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AN ENZYMATIC CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF D-AMINO ACIDS IN PLANT AND SOIL EXTRACTS*

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Abstract—A D-amino acid oxidase procedure has been adapted and coupled with radioisotopes, continuous liquid-flow scintillation monitoring, and high resolution ion exchange chromatography for quantitative determination of amino acid racemates in extracts from plants and soils. The method is 10^3 times more sensitive than previous indirect manometric techniques and yields data on individual amino acids rather than only a total value for all reactive D-amino acid components in a complex mixture. Present quantitation at the nanomole level could be extended to subnanomole levels by use and refinement of available amino acid analyzer technology. Preparation of derivatives is not required, but the technique is limited to those amino acids on which the enzyme is active. The method has been applied to the detection of D-amino acids in plant and soil extracts.

INTRODUCTION

RECENT reviews on the occurrence and importance of D-amino acids in animals,[1] microorganisms and antimetabolites[2] serve to underscore the lack of data about D-amino acids in soils and plants. Such reports suggest that D-amino acids must be present in the soils system as excretory, residual, and hydrolysis products of biological agents such as earthworms, bacteria, etc., and as probable transformation products of high temperature, high pressure reaction in geologic sediments. Questions of how to prove their presence, of what happens to them in the soil, of what effect they might have on plant growth, led to the present work. Although present concern has been with terrestrial materials, the results may have relevance to determining configuration of possible amino acids of extraterrestrial origin.

In recent studies, we found that ^{14}C labeled D-valine, D-leucine, D-alanine, D-methionine, and D-lysine (at 10^{-3} M) when applied to 18-day-old maize (*Zea mays*) or ryegrass (*Lolium* sp.) were readily absorbed and apparently metabolized within hours.[3] Maize and ryegrass grown from seed on full nutrient solution plus the same 10^{-3} M D-amino acids showed growth response ranging from no effect to severe root inhibition, blackening of root tips, chlorosis, necrotic lesions of leaves, and other physiological disorders (Aldag and Young, unpublished observations). Previous work had shown similar disturbances in

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[1] J. J. CORRIGAN, *Science* **164,** 142 (1969).
[2] M. BODANSKY and D. PERLMAN, *Science* **163,** 352 (1969).
[3] R. W. ALDAG and J. L. YOUNG, *Agron. J.* **62,** 184 (1970).

sunflowers grown on D-leucine.[4,5] To pursue these observations required a means of identifying and quantitating individual D-amino acids in complex mixtures. We report here the adaptation and application of an enzymatic oxidation procedure[6] in combination with radioisotopes, continuous liquid-flow scintillation monitoring and ion exchange chromatography for determination of nanomole quantities of amino acid racemates in plant and soil extracts.

RESULTS AND DISCUSSION

The effectiveness of D-amino acid oxidase on various reference D-amino substrates appears in Table 1. Each D-amino acid was tested in a single oxidation experiment, and mixtures of up to 5 D-amino acids (each at 50 nmole) were also oxidized together. D-Glucosamine and D-galactosamine were included because they are commonly present in soil extracts; D-asparagine was included because substantial quantities of the amides commonly appear in 'free' amino acids of plant extracts, and in detectable quantities of some soil extracts. (Pure D-glutamine was not available for the trials.) The data are similar to the

TABLE 1. EFFECTIVENESS OF D-AMINO ACID OXIDASE ON VARIOUS REFERENCE D-AMINO ACIDS UNDER GIVEN INCUBATION CONDITIONS*

Compound	Amount oxidized (%)
D-Aspartic acid	22
D-Threonine	16
D-Serine	98
D-Asparagine	Nil
D-Glutamic acid	9
^{14}C-D-Alanine	100
D-α-NH_2-*n*-Butyric acid	100
^{14}C-D-Valine	100
^{14}C-D-Methionine	100
^{14}C-D-Leucine	100
D-Tyrosine	100
D-Phenylalanine	100
^{14}C-D-Lysine	47
D-Glucosamine	12
D-Galactosamine	7

* See Experimental and text. Amount oxidized was calculated from ninhydrin reaction values for equal quantities of D-amino acid substrate before and after treatment with the enzyme and for ^{14}C labeled compounds by comparison of 'before' and 'after' radioactivity peaks. Yield of NH_3 was an additional check for reference standards.

