L-Phenylalanine Ammonia-lyase (Maize, Potato, and *Rhodotorula glutinis*). Studies of the Prosthetic Group with Nitromethane†

Evelyn A. Havir* and Kenneth R. Hanson*

ABSTRACT: Highly purified enzyme (EC 4.1.3.5) from *Rhodotorula glutinis* was shown by sodium dodecyl sulfate gel electrophoresis to have subunits which if not identical are closely similar in molecular weight. Like the enzyme from maize and potato [Havir, E. A., and Hanson, K. R. (1973), *Biochemistry 12,* 1583] it is a tetramer of molecular weight ~ 4 × 83,000. Enzyme from all three sources inactivated and labeled at the active site with $^{14}CH_3NO_2$ gave on HCl hydrolysis $^{14}CO_2$, $H^{14}CO_2H$, D- and L-[^{14}C]aspartic acid, and unidentified radioactive products. In addition, the labeled *R. glutinis* enzyme gave [1,2-$^{14}C_2$]glycine. The formation of the first three products is compatible with the hypothesis that the electrophilic prosthetic group of the enzyme contains the dehydroalanine imine system >C=N—C$^{\alpha}$(=C$^{\beta}$H$_2$)CO— and inactivation involves attack on C$^{\beta}$. The second-order rate constants for CH_3NO_2 inactivation varied with pH as a simple titration curve. The pK_a values calculated from the curves for the three enzymes differed and were lower than the pK_a of CH_3NO_2 by at least 1 pH unit. Apparently the inactivation process is enzyme catalyzed. Both inactivation and addition of the substrate amino group may occur with attack on C$^{\beta}$.

Phenylalanine ammonia-lyase (EC 4.1.3.5) catalyzes the overall elimination of $-NH_3^+$ and the (*pro*-3*S*)-hydrogen from L-phenylalanine to give *trans*-cinnamate. Whereas the enzyme from potatoes is specific for phenylalanine, that from such sources as maize and the yeast-like fungus *Rhodotorula glutinis* will act on both L-phenylalanine and L-tyrosine and both amino acids are, presumably, physiological substrates. In higher plants it is the first enzyme leading to a great variety of phenylpropanoid compounds including lignin. As such it has been the subject of numerous metabolic and physiological investigations (Camm and Towers, 1973; Creasy and Zucker, 1974). The enzyme is believed to contain an electrophilic center which combines with the amino group to form a leaving group that is better than $-NH_3^+$. After release of the cinnamate formed the amino enzyme remaining is hydrolyzed to yield NH_4^+ and regenerate the prosthetic group. Histidine ammonia-lyase (EC 4.3.1.3) apparently generates *trans*-urocanate and NH_4^+ by a similar reaction sequence. There are, however, significant differences in the properties of the two enzymes (see Discussion) and it remains to be proved that their prosthetic groups are identical (Hanson and Havir, 1972a,b).

In earlier studies we found that phenylalanine ammonia-lyase from potato and (later) maize when inactivated and specifically tritiated at the active site by sodium [^{3}H$_4$]borohydride yielded DL-[3-^{3}H]alanine and tritiated water after HCl hydrolysis. This led us to propose (Hanson and Havir, 1969, 1970) that the electrophilic center was a derivative of dehydroalanine (2-aminoacrylic acid) with the double bond activated through the substituents of either the nitrogen, or carboxyl, or both. Analogous results were obtained for histidine ammonia-lyase by Wickner (1969) and for phenylalanine ammonia-lyase from *R. glutinis* and *Sporobolomyces pararoseus* by Hodgins (1971) and by Parkhurst and Hodgins (1972). In addition, it was shown that ^{14}CN inactivated phenylalanine ammonia-lyase upon hydrolysis gave DL-[4-^{14}C]aspartic acid, a result which suggested that ^{14}CN added to the β position of a dehydroalanine residue. The studies of phenylalanine ammonia-lyase inactivated by $^{14}CH_3NO_2$ described in this paper further support our (1969) dehydroalanine hypothesis. Givot et al. (1969) studied $^{14}CH_3NO_2$ inactivated histidine ammonia-lyase and obtained evidence that the prosthetic group of this enzyme contains a dehydroalanine residue activated through nitrogen by imine formation. Although our experimental approach differed from theirs and the products are different, the results are also compatible with a dehydroalanine imine hypothesis.

Materials and Methods

General. $^{14}CH_3NO_2$ (1.81 Ci/mol) was purchased from Mallinckrodt (custom synthesis which included purification by gas chromatography). Other labeled compounds were purchased from New England Nuclear. Unlabeled nitromethane (Spectranalyzed) was obtained from Fisher Scientific, and nitroethane, nitroethanol, and nitropropionic acid from the Aldrich Chemical Co. "Glutamic oxalacetic transaminase" (EC 2.6.1.1) from pig heart (Type I) was purchased from Sigma. Agarose-1.5m and agarose-15m were purchased from Bio-Rad Laboratories as agarose Bio-Gel A-1.5m and Bio-Gel A-15m, both 200–400 mesh. Dowex-1-acetate was purchased from Bio-Rad.

Radioactivity was determined in a Searle Analytic Isocap/300 liquid scintillation counter using an ethanol–toluene based scintillation fluid unless otherwise indicated.

Paper electrophoretic separations were performed on Whatman 3MM paper with a 45-cm flat plate system (Savant). Runs were carried out at 15° and 50–70 V/cm for 20–40 min with the following buffers: pH 1.9, formic and acetic acids (30 and 120 ml/l.); and pH 3.5, 4.4, and 5.3,

† From the Department of Biochemistry, The Connecticut Agricultural Experiment Station, New Haven, Connecticut 06504. *Received September 27, 1974.* The investigation has been supported in part by Grant GB-42247-X from the National Science Foundation. A preliminary report of this work was presented at the 9th International Congress of Biochemistry, Stockholm, 1973.

pyridine and acetic acid (2.2 and 22 ml/l., 4.0 and 8.0 ml/l., and 6.67 and 2.67 ml/l.).

