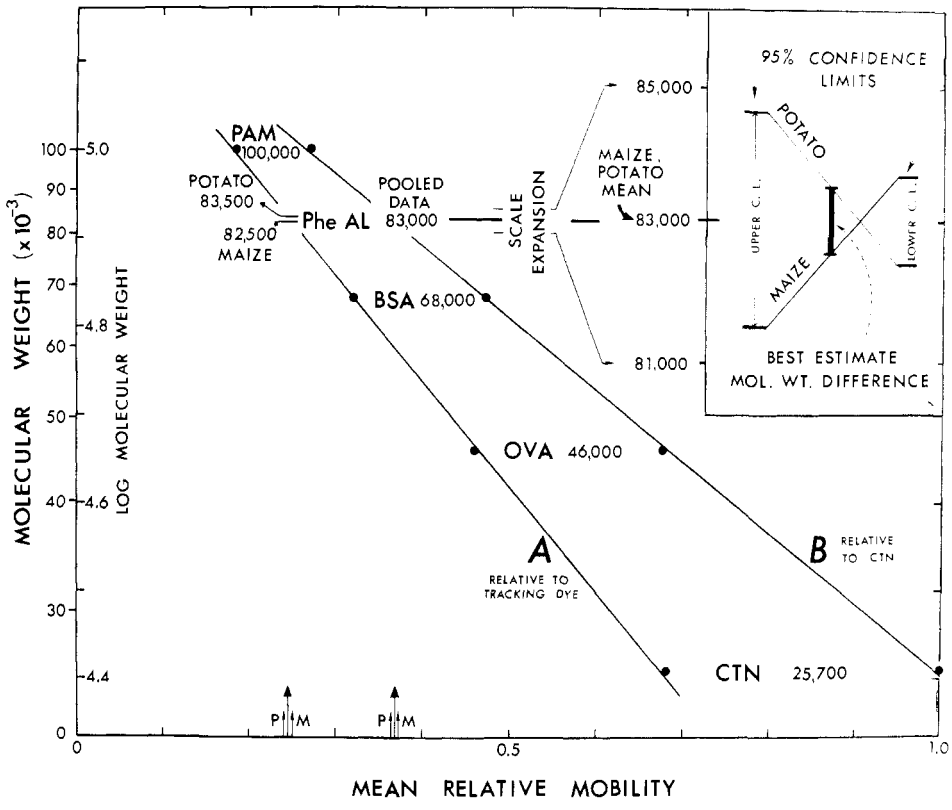

FIGURE 5: Estimations by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the subunit molecular weights of the maize and potato enzymes and (inset) of the difference in their molecular weights. Mobilities were calculated relative to tracking dye (A) and also to chymotrypsinogen (B). Experimental points represent the means of at least ten determinations; SE of means are <0.0025 (A) and <0.005 (B). For molecular weight references, see Weber and Osborn (1969); however, for ovalbumin, see Castellino and Barker (1968) and also Dunker and Rueckert (1969). Best estimates of the log molecular weights of the phenylalanine ammonia-lyase subunits and the 95% confidence limits of these estimates were calculated for the separate maize and potato data, and for the pooled data. Both regression equations for A and B were used taking $N = 4$, *i.e.*, 2 degrees of freedom in s^2 (Bliss, 1967; p 439). The derived confidence limits in the molecular weights are slightly skewed about the best estimates of the subunit molecular weights. The values shown are the rounded-off means for the two methods of calculation. The inset shows the 95% confidence limits of the estimated molecular weight difference, potato − maize (= 1100), calculated for 23 degrees of freedom from the error variance s_d^2 of the difference in mean relative mobilities ($N = 15$ and $N = 10$), assuming that the variance for the two groups being compared was the same (Bliss, 1967; p 213). Conversely, a t test of the significance of the apparent mobility difference was applied for 23 degrees of freedom. Abbreviations: Phe AL, phenylalanine ammonia-lyase; CTN, chymotrypsinogen; OVA, ovalbumin; BSA, bovine serum albumin; PAM, paramyosin.