[4] F. SCHEFFER, R. KICKUTH and R. ALDAG. *Naturwiss.* **54,** 144 (1967).
[5] F. SCHEFFER, R. KICKUTH and R. ALDAG. *Z. Pflanzen Ernaehr. Dueng. Bodenk.* **116,** 25 (1967).
[6] P. BOULANGER and R. OSTEUX, In *Methods of Enzymatic Analysis* (edited by H. BERGMEYER), p. 371, Academic Press, New York (1963).

results of Scannone *et al.*, as determined on a number of D-, L-, and DL-compounds.[7] D-Aspartic acid, D-glutamic acid, D-threonine, D-asparagine and the D-amino sugars were all oxidized rather poorly. Nearly half the D-lysine was converted in 60 min although D-lysine is reportedly very slowly and poorly converted.[7,8] The other amino acids tested were essentially completely oxidized.

The extent of conversion was reflected in disappearance of the ninhydrin peak, and corresponding appearance of an ammonia peak; the resulting alpha-keto acid analog is ninhydrin negative but elutes from the ion exchange column shortly after buffer breakthrough. As indicated under Experimental, the use of the ^{14}C-labeled compounds permitted an additional check by determining ^{14}C loss from the amino acid peak and ^{14}C appearance in the alpha keto acid analog. ^{14}C-D-methionine as an internal check was used in this way (see later discussion and Fig. 1). Methionine also served as an indicator of undesirable chemical degradation, i.e. by appearance or absence of its sulfoxides. The ammonia level increase was a useful check in pure systems, but the relative quantities of ammonia in plant and soil extracts were usually too high for using ammonia increase as a measure of oxidation of D-amino acids in these extracts.

The purity of the enzyme obviously affects the sensitivity and capacity of the method. As indicated, (see Experimental) interfering levels of 'free' amino acids were present in the crude D-amino acid oxidase and in the twice crystallized catalase preparations from Worthington. Trace amounts (generally less than 1 nmole) of some common amino acids (asp, ser, thr, glu, gly, ala, val, meth, ileu, leu) were occasionally detectable in early lots of Sigma twice crystallized catalase or DAOFF preparation. These were considered negligible for present purposes; nevertheless, subtraction of any 'blank' values was made to enhance data reliability.

Extensive kinetic experiments were not run with the DAOFF enzyme but it was demonstrated for the reaction conditions employed, that doubling the concentration of D-amino acid oxidase doubled the amount of substrate oxidized. The quantities described for the oxidation procedure yielded levels of amino acids conveniently handled by the amino acid analyzer. The first run to establish 'before oxidation' amino acid concentrations permitted adjustment, if necessary, in amount of enzyme or sample used, i.e. to balance requirements for sufficient enzyme (and FAD),[8] as essential, yet to avoid expensive excess.

Sensitivity permitted detection at least to 5 nmole of D-amino acid in a racemic mixture. This is equivalent to the catalase inhibition method[7] and a thousand times more sensitive than manometric techniques which call for 10 μmole of D-amino acid as a minimum.[6] Obvious limitations of the method include the failure of the enzyme to oxidize all D-amino acids and the need for two chromatographic runs, i.e. before and after oxidation. Other available techniques employing gas and liquid chromatography suffer similar and other limitations.[9–11] The derivative-type sample preparation is effective for a limited number of amino acids but several different derivative procedures, preparations, and chromatograms are necessary. Resolution of D- and L-configurations as paired peaks in one run is a distinct advantage of these methods on simple mixtures, but simultaneous resolution of complex

[7] H. SCANNONE, D. WELLNER and A. NOVOGRODSKY, *Biochem.* **3**, 1742 (1964).
[8] M. DIXON and K. KLEPPE, *Biochim. Biophys. Acta* **96**, 368 (1965).
[9] E. GIL-AV, R. CHARLES and G. FISCHER, *J. Chromatog.* **17**, 408 (1965).
[10] G. E. POLLOCK and V. I. OYAMA, *J. Gas Chromatog.* **4**, 126 (1966), and G. E. POLLOCK and A. H. KAWAUCHI, *Anal. Chem.* **40**, 1356 (1968).
[11] J. M. MANNING and S. MOORE, *J. Biol. Chem.* **243**, 559 (1968).