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 (Havir and Hanson, 1970). Specific activities were determined as before (Havir and Hanson, 1973).

Enzyme Purification. Enzyme for nitromethane-inactivation studies was purified from maize and potato as described (Havir and Hanson, 1973) employing an agarose-15m column (4 × 45 cm) for the final stage; specific activities: maize, 275 mU/mg; potato, 428 mU/mg. Both preparations showed the presence of contaminant proteins upon sodium dodecyl sulfate gel electrophoresis.

Enzyme from *R. glutinis* was purchased from PL Biochemicals as a frozen $(NH_4)_2SO_4$ suspension. The enzyme (50 U, specific activity 220 mU/mg), in 0.2 *M* borate (Na^+) buffer, pH 8.7 (25 ml), was chromatographed in the same buffer on a large agarose 1.5m column (10.8 × 66 cm; void volume 2600 ml; initial volume, 3280 ml; peak volume, 3300 ml, i.e., 21% retardation; flow, 6 × 20 ml/hr). Combined high specific activity fractions (>800 mU/mg) were rechromatographed on a smaller agarose 15m column (8.9 × 47 cm, compacted to 44 cm during operation; from rest, void volume, 1160 ml; initial volume, 1720 ml; peak volume, 1920 ml, i.e., 44% retardation; flow 6 × 20 ml/hr). (Both columns were fabricated from cast acrylic tubing.) The overall yield of purified enzyme (specific activity >1.5 U/mg) was 36% (18 U, 12.8 mg). This material showed only minor bands of contaminant proteins on sodium dodecyl sulfate gel electrophoresis.

Labeling the Active Site with $^{14}CH_3NO_2$. The labeling of potato (428 mU/mg of protein), maize (275 mU/mg of protein), and *R. glutinis* (1500 mU/mg of protein) enzymes was carried out as described previously (Havir and Hanson, 1973) using $^{14}CH_3NO_2$ (1.81 Ci/mol). The concentration of nitromethane used for the inactivation of the *R. glutinis* enzyme was half that used for the potato enzyme.

HCl Hydrolysis of $^{14}CH_3NO_2$ Labeled Enzyme, Separation, and Identification of Hydrolysis Products. Lyophilized samples of $^{14}CH_3NO_2$ labeled enzyme (0.2–1 mg) from all three sources were hydrolyzed with 6 *N* HCl (0.5 ml) at 110° for 18 hr in evacuated sealed tubes. The hydrolysis products were separated as follows.

(a) TRAPPING $^{14}CO_2$. In order to trap released $^{14}CO_2$ and possibly other volatile products the drawn out tip of each tube was broken by allowing a weight to fall on the tube. This operation was performed in a 2 × 50 cm Kontes Chromaflex column under a stream of N_2 gas which passed into 3 *N* ethanolamine (2 hr). To confirm the identity of CO_2 a solution of $Ba(OH)_2$ (0.25 *N* containing 2 g of $BaCl_2$/100 ml) was employed in place of ethanolamine in one experiment. The $Ba^{14}CO_3$ was collected and acidified in a closed Warburg flask, and the released CO_2 was absorbed by a paper wick moistened with 3 *N* ethanolamine in the center well.

(b) SUBLIMATION AND IDENTIFICATION OF $H^{14}COOH$. After removal of the volatile fraction, the HCl solution was sublimed, in a Y-assembly (Hanson and Havir, 1970). The sublimate was steam distilled until ten times the initial volume had been collected. At least 75% of the radioactivity was in the distillate. This was made alkaline with KOH and concentrated on a rotary evaporator. The major portion of the radioactivity was shown to be present as $H^{14}COOH$ as follows: by descending chromatography on Whatman 3MM paper in 1-butanol–H_2O–95% ethanol–diethylamine (80:20:10:1) (Zelitch, 1965); by ion exchange chromatography on a column (0.7 × 6 cm) of Dowex-1-acetate (×8, 200–400 mesh), upon eluting the column with water (10 ml) and 2 *N* acetic acid the radioactivity was located in the formic acid containing fractions (elution volumes acetic, 6–8 ml, glycolic, 12–14 ml, and formic, 34–38 ml); by specifically decomposing the $H^{14}COOH$ recovered from the column to $^{14}CO_2$ with mercuric acetate (Wood and Gest, 1957) as follows: to an eluate sample (5 ml) was added $Hg(CH_3COO)_2$ (4 ml, 0.3 *M*), the solution was heated for 1 hr under reflux during which time the $^{14}CO_2$ released was trapped in 3 *N* ethanolamine using a slow stream of N_2 as carrier. The yield of $^{14}CO_2$ was >80%.

(c) ION EXCHANGE FRACTION OF RESIDUE. The radioactivity in the residues was shown by paper electrophoresis at pH 5.45 to be associated only with material having a net charge of zero or −1. Each residue was therefore fractionated on a column of Dowex-1-acetate (as used under (b)) by elution with water (15 ml) and 0.25 *N* acetic acid. Most of the radioactivity was present in the initial water fractions, and in the aspartic acid containing fractions (acetic elution volumes, 14–28 ml). The small amounts of radioactivity preceding the aspartic acid fraction were insufficient for characterization. Essentially all of the applied radioactivity was recovered.

