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THE OCCURRENCE OF D-ALANINE AND D-ALANYL-D-ALANINE IN *PHALARIS TUBEROSA*

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Plant and source. Five strains of *Phalaris tuberosa* L. (Gramineae) designated as "low alkaloid", "high alkaloid", Sirocco, Seedmaster and G.B. 81, growing in experimental plots at the Division's Field Station at O'Halloran Hill, South Australia. *Uses.* Pasture grass. *Previous work.* Alkaloids [1,2]. *Present work.* D-Alanyl-D-alanine was isolated from the leaves of all 5 strains of *P. tuberosa*, its content being approx 0·2 mg/g of the dry weight of the grass. Little seasonal variation of this value was observed throughout the growth cycle of the grass between March and December 1973, and only this (D-D) stereoisomeric form of the dipeptide was obtained. The leaves of one strain (Seedmaster) were estimated to contain 0·4 mg/g of free α-alanine on a dry wt basis and of this 7% was shown to be the D-isomer. The results of one experiment indicated that a trace of D-alanine may also be incorporated in the leaf protein of this strain or be bound in other insoluble conjugated form.

Other grasses collected near Adelaide (identified by Dr. Hj Eichler, S. A. State Herbarium, North Terrace, Adelaide) were examined. Perennial ryegrass [*Lolium perenne* L. (S. Lat.)], oat [*Avena sativa* L.] and the Common Reed [*Phragmites australis* (Cav.) Trin. ex Steud.] were found to contain alanylalanine but in lesser amounts than in *P. tuberosa*. The stereochemical configuration of the dipeptide from these other sources was not determined. The dipeptide was shown to be absent, however, from extracts of wheat [*Triticum vulgare* (Vill)], barley [*Hordeum leporinum* (Link)] and "many-flowered millet grass" [*Oryzopsis miliacea* (L.) Aschers and Schweinf.] but these grasses were each sampled on one occasion only and the possibility of a seasonal occurrence of the dipeptide in their tissues cannot be ruled out.

Comments. The first and only other example of the occurrence of D-alanyl-D-alanine in plant tissues was provided recently by Noma *et al.* [3] who isolated it from leaves of *Nicotiana tabacum* (Hicks) in a yield of 0·3 μg/g of fr. wt. The experiments of Aldag *et al.* [4] indicate that D-alanine probably occurs in the free state in the roots of maize (*Zea mays*). D-Alanine and its dipeptide have no obvious metabolic function in plant tissues and it may be that they are simply absorbed and concentrated from the soil where they occur in traces as a result of bacterial activity [5, 6].

EXPERIMENTAL

Isolation and identification of the dipeptide. Extracts of the grass leaves [7] yielded the free amino acid fraction by ion-exchange chromatography [8]. The total free amino acids averaged 6 mg/gm of the dry wt of the samples. The alanyl-alanine content of these fractions separated cleanly by electrophoresis on cellulose TL using 0·5 M acetic acid as electrolyte. Identification was based on the following criteria: (a) *acid hydrolysis* of a sample yielded α-alanine as the only detectable amino acid; (b) *chromatography* on Si gel TLC using $CHCl_3$–MeOH 17% NH_3-(2:2:1) and PrOH–34% NH_3 (7:3) showed it to be indistinguishable from a commercial sample labelled DL-alanyl-DL-alanine. The mobilities in the two solvent systems relative to L-alanine were 1·25 and 1·35 respectively; (c) *column chromatography.* Dipeptide was eluted at a characteristic rate from a column of the J.E.O.L. cation-exchange resin LCR-2 at 52° using Na citrate pH 4·25 as eluent at a pressure of 18–20 kg/cm^2. It appeared as a peak between those of isoleucine and leucine; (d) *electrophoresis.* The dipeptide was also indistinguishable from the commercial sample on high-voltage paper electrophoresis [9, 10] in (i) HCOOH (1·5 M) and HOAc (2·0 M), mobility 1·08 that of L-serine and (ii) borate buffer (pH 9·2) mobility 0·70 that of L-glutamic acid. This latter system was best for rapidly establishing the presence of the dipeptide in the free amino acid fraction of grass extracts. The dipeptide separated cleanly

from the mixtures and was quantitatively estimated by the method of serial dilution against standards prepared from the commercial sample; (e) *enzymic assays*. The dipeptide was hydrolysed and the resulting alanine treated with D-amino acid oxidase (E.C. 1.4.3.3.) [11]. No alanine could be detected subsequently in the reaction mixture. The specificity of the D-amino acid oxidase was proven by showing that reaction proceeded readily with D-alanine but not at all with the L-isomer. Glutamate–pyruvate–transaminase (E.C. 2.6.1.2.) failed to react with the hydrolysate under conditions prescribed for the determination of L-alanine [12]. The results of these tests, one showing the presence of D-alanine in the hydrolysate and the other showing the absence of L-alanine, mutually support the conclusion that the dipeptide is the D-D stereoisomer.

The free D-alanine content of P. tuberosa. Fresh leaves of the Seedmaster strain were crushed in a press and the expressed sap centrifuged and subjected directly to electrophoresis on cellulose thin layers impregnated with borate buffer (pH 9·2). The alanine band was extracted with H_2O from the cellulose, the extract concentrated, and the alanine again subjected to TL electrophoresis, this time using 0·5 M AcOH as the electrolyte. The alanine extracted from the appropriate band was thus obtained free of serine which, if present in the D-form, would have invalidated the subsequent enzymic assay using D-amino acid oxidase [11]. By means of this assay the alanine was shown to be 93% the expected L-isomer and 7% D.

The content of bound D-alanine in P. tuberosa. A sample of the Seedmaster strain was shredded under EtOH and the residual fibre and precipitated protein washed with EtOH and then H_2O. The dried fibre was hydrolysed and the liberated alanine isolated as before. Enzymic assays showed the presence of 1·2% D-alanine in the total alanine recovered, but approx. half this can be accounted for by racemization of the L-form under the conditions of hydrolysis. A small fraction of the total alanine present in the bound form in the grass as protein or other conjugate may therefore be of the D-form.

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