amino acid mixtures is not yet sufficient to avoid ambiguity from over-running or overlapping peaks without multiple runs, even with high resolution ion exchange columns. At present, the enzymatic and derivative-type approaches are best considered complimentary.[11]

The reliability of the method obviously is dependent on the specificity of the D-amino acid oxidase and on the purity of reference D-amino acids. Specificity of the enzyme at nanomole sensitivity levels appears well established,[7] at least for the common amino acids with which we have been dealing. A possible exception apart from the previously known slight attack by the enzyme on L-proline, is an apparent very slight attack on L-alanine, detectable at high (micromolar) concentrations of L-alanine. Detection of < 10 nanomoles of D-isomer contaminant in micromolar concentrations of commercial L-amino acid salts further illustrates the specificity and effectiveness of the enzyme.[7] Purity of the D-amino acids used in the current work was determined by Cal Biochem through standard chromatographic and optical rotation methods for the cold (unlabeled) compounds. In the case of radioactive D-amino acids, not enough was available for rotation measurements but possible 'L" isomer contamination was tested by isotope dilution analysis with authentic D-amino acids (personal communication with Cal Biochem). Purity of the compounds was further confirmed by the enzymatic oxidation experiments as previously illustrated in Table 1.

Application of Method in Plant and Soil Extracts

Figure 1 illustrates results of the method on 'free' amino acids extracted from ^{14}C-D-valine treated corn roots. Effectiveness of the enzyme was apparent from the absence of any radioactivity, at 80–90, and 274 min; no methionine oxides or parent ^{14}C-D-methionine were present after the D-amino acid oxidase incubation. The sharp increase in radioactivity at 24 min (top chart) after enzyme oxidation resulted from ^{14}C-D-valine and ^{14}C-D-methionine conversion to their respective alpha keto acid analogs. The sharp increase in NH_3 (545 min, center chart) also resulted from these conversions.

Overlaying the bottom and center charts in Fig. 1 clearly showed (as did area integration) some changes in a few other peaks, i.e. glutamic acid, glutamine and alanine. The loss of glutamine showed up as an increase in glutamic acid as might be expected from the acid lability of glutamine (enzyme action was terminated and enzyme precipitated with TCA). The decrease in alanine indicates presence of some D-alanine in the extract, the origin of which is not yet certain.

Figure 2 illustrates an application of the method to an extract of 'free' amino acids from soil. This particular conifer soil extract had high levels of common amino acids with which to work. Of these, the swamping concentration at the valine position proved most startling, not only because of its exceptionally high value but because > 90 per cent was removed by incubation with D-amino acid oxidase. Conceivably, seed material, mycorrhizal, cell-wall or other microbial residue could have been the source.[12] The procedure, thus, appeared promising for extracts of at least some soils.

An important concern at this stage was to establish utility of the procedure for other soil extracts. With the aid of ^{14}C-D-methionine as an internal check, the enzyme was found effective on 'free' amino acids in soil extracts from a number of soils but not in all.

[12] With regard to this anomolous result, we have observed other instances of unusually high levels of a particular amino acid (e.g. tyrosine and alanine from soils and microbially produced humic acid-like preparations). Of the unusual amino acids found in plant tissues, Fowden concluded that often they represent a major component of the nonprotein nitrogen fraction of plants. L. FOWDEN, *Utilization of Nitrogen and Its Compounds by Plants*, p. 285, Academic Press, New York (1959).