The identity of the [^{14}C]aspartic acid was further established by paper electrophoresis at pH 5.3 (net charge −1), and at pH 3.5 (large separation aspartic and glutamic acids), by paper electrophoresis of its dansyl derivative at pH 4.4 [a clear separation of Dns-aspartic and Dns-glutamic acids were obtained (Gray, 1972)], by a degradative procedure (next section), and by thin-layer and paper chromatography.

The radioactivity present in the water eluate was resolved into at least three components on paper electrophoresis at pH 1.9. Appreciable radioactivity with a mobility corresponding to glycine was found in the sample from the *R. glutinis* enzyme, but no such radioactivity was present in the samples from the maize and potato enzymes. Electrophoresis at pH 1.9 in the presence of [^{3}H]alanine showed that the compound was separable from alanine. The compound moved with glycine upon TLC on ChromAR sheet 500 (Mallinckrodt) using 1-butanol–acetic acid–water (8:2:2 v/v). The identity of the eluted [^{14}C]glycine was further established as follows. After treatment (15 min) of a sample with nitrous acid (final concentration, 2 *M* $NaNO_2$, 1 *N* H_2SO_4) the radioactivity was recovered from a Dowex-1-acetate column in the glycolic acid region (as under $H^{14}COOH$ identification above) and migrated with glycolic acid upon paper chromatography (as under $H^{14}COOH$ above). A sample diluted with unlabeled glycine upon treatment with ninhydrin (Aronoff, 1956) gave both $^{14}CO_2$ and $H^{14}CHO$. The CO_2 derived from C-1 was trapped in ethanolamine (N_2 as carrier). This accounted for 30–50% of the radioactivity (controls with [1-^{14}C]glycine and [1,2-$^{14}C_2$]glycine). The volatile material in the residual solution was sublimed in the Y-assembly into a tube containing 0.4% dimedone solution. The crystalline precipitate that formed overnight at 21° was collected, washed, dissolved in dilute KOH, and shown to be radioactive.

Examination of [^{14}C]Aspartate from $^{14}CH_3NO_2$ La-

Table I: Direct Estimates of Molecular Weight Differences between Enzyme Subunits from Different Sources by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.

Comparison and Sources of Enzyme	Best Estimate Mol Wt Diff.[a]	95% Conf. Lts.[b]
R. glutinis minus maize[c]	−100	−1300 to +1100
Potato minus maize[d]	+1100	−1400 to +3600

[a] Subunit molecular weights ~83,000. [b] In rounding off the skewedness of the limits about the best estimate has disappeared. [c] This paper: samples preparation and electrophoresis as before. Three replicate experiments were performed using an Isco Model 1270 apparatus (with 7.5% gels, 0.6 × 7 cm) each having 5 gels with maize enzyme (25 μg), 5 with *R. glutinis* enzyme (25 μg), and three with a mixture of standard proteins. Mobilities were calculated relative to the tracking dye. An analysis of variance on the 30 relative mobilities for the samples permitted the total variance to be apportioned between experiments, sources of enzyme, and unassigned error. The 95% confidence limits in relative mobilities were calculated for 26 degrees of freedom and these limits were converted to molecular weight values. The design of the experiment allows greater precision than in the potato–maize comparison. [d] Havir and Hanson (1973).

beled Enzyme. The L-[^{14}C]aspartate content of samples of [^{14}C]aspartate isolated from the ion exchange column was determined by treating the sample (e.g., 1,800 dpm) and 2-ketoglutaric acid (7 μmol) in phosphate (K^+) buffer pH 7.6 (20 μmol) with glutamic–oxaloacetic transaminase (20 μl of $(NH_4)_2SO_4$ suspension, 150 μg of protein, 32 units) for 3 hr at 20°; final volume 1.2 ml. (Preliminary experiments showed that the reaction was essentially complete in 30 min.) An initial sample (400 μl) was withdrawn for radioactivity measurements. After 3 hr the balance of the reaction mixture was transferred with water washing (1.5 ml) to a column (0.5 × 2 cm) of Dowex-1-acetate (×8, 200–400 mesh) in a Pasteur pipet. The D-[^{14}C]aspartic acid remaining was eluted with 4 *N* acetic acid (complete in 5 × 0.5 ml). The L content of the sample was calculated by difference. Controls with DL-[4-^{14}C]aspartic acid (~2000 dpm) gave values in the range 47–53% L and recoveries from the column were >90% if enzyme was omitted.

The radioactivity present at C-4 of the L-[^{14}C]aspartate was determined by carrying out the above reaction in a Warburg flask with two side arms. One side arm contained 0.75 *M* phthalate (K^+) buffer, pH 5 (400 μl), the other 33.3% $Al_2(SO_4)_3$ (400 μl), and the center well a filter paper wick moistened with 3 *N* ethanolamine (50 μl) to trap $^{14}CO_2$. After the initial ⅓ aliquot had been withdrawn, the flask was closed with serum stoppers and left at 20° for 3 hr. The contents of the side arms and main compartment were then mixed and the flask was shaken gently overnight. The wick was added to a vial of scintillation fluid and the vial shaken for 1 hr prior to counting. A correction factor of 1.25 was applied on the basis of controls with DL-[4-^{14}C]aspartic acid. Preliminary experiments showed the decomposition of oxaloacetate by Al^{3+} (Krebs, 1942; Umbreit et al. 1949, p 176) was complete in < 1 hr. To measure the radioactivity in the $Al_2(SO_4)_3$ containing residue the usual scintillation fluid was replaced by Aquasol (New England Nuclear).