per tetrameric enzyme of mol wt 330,000 (column 4) we need to know the specific activity of pure, fully active enzyme (column 3). Maize enzyme of specific activity *ca.* 400 mU/mg and having only a trace amount of contaminating proteins (by sodium dodecyl sulfate electrophoresis) has been obtained in a number of preparations. Calculations using this figure indicate that there are two active sites per tetramer. If we consider only the specific activity of our recent best preparations of potato enzyme, we arrive at the same result. Earlier preparations (Havir and Hanson, 1968a, 1970) had specific activities as high as 790 mU/mg—a value which could imply that there are four active sites per molecule, *i.e.*, that the values in column 4 are averages for incompletely activated, fully activated, and partly inactivated enzyme. In view of the unusual kinetic properties of the potato enzyme (Havir and Hanson, 1968b) this inference may be unjustified. Unfortunately, we do not have the high specific activity enzyme available in order to study the matter in greater detail.

The values for k_{cat}, the turnover number per active site, listed in column 2, are independent of any assumptions about the number of active sites per molecule. They are very much less than those observed for histidine ammonia-lyase (170 sec^{-1}). For references and a discussion concerning the rate limiting step for these enzymes, see Hanson and Havir (1972b).

Amino Acid Composition. The results of amino acid analyses of electrophoretically homogeneous maize enzyme are recorded in Table II. The moles of each amino acid in the sample are expressed relative to the calculated sum of the residue weights. The true weight of the enzyme sample includes the tryptophan content and the weight of the prosthetic group. A provisional estimate of the former was calculated by the spectrophotometric method of Edelhoch (1967). In Table III, the results are reexpressed in terms of residues per 100 residues in order to allow a comparison with the composition of highly purified wheat enzyme (Nari *et al.*, 1972) and our own preliminary results for the potato enzyme. All resemble the "average protein" of Smith (1966).

Even if there are two rather than four active sites per tetramer, it is possible that the subunits of the maize enzyme prior to the formation of the prosthetic group are chemically identical, or only differ in amide composition or in one or two amino acid residues. We have, therefore, used the statistical data from our replicate analyses to calculate the probability density function (Y_w) for the protein in the mol wt 83,000 region (Jurášek and Whitaker, 1967). Possible best fits of the amino acid composition are indicated by the peaks

TABLE II: Amino Acid Composition of Phenylalanine Ammonia-lyase (Maize).

Amino Acid	Mol of Amino Acid per 100 kg of Protein*[a,b]	Wt of Protein*[a] Giving 1 mol of Amino Acid ($W_a \pm \Delta W_a$)[c]	Peak B Composition (Residues per Molecule)[d]
Lys	56.20 ± 0.09 (4)	1779 ± 3	24
His	20.75 ± 0.10 (4)	4820 ± 23	17[e]
Arg	42.59 ± 0.19 (4)	2348 ± 10	34
Asx (as Asp)	88.01 ± 0.63 (6)	1138 ± 8	70–71
Thr	45.6 ± 1.6 (6 ex)	2192 ± 77	(36–37)[f]
Ser	64.4 ± 5.4 (6 ex)	1553 ± 130	(51–52)[f]
Glx (as Glu)	97.50 ± 0.35 (6)	1025 ± 4	78
Pro	42.78 ± 0.14 (4)	2337 ± 7	34
Gly	84.98 ± 0.43 (4)	1177 ± 6	68
Ala	109.56 ± 0.36 (6)	913 ± 3	88
Cys + $^1/_2$[h] [as Cys(O_3H)]	14.3 ± 0.8 (2)	7439 ± 416	(11)[f]
Val	76.11 ± 0.14 (4)	1314 ± 3	61
Met	14.83 ± 0.34 (6)	6740 ± 154	12
[as Met(O_2)]	[14.3 ± 0.8 (2)]		
Ile	49.79 ± 0.16 (4)	2008 ± 6	40
Leu	90.16 ± 0.40 (4)	1109 ± 5	72
Tyr	18.2 ± 1.6 (6 ex)	5480 ± 483	(14–15)[f]
Phe	27.62 ± 0.13 (6)	3620 ± 16	22
Trp	Not used in calcn		(9)[f]
NH_3	Not determined		
Sum	943.4		
	Peak B: approximate no. of residues per subunit		764
	Corresponding subunit mol wt[g]		81,570