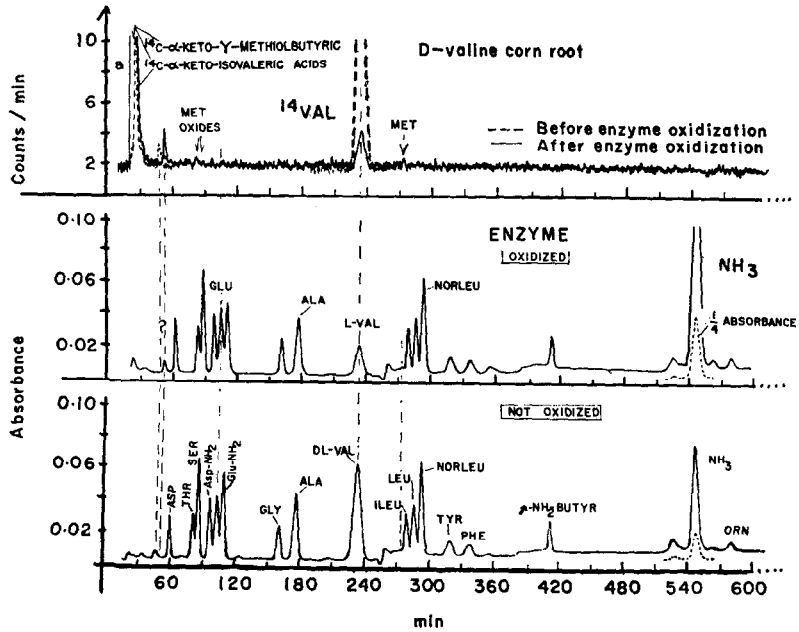

FIG. 1. ILLUSTRATION OF A COMBINATION ENZYMATIC CHROMATOGRAPHIC DETERMINATION OF D-AMINO ACIDS IN PLANT EXTRACTS.
Substrate was 'free' amino acids (80% ethanol extractables) from root tissue of ^{14}C-D-valine treated maize. 10 nmole of ^{14}C-D-methionine were added to the extract as an internal check just prior to addition of D-amino acid oxidase.

Chromatographic interferences sometimes occurred in the tyrosine-phenylalanine regions. Also, with some samples, partial to nearly complete inactivation of the D-amino acid oxidase occurred, as in a sample from a red Alder site (836). These tended to be dark, densely colored concentrates which failed to completely dissolve in the pH 8·3 Tris buffer. Removal of heavy metals, particularly iron and aluminum, by an acetylacetone extraction gave little improvement. 'Desalting' through a miniature ion exchange column removed whatever enzyme inhibitory components were present in most samples.

Because of their presumed lesser biological reactivity, more measurable levels of D-amino acids should have accumulated in the 'bound' or 'hydrolyzable' nitrogen fraction in soils. Unfortunately, these bound amino acids are also a much more severe analytical challenge, not so much from the enzyme oxidation step as from the possible chemical racemization during hydrolysis. Initial trials, again with ^{14}C-D-methionine as a check, showed the enzyme procedure worked well on water solutions of amino acids recovered from HCl hydrolysates after *in vacuo* removal of the concentrated HCl. As a result, D-amino acids were found in acid hydrolysates from soil. This appeared promising providing strong acid hydrolysis (in contrast to known damaging effects of alkaline hydrolysis) does not cause significant racemization. However, subsequent initial trials with ^{14}C-labeled D-alanine, D-valine and D-leucine indicated 22, 19, and 5 per cent racemization after 6 hr refluxing in 6N HCl. Moreover, the extent of racemization is variable, depending on, among other things, the particular peptide linkages or neighboring residues involved.[11] Hence, as with an earlier