Second-Order Rate Constants for Nitroalkane Inactivations. (a) AT PH 8.7 FOR DIFFERENT NITRO COMPOUNDS. Initial mixture, enzyme (75–100 mU) in 0.2 *M* borate (Na^+) buffer (pH 8.7), 5.5 ml, at 30°. Initial spectrophotometric assay, a sample (1 ml) from the mixture was added to L-phenylalanine (1.2 ml, 0.0167 *M*) in a cuvet and the absorbance change at 30° followed at 290 nm for 10 min. Reaction: to initiate the inactivation a total additional volume of 100 μl was added, water then nitroalkane solution (25–100 μl). At 1-, 2-, or 3-min intervals, as appropriate to the rate of inactivation, samples (1 ml) from the reaction mixture were added to L-phenylalanine solution for assay as in the initial assay. The phenylalanine present during the assay was sufficient to completely protect the enzyme against further inactivation by the reagent. For each nitroalkane concentration the half-times ($t_{1/2}$) for inactivation were determined from a plot of the log of remaining enzyme activity against reaction time. The second-order rate constants were calculated as the slope of a plot of pseudo-first-order rate constants ($0.693/t_{1/2}$) against nitroalkane concentration (e.g., see Jencks, 1969).

(b) VARIATION WITH PH FOR CH_3NO_2 INACTIVATION. The initial mixture contained 0.2 *M* borate (Na^+) buffer in the range pH 6.9–10.6. A single concentration of nitromethane was used for each enzyme (maize, 0.21 m*M*; potato, 2.75 m*M*; *R. glutinis*, 2.06 m*M*), otherwise the procedure was as described above. The second-order rate constants were calculated as the pseudo-first-order rate constant divided by the standard nitromethane concentration.

Results

Subunit Structure of R. glutinis Enzyme. Previous evidence showed that the maize enzyme and the lowest molecular weight form of the potato enzyme are tetrameric with subunits similar if not identical in size and having molecular weights of ~83,000 (Havir and Hanson, 1973). The *R. glutinis* enzyme likewise gave a single protein staining band on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Table I shows the results of direct comparisons of the subunit molecular weights of the *R. glutinis* and potato enzymes with those of the maize enzyme. The observed differences are not statistically significant and the molecular weights estimated in these experiments did not differ significantly from our earlier determinations. We therefore consider 83,000 also to be the best estimate of the subunit molecular weight of the *R. glutinis* enzyme. The undissociated *R. glutinis* enzyme had, if anything, a slightly greater Stokes' radius than the undissociated maize enzyme (determined by repeated agarose-15m chromatography of both enzymes on the same column). The difference was ~1 Å (95% conf. Lts. 0–2 Å), the absolute value being ~63 Å (see Marsh et al., 1968). We conclude that the *R. glutinis* enzyme like the others is a tetramer of molecular weight ~330,000 with subunits closely similar in size. As appreciably lower estimates of the *R. glutinis* enzyme's molecular weight have been given (Hodgins, 1971), we wish to emphasize the importance of making determinations in the same laboratory using comparative methods.

The molecular weight of the wheat enzyme is similar to the above three enzymes (~320,000); the observation that there are two sizes of subunit may indicate the protein was damaged (Nari et al., 1972). In contrast to these enzymes from eukaryotic organisms the enzyme from *Streptomyces verticilatus* is reported to have a molecular weight ~226,000 (Emes and Vining, 1970). Histidine ammonia-lyase from bacteria has much smaller subunits (~50,000) but it also is tetrameric and the subunits are chemically similar and could be identical (Hassall and Soutar, 1974; Klee and Gladner, 1972).

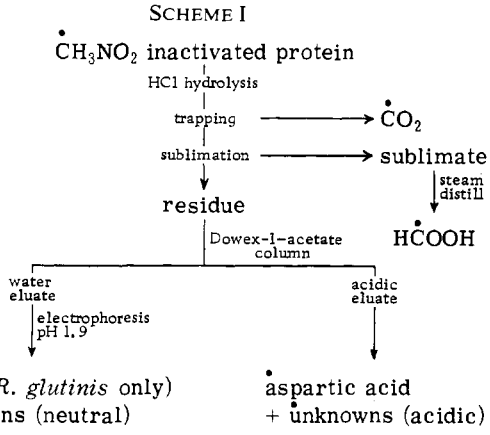
Labeling the Active Site with $^{14}CH_3NO_2$. Enzyme sam-

Table II: Hydrolysis Products from $^{14}CH_3NO_2$ Labeled Phenylalanine Ammonia-lyase.[a]

	Source of Enzyme		
	Maize (%)	Potato (%)	*R. glutinis* (%)
Composition of Hydrolysate (total 100%)[b]			
Volatile ($^{14}CO_2$)	10 (30)	10 (40)	5
Sublimate ($H^{14}COOH$)	30 (20)	20 (20)	50
Residue	60 (50)	70 (40)	45
Composition of Residue (total 100%)			
Dowex-1-acetate separation: acidic eluate			
[^{14}C]Aspartic acid	30	30	7
Unidentified (acidic)	15	30	13
Water eluate, then electrophoresis pH 1.9			
[^{14}C]Glycine	0	0	65
Unidentified (neutral)	55	40	15
Analysis of [^{14}C]aspartic acid by transaminase method (total 100%)			
As L-enantiomer	47	40	35
$^{14}CO_2$ release (C-4 of L enant. only)	45	35	36
Analysis of [^{14}C]glycine by ninhydrin method (total 100%)			
Carboxyl carbon			>26

[a] Fractionation as in Scheme I; for experimental details see Materials and Methods. [b] Figures not in parentheses are typical values for enzyme inactivated in 1973, those in parentheses for enzyme inactivated in 1972.