[a] Protein* = sum of residue weights of amino acids listed, *i.e.*, tryptophan excluded. [b] Listed as mean ± standard error of mean (number of hydrolyses = N). The analytical results were pooled on a sample basis, then calculated relative to 100 kg of protein*. For $N = 6$, data for the 24-, 48-, and 72-hr hydrolysis samples were pooled, but for $N = 4$, the 48- and 72-hr values only were pooled (24-hr data were less reliable); ex = extrapolated values. [c] Mean ± standard error of mean (for calculations, taken to be the standard deviation). The true molecular weight of the protein, or its subunits, should be a multiple of these estimates of the minimal molecular weight. [d] See Figure 7. [e] Peak B does not fall within the 95% confidence limits for 17 histidine residues (see text). [f] Numbers subject to considerable uncertainty as the standard errors in column 3 are rough estimates. [g] Protein* molecular weight (Figure 7) plus weight of nine tryptophan residues. [h] Cys + $^1/_2$, cysteine + cystine.

in Y_w (Figure 7). The distribution of peaks was not greatly changed on making minor changes in the standard deviations ΔW_a used in the calculation. The composition for peak B falls outside the 95% confidence limits for the histidine value, but seems to give the best fit in other respects and we have, therefore, listed this as the composition of an average subunit (Table II, column 4). If there are two different types of subunit, the smallest integral composition will be approximately twice that for peak B. Experiments to distinguish between the various alternatives are in progress.

Absorption Spectrum. In examining a number of preparations of highly purified enzyme from both sources, we have observed an extended shoulder in the absorption spectrum in the region 310–350 nm, the ratio A_{315}/A_{277} being ~0.1. High absorbancies have been observed in this region for phenylalanine ammonia-lyase from *Rhodotorula glutinis* (Hodgins, 1971) and histidine ammonia-lyase (Rechler, 1969; Klee, 1970). The absorbance at 315 nm for the maize enzyme was essentially the same in borate buffer, pH 8.7, and 6 M guanidine hydrochloride at pH 6.5 (*cf.* Edelhoch, 1967). Reduction of the enzyme with borohydride produced no effect on the absorbance at 315 nm and only a small lowering

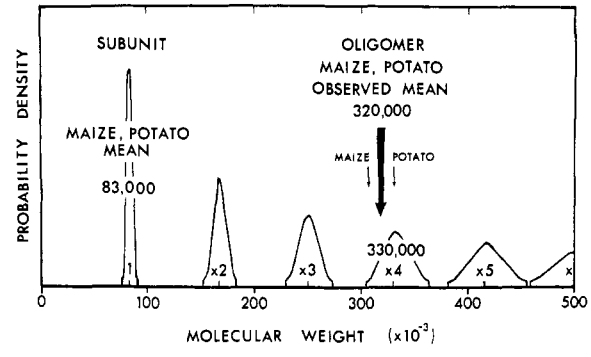

FIGURE 6: Demonstration that the number of subunits per enzyme molecule is 4. Curve 1 of area 0.95 is the probability distribution function about the best estimate of the mean molecular weight of the potato and maize subunits and is truncated at 75,000 and 91,000 (see text). The area under the curve between designated limits is the probability that the molecular weight lies between these limits. Curves ×2, ×3, etc., are multiples of curve 1 having equal areas to that curve. The arrows mark the provisional estimates of the molecular weights of the maize and potato enzymes obtained previously (Marsh *et al.*, 1968; Havir and Hanson, 1968a) and the mean of these estimates.