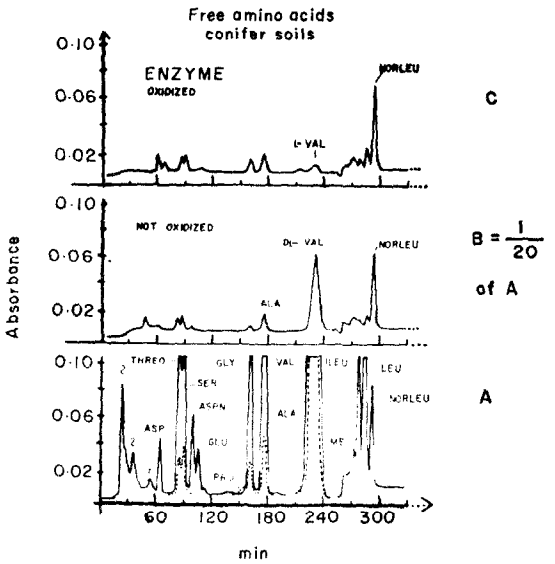

FIG. 2. 'FREE' AMINO ACIDS EXTRACTED FROM A CONIFER AREA SOIL (CHART A). A 0·05 ALIQUOT OF 'A' WAS RERUN WITHOUT ENZYME OXIDATION TO CONFIRM PRESENCE OF THE SWAMPING CONCENTRATION AT THE VALINE POSITION (230 min, CHART B). AN IDENTICAL 0·05 ALIQUOT SUBJECTED TO D-AMINO ACID OXIDASE SHOWS THE PEAK AT THE VALINE POSITION WAS LARGELY D-CONFIGURATION COMPOUND (CHART C).
Note that internal standard 'norleucine' is added to the samples at the time of application to the chromatography column and that a number of easily detectable components in A could be missed entirely or disregarded as inconsequential 'traces' in B if analyses were limited to assay for more prominent components only.

report,[13] it remains to be seen whether or not the D-amino acids found in the soil hydrolysates came from the soil or were racemic artifacts of the procedures.

EXPERIMENTAL

Materials. ^{14}C-labeled and unlabeled D-amino acids from Calbiochem. Corp. California. Catalase (Beef Liver, 2× crystallized), and Tris buffer from Sigma Chem. Corp., St. Louis, Missouri. D-amino acid oxidase (hog kidney) Code: DAOFF from Worthington Biochem. Corp., New Jersey, U.S.A. Pure enzymes are essential for the procedure. Worthington offers two D-amino acid oxidase preparations; a DAO and DAOFF. The DAO preparation with a minimum specific activity of 0·025 units/mg is relatively crude but requires no additional coenzyme for full activity. Our DAO sample (and some catalase preparations from both Worthington and Sigma) showed high levels of 'free' amino acids which made them unuseable in combination with a chromatographic assay. The DAOFF preparation is purified as an intact flavoprotein by the method of Massey *et al.*[14] except that at 'stage 4', the preparation is isolated electrophoretically rather than by adsorption on calcium phosphate gel. The salt-free lyophilized powder had a minimum specific activity of 2 units/mg.

D-*Amino Acid Oxidation Assay Procedure*

Reaction quantities and conditions were adapted from Scannone *et al.*[7] The most important difference is that we determined activity or specificity of the enzyme by sensitive (nmole-level) chromatographic quantitation of the D- and/or L-amino acid substrates, both before and after enzymatic incubation. Values for individual compounds are thus obtained, although the oxidation is performed on a complex mixture. Others have

[13] J. M. BREMNER, *Biochem. J.* **47**, 538 (1950).
[14] V. MASSEY, G. PALMER and R. BENNET, *Biochim. Biophys. Acta* **48**, 1 (1961).

used the indirect methods of measuring the inactivation of catalase by 3-amino-1,2,4-triazole in the presence of H_2O_2 generated by the amino acid oxidase reaction[7] (nmole sensitivity), or of measuring O_2 consumption in a Warburg apparatus[6] (μmole sensitivity). These indirect assays give only a total value for reactive D-amino acid content which is seriously limiting where knowledge of individual D-amino acid content is desired.

For plant extracts, the reaction mixture contained: Tris HCl buffer (150 μmole, pH 8·3 at 37°), catalase (6000 units), D-amino acid oxidase (0·5 units) and D-amino acid ($\approx$50–500 nmole) in a total vol. 0·5–1·0 ml. An exact final volume in this range is not critical if the total supernatant is applied to the analyzer; effective reduction in enzyme concentration or enzyme-substrate contact at much larger volumes (2–10 ml) can lead to errors from variable or incomplete oxidation. To minimize degradation of the enzyme, which shows instability in dilute solution, the oxidase was dissolved and diluted and the catalase was diluted just prior to use. The mixture was conveniently incubated in a 10-ml centrifuge tube at 37° with continuous shaking for 1 hr and the enzyme activity terminated by adding 50 μl of 10% trichloroacetic acid. After 1 hr, the precipitate was sedimented by centrifugation at 2000 *g* for 30 min.

The supernatant was assayed by quantitative transfer to and elution through an automated amino acid analyzer.[15] For amino acids that are rapidly and completely oxidized by the D-amino acid oxidase, the difference in concentration between identical paired incubated samples, one being a control lacking only the oxidase enzyme replaced by equivalent volume of buffer, equals the amount of D-configuration isomer in the sample. Difference values obtained when the control tube is not carried through identical incubation procedures may yield false positive results should any reaction (condensation, adsorption, coprecipitation, etc.) occur with unknown components in the extracts—a greater hazard with some soils than with plant extracts. For D-amino acids on which the enzyme is not 100% effective under given incubation conditions, estimates can be made by applying a percentage activity factor as previously determined for the enzyme on the specific amino acid in question.