SCHEME I



ples were labeled with $^{14}CH_3NO_2$ as before (Havir and Hanson, 1973). The loss of enzyme activity was directly proportional to the incorporation of radioactivity. For the maize and potato enzymes the level of incorporation determined was stable after 5 hr of dialysis (~5.5 nmol/U) whereas the *R. glutinis* enzyme fell from an incorporation of 2.2 nmol/U at 5 hr to 1 nmol/U at 24 hr. Dialysis against 0.1 m*M* cinnamate failed to prevent this slow loss of radioactivity, but when inactivated enzyme dialyzed for 5 hr was stored at −10° for weeks or months the radioactivity released on lyophilization was <5%. If the specific activity of the enzyme used in these experiments approximated to that of fully active enzyme, the incorporations were 0.51, 0.78, and (after 5 hr dialysis) 1.3 molecules/tetramer for the maize, potato, and *R. glutinis* enzymes, respectively. The incorporation into the maize and potato enzymes is less than the best values calculated from previous experiments (1.6 and 2.1 molecules/tetramer). Some alternative explanations for the variability have been discussed (Havir and Hanson, 1973).

Hydrolysis Products of $^{14}CH_3NO_2$ Labeled Inactivated Enzyme. The distribution of radioactivity following HCl hydrolysis at 110° is summarized in Scheme I and Table II. Although the separation into volatile, sublimate, and residue fractions could be performed reproducibly with recoveries normally >80%, samples of maize and potato enzyme inactivated in December, 1972, and hydrolyzed in June, 1973, showed consistently higher amounts of the volatile and lower amounts of the residue fraction than those prepared in December, 1973, and hydrolyzed in May, 1974. When a sample of labeled *R. glutinis* enzyme was hydrolyzed for different times or with 1.2 *N* and 3 *N* HCl for 2–6 hr the distribution of radioactivity was essentially the same as under the standard conditions.

The major and probably only radioactive components in the volatile and sublimate fractions were respectively $^{14}CO_2$ and $H^{14}COOH$. The major single component in the residue derived from the maize and potato enzymes was [^{14}C]aspartic acid, but this was accompanied by one or more acidic compounds and 3 or 4 compounds that were eluted from the Dowex-1-acetate column by water. [^{14}C]Glycine or β-[^{14}C]alanine could not be detected in the water eluate. Although [^{14}C]aspartic acid was recovered from the *R. glutinis* enzyme residue, the major component was [^{14}C]glycine.

The [^{14}C]aspartic acid, identified by standard methods, was shown to be a mixture of D- and L-[4-^{14}C]aspartic acids by determining the fraction that could be converted enzymically to [^{14}C]oxaloacetate. The sample from the maize enzyme appeared to be a racemic mixture whereas the samples from potato and *R. glutinis* appeared to contain somewhat more D than L enantiomer (Table II). In separate experiments the [^{14}C]oxaloacetate was specifically decomposed to $^{14}CO_2$ with Al^{3+} ions. As agreement was observed in each case between the yield of $^{14}CO_2$ and the L content of the sample, essentially all of the radioactivity of the L enantiomer was at C-4. The presence of ^{14}C at C-4 of the D enantiomer is assumed but not proven.

The [^{14}C]glycine from the *R. glutinis* enzyme was identified by standard procedures. Ninhydrin degradation showed that <50% of the radioactivity was at C-1. [^{14}C]Formaldehyde dimedone adduct was prepared from the steam distilled residue to confirm the presence of radioactivity at C-2.

Kinetics and Structural Dependency of Nitroalkane Inactivation. If the inactivating species is the aci-ion of a nitro compound $(RCHNO_2)^-$, one would expect the more alka-

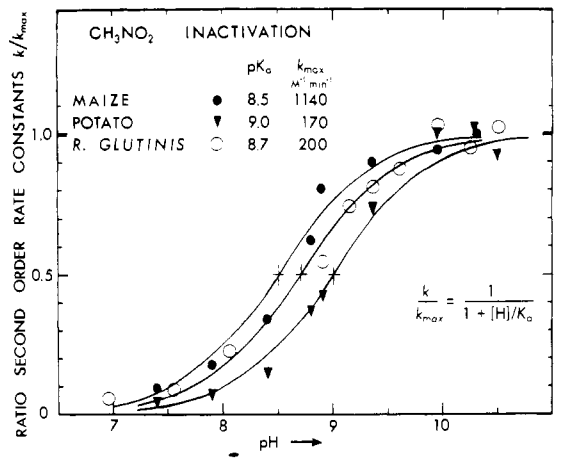


FIGURE 1: pH dependence of the second-order rate constants for nitromethane inactivation of phenylalanine ammonia-lyase from maize, potato, and *R. glutinis*.

line the system, the greater the ease of forming the electrophilic reagent. Figure 1 shows that although the inactivation process behaves as a simple titration curve, the observed pK_a varies with the enzyme. As the pK_a of CH_3NO_2 is ~10.3 (Wheland and Farr, 1943; Pearson and Dillon, 1950), the environment of the bound reagent in all three cases has shifted its effective pK_a from that of CH_3NO_2 by at least 1 pH unit. The rate of inactivation by the fully ionized species as given by k_{max} also varies with the enzyme. Both results suggest that the inactivation is catalyzed by the enzyme.

The pK_a for nitroethane is ~8.6 (Dixon and Bruice, 1970; Freeman and Lin, 1971; Pearson and Dillon, 1950); however, this compound is a much less effective reagent at pH 8.7 than nitromethane (Table III). Nitroethanol and nitropropionic acid are potentially useful as both contain an additional functional group which could be linked to other structures, markers, or reagents.