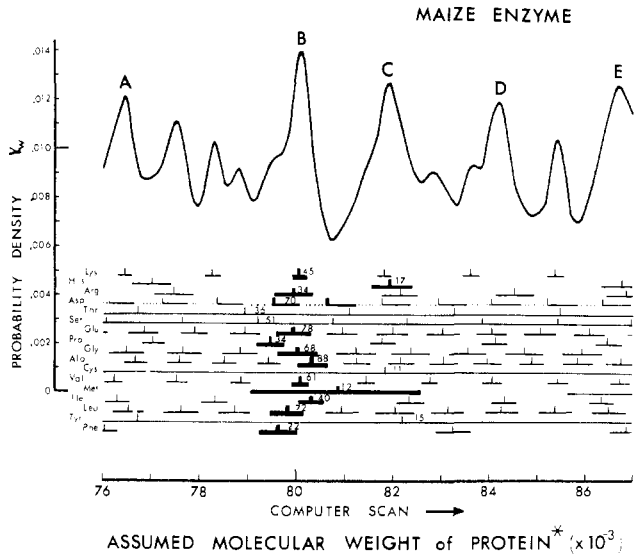

FIGURE 7: Computer search by the method of Jurášek and Whitaker (1967) for the most probable molecular weight of a subunit of the maize enzyme using the data of Table II. Each estimate of the minimal molecular weight of the protein is described by a normal distribution curve $Y_a = f(W)$ of unit area having a mean and standard deviation $W_a \pm \Delta W_a$ (Table II, column 3). The vertical lines in the lower part of the figure show multiples of W_a and the horizontal lines the appropriate multiples of $2 \times \Delta W_a$. The function Y_w was computed as the sum for all amino acids (a = 1–17) of the sum for all multiples of Y_a for each amino acid over the protein* mol wt range 70,100–93,000. The peaks (not shown) at 72,300 and 74,800 were similar in height to peak B.

of the absorbance in the 280-nm region either in pH 8.7 buffer or in the presence of 6 M guanidine hydrochloride, pH 6.5. There is, therefore, no present indication that the chromophoric system and the prosthetic group are one and the same. It is possible that for the maize and potato enzymes the absorbance shoulder arises from random phenolic coupling to the enzyme during purification. Such coupling could interfere with studies of amino acid composition, peptide mapping, etc.

Discussion

The main conclusion of this study is that the maize enzyme and the major species of the potato enzyme each consist of four subunits with molecular weights close to 83,000. The argument is summarized in Figure 6. If the subunits for a given enzyme differ in number of residues they do so by less than 5%. There is a small probability that the subunits of the maize enzyme on the average have a lower molecular weight than those of the potato enzyme. If all four subunits of the enzymes have equal numbers of residues, the maize and potato enzymes may differ by only a few residues per subunit.

There is no indication that the maize enzyme exists as higher molecular weight forms either in the crude tissue extracts or in stored preparations of the purified enzyme. The higher molecular weight forms of the potato enzyme may well be present *in vivo*. They occur in preparations of partially purified enzyme and are also formed in storage at −20°. Phenylalanine ammonia-lyase from mustard seedlings (*Sinapis alba*, L.) also forms high molecular weight aggregates *in vitro* (Schopfer, 1971). Although the enzyme may associate with contaminant proteins, aggregation may arise from self-association through intraenzyme disulfide bridge formation

TABLE III: Comparison of Amino Acid Compositions in Terms of Residues per 100 Residues.

Amino Acids[a]	Phenylalanine Ammonia-lyase			"Average protein"[e]
	Maize[b]	Potato[c]	Wheat[d]	
Hydrophilic, basic				
Lys	5.88	6.68	5.14	5.9 ± 3.0
Arg	4.46	4.18	4.10	4.0 ± 2.3
His	2.17	2.31	2.12	1.8 ± 1.3
Hydrophobic, aromatic				
Phe	2.89	3.64	3.78	3.4 ± 1.5
Tyr	1.91	2.97	2.43	3.2 ± 2.0
Trp	1.18	*f*	*f*	0.7 ± 1.2
Hydrophobic, not aromatic				
Leu	9.44	9.85	7.99	7.4 ± 2.8
Val	7.97	8.41	7.36	6.0 ± 2.2
Ile	5.22	5.20	5.42	3.8 ± 1.8
Met	1.55	2.06	1.98	1.3 ± 0.7
Ambivalent				
Ala	11.47	8.88	10.18	7.6 ± 3.5
(Gly)	8.90	6.75	8.37	6.8 ± 3.2
Ser	6.75	5.23	6.84	6.8 ± 2.7
Thr	4.78	2.97	5.24	5.2 ± 2.3
Pro	4.48	4.76	5.49	4.4 ± 2.1
Cys + $^1/_2$[h]	1.50	*g*	1.77	1.0 ± 5.4
Ambivalent (Gln, Asn) plus hydrophilic, acidic (Glu, Asp)				
Glx	10.21	11.60	10.20	9.5 ± 3.6
Asx	9.21	9.54	10.10	9.0 ± 3.0