A double check on extent of enzymatic conversion and specificity was available for those D-amino acids containing a ^{14}C label. The chromatographic effluent from the ion exchange column was diverted through a continuous liquid-flow scintillation detector and then fed back to the amino acid analyzer for automatic ninhydrin reaction and colorimetric analysis. Radioactivity passing the scintillation detector was continuously monitored with a dual channel linear scaler (Packard 320E), the output ending as a continuous trace on a double-pen recorder. Overlaying the radioactivity and ninhydrin charts readily showed coincident or non-coincident ninhydrin positive and ^{14}C peaks.

The purity, effectiveness and possible degradation of the enzyme were checked with each series of oxidations by including a control tube of ^{14}C-D-methionine (10 nmole). For treatments where labeled methionine was not otherwise involved, 10 nmole of ^{14}C-D-methionine were also included in the incubation mixture as an internal standard or check.

Plant and Soil Extracts

Extracts from plants were obtained by serial 5-minute extractions of lyophilized tissue (0·1–0·4 g) with 3 or more aliquots (*ca.* 60-ml each) of 80% boiling ethanol. After concentration *in vacuo* of the combined aliquots, samples were split and the 'free' amino acids picked up in water or lithium citrate diluting-buffer, as appropriate.[3] Ryegrass and corn as source materials for extracting ^{14}C labeled D-amino acids from plants are described elsewhere.[3]

Extracts from soils were obtained by a somewhat different procedure: 25 g soil wetted with 5–10 ml of 20% ethanol in 50-ml beaker was thoroughly mixed and dispersed by 5-min ultrasonication, then transferred to a 60-ml vol. thimble and extracted with 150 ml of 20% ethanol for 20 hr in a Soxhlet; this extract was concentrated by rotary evaporation at 35° and the amino acids taken up in 2–5 ml final volume of 80% ethanol. Any residue not soluble in 80% ethanol was filtered out with an 'F' grade sintered glass frit.

Soils for this series of tests included samples from forest area sites (Oregon Coast Range) and one from an agricultural area (Willamette Valley). Organic matter of the A horizons ranged from 6 to 33%, pH from 4·3 to 5·8, total N from 0·29 to 1·19% and C:N from 18 to 36. The soil under both the adjacent conifer and alder forests is quite acid, is known as Astoria-like Sols Bruns Acides, and has developed from deeply weathered siltstone. Tree cover has been maintained for 40 years in adjacent plots as pure stands of conifer (mainly *Pseudotsuga menziesii*) and red alder (*Alnus rubra*). There is dense understory of salmonberry (*Rubus spectabilis*) and blackbead elder (*Sambucus melancarpa*) on the alder site and little understory on the conifer site. The valley soil is slightly acid and developed on recent (post pleistocene) alluvium mixture of igneous

[15] G. MULLER, *Deut. Klin. Woch.* **41**, 1018 (1963), has used a similar approach on a μmole level for measuring D-amino acids in urine excreted by human patients. However, his procedure calls for relatively enormous amounts of enzyme (40 mg/sample), a very long incubation time (48 hr), and use of O_2 bubbling tubes rather than catalase for elimination of peroxide.

and sedimentary materials; dominant vegetative cover was maple, ash and poison oak. More detailed information is published elsewhere.[16,17]

Acknowledgements—We thank the Oregon State University Research Council for partial financial assistance to the senior author and the OSU Science Research Institute for loan of the continuous liquid-flow radioactivity monitor and scaler.

[16] J. F. FRANKLIN, C. T. DYRNESS, D. G. MOORE and R. F. TARRANT, *Proceedings of a Symposium, Biology of Alder*, pp. 157–172, Fortieth Annual Meeting, Northwest Scientific Association, Pullman, Washington, 14–15 April 1967 (1968).

[17] J. L. YOUNG and R. A. CATTANI, *Soil Sci. Soc. Am. Proc.* **26**, 147 (1962).