Discussion

The finding that the higher plant and fungal enzymes examined have similar subunit sizes and tetrameric structures suggests that these properties have been conserved in both kingdoms during evolution (the separation of plants and fungi occurred some 1.5×10^9 years ago; Ramshaw et al., 1972). This hypothesis, which requires much more detailed testing, is of particular importance in relation to the study of the regulation and variation of phenylpropanoid metabolism in higher plants.

An exchange of genetic information between plants and fungi is a conceivable explanation for the observed uniformity; however, the exterior amino acids of the plant and fungal enzymes differ significantly: rabbit antisera to the purified *R. glutinis* enzyme used in this study failed to cross react with the enzyme from various higher plants tested (unpublished experiments by T. S. Handwerker and L. L. Creasy, Cornell University, Ithaca, N.Y.). The finding that [1,2-$^{14}C_2$]glycine was obtained upon hydrolysis of the nitromethane labeled *R. glutinis* enzyme but not from the others may indicate that there are important structural differences between the plant and fungal enzymes in the active site region.

All of the enzymes after $^{14}CH_3NO_2$ labeling yielded D- and L-[4-^{14}C]aspartic acid, $H^{14}COOH$, and $^{14}CO_2$ upon

Table III: Second-Order Rate Constants for Nitroalkane Inactivation of Phenylalanine Ammonia-lyase at pH 8.7.[a]

Nitroalkane	Source of Enzyme: Maize (M^{-1} min^{-1})	Potato (M^{-1} min^{-1})	*R. glutinis* (M^{-1} min^{-1})
O_2NCH_3	610	55	100
$O_2NCH_2CH_3$	14	1.3	9
$O_2NCH_2CH_2OH$	75	1.3	27
$O_2NCH_2CH_2COO^-$	16	12	24–34

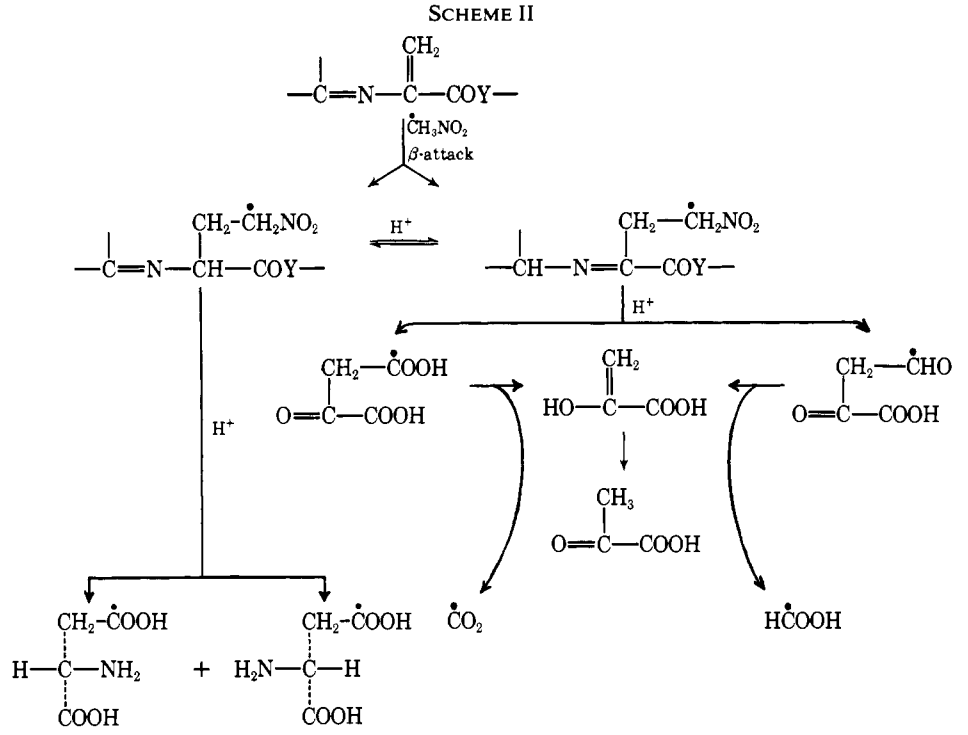
[a] See also Figure 1. The second-order rate constants were determined as described under Materials and Methods. The range of nitroalkane concentrations (m*M*) used for maize, potato, and *R. glutinis* enzymes respectively were: O_2NCH_3: 0.14–0.45, 0.46–1.43, and 0.46–1.83; $O_2NCH_2CH_3$, 2.7–5.5, 8.3–24.2, and 20–80; $O_2NCH_2CH_2OH$, 1.8–6.2, 3.0–20.0, and 12.5–37.5; $O_2NCH_2CH_2COO^-$, 2.4–7.1, 3.0–7.2, and 4.8–7.1.

acid hydrolysis. The formation of the first of these indicates that the nitromethane attacked the β carbon of a structure containing the alanine skeleton as carboxyl groups are the usual product of acid hydrolysis of nitroalkyl aci-salts (Meyer and Wurster, 1873; Sowden, 1951). Inactivation of the enzyme involves either β displacement of an unknown group or β addition to the double bond of a dehydroalanine residue [>N—C$^\alpha$(=C$^\beta$H$_2$)CO—]. Either process would generate the partial structure >NCH($CH_2{}^{14}CH_2NO_2$)-CO—. If a β displacement takes place, one must assume that the α center initially has a defined chirality but adjacent groups promote complete or partial racemization on hydrolysis. If β addition occurs, stereoselectivity of protonation at C$^\alpha$ seems likely, but the substituents that activate the double bond toward nitromethane attack could promote complete or partial racemization upon hydrolysis. [The [3-^{3}H]alanine isolated upon hydrolysis of the NaB^3H_4 inactivated maize and potato enzymes (Hanson and Havir, 1970) and the [4-^{14}C]aspartic acid isolated after hydrolysis of the $^{14}CN^-$ inactivated *R. glutinis* enzyme (Hodgins, 1971) were both racemic.]