[a] Groupings, according to Dickerson (1972); order, that of abundance in "average protein" (Smith, 1966). [b] From Table II. [c] Provisional values, this study. [d] Calculated from Nari *et al.* (1972). [e] Mean values, calculated by grouped frequency analysis and standard errors of means of 80 proteins (Smith, 1966). [f] Assumed to be 1.18 residues per 100 for calculation purposes. [g] Assumed to be 1.35 residues per 100 for calculation purposes. [h] Cys + $^1/_2$, cysteine + cystine.

[*cf.* histidine ammonia-lyase (Klee, 1970; Soutar and Hassall, 1969)].

The bulk of the evidence on [^{14}C]nitromethane labeling at the active sites indicates that there are two active sites per tetramer (Table I). Hodgins (1972) reports that 2 out of 4.5 molar equiv of –SH was essential for the activity of phenylalanine ammonia-lyase from *Rhodotorula glutinus*. The finding is relevant if this fungal enzyme and the enzymes from higher plants have similar subunit structures. We think it unwise at the present time to discount the possibility that the fully active enzyme has four active sites and that the results of Table I are average values. As the enzyme contains an unusual prosthetic group, it must be formed *in vivo* from a precursor protein through the agency of a secondary enzyme. It could be that plant tissues always contain a reserve of precursor protein or partly activated enzyme and that the enzyme is only fully activated in response to a particular demand on the system. The observations of Sacher *et al.* (1972) and Zucker (1968) suggest that there is *de novo* synthesis of

both enzyme and precursor during light exposure of potato tuber slices but do not indicate the rate or extent of conversion of precursor to active enzyme.

Symmetry theory (Hanson, 1966, 1972; Klotz *et al.*, 1970) makes it unlikely that the tetrameric enzyme contains an odd number of distinct polypeptide chains; therefore, the protein derives from either one or two structural genes. If there are, in fact, only two active sites per fully active tetramer, the enzyme must have C_2 symmetry. There may, however, be only one structural gene as, *e.g.*, a precursor tetrameric protein having identical subunits and D_2 symmetry could be modified by a secondary enzyme to yield an enzyme with two prosthetic groups and C_2 symmetry. A recent preliminary account of studies of phenylalanine ammonia-lyase purified from wheat indicates that this enzyme of mol wt 325,000 (by ultracentrifugation) is composed of two subunits of 75,000 and two of 85,000 mol wt (Nari *et al.*, 1972). This result supports a two-gene hypothesis and suggests that there should be two active sites per tetramer. It is somewhat puzzling, however, that the wheat enzyme has two sizes of subunit when the potato and maize enzymes, even though they differ in many respects, have apparently one size of subunit. It is possible that the smaller wheat protein is an artifact formed from the mol wt 85,000 protein by proteolysis during purification or by cleavage of a labile linkage in the presence of sodium dodecyl sulfate and mercaptoethanol. Histidine ammonia-lyase from *Pseudomonas* sp. ATCC 112995 has been found to contain such a labile linkage (Klee, 1970). It may be relevant that this enzyme, which probably has two active sites, appears to have four subunits of mol wt 55,000 which have either identical or closely related polypeptide chains (Klee, 1972; Klee and Gladner, 1972). To distinguish between the two- and one-gene hypotheses for phenylalanine ammonia-lyase further comparisons between enzymes obtained from diverse sources will be necessary as well as detailed studies of the enzyme and its active site. Such studies should eventually indicate whether the histidine and phenylalanine ammonia-lyases are related by divergent or convergent evolution.

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