Additions to dehydroalanine residues can be α or β depending on the attacking group and conditions (Hanson and Havir, 1972b). Upon hydrolysis [1-^{14}C]alanine should be formed from an α adduct along with $^{14}CO_2$ but we have not detected this compound.

The formation of $^{14}CO_2$ and $H^{14}COOH$ can be explained (Scheme II) if the dehydroalanine residue is activated through Schiff base formation as suggested by Givot et al. (1969) for the prosthetic group of histidine ammonia-lyase. The postulated conversion of R-$^{14}CH_2NO_2$ to R-^{14}CHO is known to occur under certain conditions of hydrolysis (Nef, 1894). The same products would be generated if the imine were not present in the prosthetic group itself but were formed either spontaneously or during hydrolysis after β addition of $^{14}CH_3NO_2$. The scheme also accounts for racemization at C$^\alpha$. If β addition of $H^{14}CN$ to the same partial structure is considered, then racemic [4-^{14}C]aspartic acid isolated after hydrolysis should be accompanied by $^{14}CO_2$. We suggest that the volatile radioactivity not accounted for in the experiments of Hodgins (1971) with $H^{14}CN$ labeled *R. glutinis* enzyme was $^{14}CO_2$. Givot et al. (1969) in studying $^{14}CH_3NO_2$ inactivated histidine ammonia-lyase included a reductive step before HCl hydrolysis, thus a general comparison of the two investigations is not possible. They did, however, interpret the formation of β-[1-^{14}C]alanine exclusively in terms of hydrolysis. In this respect our

SCHEME II



results differ as we have been unable to detect the compound in our hydrolysis residues.

In the case of the *R. glutinis* enzyme, it is not apparent how [^{14}C]glycine could derive from a simple $^{14}CH_3NO_2$ adduct to a dehydroalanine residue. The condensation of $^{14}CH_3NO_2$ with a molecule that had already reacted with the enzyme could result in a ^{14}C–^{14}C labeled product, but hydrolysis should not reduce a nitro group in such an adduct to the $-NH_2$ level. We tentatively suggest, therefore, that in each molecule of [^{14}C]glycine one carbon comes from $^{14}CH_3NO_2$ and the other from a part of the active site that is not a dehydroalanine residue.

A possible explanation for the formation of [1-^{14}C]glycine is as follows:

$$RN{=}CH{-}CONH{-} \rightarrow RNH{-}CH(^{14}CH_2NO_2){-}CONH{-} \rightarrow H_2NCH(^{14}COOH)COOH \rightarrow H_2NCH_2\,^{14}COOH \text{ or } ^{14}CO_2$$

The same initial adduct could yield $H_2N^{14}CH_2COOH$ if an intermediate in the hydrolysis sequence is symmetrical or can rearrange but the obvious candidates [e.g., $H_2NCH_2{}^{14}C(OH){=}NOH$] do not have the required properties. Alternative degradative studies of $^{14}CH_3NO_2$ labeled *R. glutinis* enzyme are in progress. If the explanation for [1-^{14}C]glycine is correct, then the Schiff base could be that shown in Scheme II, i.e., the partial structure is $O{=}C(NH{-}){-}CH{=}N{-}CH({=}CH_2)COY{-}$.

The reactive group revealed by chemical studies of phenylalanine ammonia-lyase need not be the form of the prosthetic group active during catalysis. [Note that enzymes other than ammonia-lyases upon chemical inactivation can give products explicable in terms of a dehydroalanine residue (Cohn and Phillips, 1974).] Figure 1 implies that inactivation by CH_3NO_2 is catalyzed by the enzyme. This is most readily explained if the CH_3NO_2 molecule fits into the position occupied by the amino group of phenylalanine during normal catalysis and the prosthetic group is the *same* in both cases. If the fit is exact, then both inactivation and catalysis must involve attack on C^{β}. In the case of the *R. glutinis* enzyme the $CH_2NO_2^-$ ion once formed may have sufficient freedom of motion to attack not the nearest but a more reactive center.

Lastly, it must be asked whether the dehydroalanine-imine hypothesis can be reconciled with the spectral data on phenylalanine and histidine ammonia-lyase. The low intensity shoulder in the uv absorption spectrum above 300 nm was unchanged for phenylalanine ammonia-lyase after $NaBH_4$ reduction (Havir and Hanson, 1973) whereas that of histidine ammonia-lyase was changed (Klee, 1970). We find that phenylalanine ammonia-lyase from maize and *R. glutinis* is protected against inactivation at pH 10.5 in carbonate (Na^+) buffer (0.05 *M*) by L-cysteine (5 m*M*) or mercaptoethanol (5 m*M*) to about the same extent. The spectrum of *R. glutinis* enzyme was unchanged in the region examined (>300 nm) after inactivation at pH 10.6 in the presence of 5 m*M* L-cysteine for 18 hr. In contrast, histidine ammonia-lyase, though stable at pH 10.6, developed a strong absorption band at 340 nm when inactivated under these conditions (Klee, 1974). If the prosthetic group is indeed the same in the two enzymes, the chemically induced spectral changes for histidine ammonia-lyase may be related to the presence of a divalent metal ion at the active site. The dehydroalanine imine system itself if it absorbed with low intensity at 280 nm or below might be difficult to detect. In view of the above and the large difference in the subunit sizes of the two enzymes, it is important to use the analogy between these ammonia-lyases with restraint and to assemble the evidence concerning the active site of each enzyme separately.

Acknowledgment

We should like to thank Katherine A. Clark and Mr. George Smith for skillful technical assistance.

References

Aronoff, S. (1956), Techniques of Radio-Biochemistry, Ames, Iowa, The Iowa State College Press, p 185.

Camm, E. L., and Towers, G. H. N. (1973), *Phytochemistry 12,* 961.
Cohn, M. S., and Phillips, A. J. (1974), *Biochemistry 13,* 1208.
Creasy, L. L., and Zucker, M. (1974), *Recent Adv. Phytochem. 8,* 1.
Davis, L., and Metzler, D. E. (1972), *Enzymes, 3rd Ed. 7,* 33.
Dixon, J. E., and Bruice, T. C. (1970), *J. Am. Chem. Soc. 92,* 905.
Emes, A. V., and Vining, L. C. (1970), *Can. J. Biochem. 48,* 613.
Freeman, F., and Lin, D. K. (1971), *J. Org. Chem. 36,* 1335.
Givot, I. L., Smith, T. A., and Abeles, R. H. (1969), *J. Biol. Chem. 244,* 6341.
Gray, W. R. (1972), *Methods Enzymol. 25,* 121.
Hanson, K. R., and Havir, E. A. (1969), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 28,* 602.
Hanson, K. R., and Havir, E. A. (1970), *Arch. Biochem. Biophys. 141,* 1.
Hanson, K. R., and Havir, E. A. (1972a), *Enzymes, 3rd Ed. 7,* 75.
Hanson, K. R., and Havir, E. A. (1972b), *Recent Adv. Phytochem. 4,* 45.
Hassall, H., and Soutar, A. K. (1974), *Biochem. J. 137,* 559.
Havir, E. A., and Hanson, K. R. (1968), *Biochemistry 7,* 1904.
Havir, E. A., and Hanson, K. R. (1970), *Methods Enzymol. 17A,* 575.
Havir, E. A., and Hanson, K. R. (1973), *Biochemistry 12,* 1583.
Hodgins, D. S. (1971), *J. Biol. Chem. 246,* 2977.
Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N.Y., McGraw-Hill, pp 558–564.
Klee, C. B., and Gladner, J. A. (1972), *J. Biol. Chem. 247,* 8051.
Krebs, H. (1942), *Biochem. J. 36,* 303.
Meyer, V., and Wurster, C. (1873), *Ber. 6,* 1168.
Marsh, H. V., Jr., Havir, E. A., and Hanson, K. R. (1968), *Biochemistry 7,* 1915.
Nari, T., Mouttet, Ch., Pinna, M. H., and Richard, J. (1972), *FEBS Lett. 23,* 220.
Nef, J. U. (1894), *Justus Liebigs Ann. Chem. 280,* 263.
Parkhurst, J. R., and Hodgins, D. S. (1972), *Arch. Biochem. Biophys. 152,* 597.
Pearson, R. G., and Dillon, R. L. (1950), *J. Am. Chem. Soc. 72,* 3574.
Ramshaw, J. A. M., Richardson, D. L., Meatyard, B. T., Brown, R. H., Richardson, M., Thompson, E. W., and Boulter, D. (1972), *New Phytol. 71,* 773.
Sowden, J. C. (1951), *Adv. Carbohydr. Chem. 6,* 291.
Umbreit, W. W., Burris, R. H., and Stauffer, J. F. (1949), Manometric Techniques, Minneapolis, Minn., Burgess, p 175.
Wheland, G. W., and Farr, J. (1943), *J. Am. Chem. Soc. 65,* 1433.
Wickner, R. B. (1969), *J. Biol. Chem. 244,* 6550.
Wood, H. G., and Gest, H. (1957), *Methods Enzymol. 3,* 2854.
Zelitch, I. (1965), *J. Biol. Chem. 240,* 1869.

The Mechanism of Microsomal and Mitochondrial Nitroreductase. Electron Spin Resonance Evidence for Nitroaromatic Free Radical Intermediates†

Ronald Paul Mason* and Jordan L. Holtzman

ABSTRACT: Electron spin resonance spectra are observed during the enzymatic reduction of many nitrophenyl derivatives by rat hepatic microsomes or mitochondria. The spectra indicate that nitroaromatic anion radicals are present and are freely rotating in aqueous solution at a steady-state concentration of 0.1–6 μM. The rate of formation of *p*-nitrobenzoate (NBzO) dianion radical in microsomal incubates is consistent with the radical being an obligate intermediate in the reduction of NBzO to *p*-aminobenzoic acid. A model system consisting of NBzO, NADPH, and FMN, but no heme-containing compounds, also reduced NBzO to the NBzO dianion free radical. The steady-state concentration of the anion radicals in microsomal systems is not altered by CO. This observation, together with the results from the model system, suggests that the formation of nitroaromatic anion radicals is mediated through a flavine and not cytochrome P-450. The oxidation of the anion radical intermediate by O_2 to the parent nitro compound is proposed to account for the well-known O_2 inhibition of microsomal nitroreductase.

Fouts and Brodie (1957) were the first to report the presence of nitroreductase in hepatic microsomes. This activity was found to be dependent on either NADH or NADPH, was inhibited by oxygen, and was markedly stimulated by the addition of flavines (Gillette, 1971; Weisburger and Weisburger, 1971). The system exhibited little substrate specificity, being able to reduce not only nitrobenzene and *p*-nitrobenzoate, but also a wide range of other nitro com-

† From the Veterans Administration Hospital, Minneapolis, Minnesota 55417, and the Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455. *Received April 19, 1974.*