Phytochemistry, 1972, Vol. 11, pp. 2271 to 2276. Pergamon Press. Printed in England.

AMINO ACID COMPLEMENT OF PLANTS

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(*Received* 10 *February* 1972)

Key Word Index—*Beta vulgaris*; Chenopodiaceae; sugar beet; azetidine-2-carboxylic acid; γ-*N*-acetyl-α,γ-diaminobutyric acid; γ-*N*-lactyl-α,γ-diaminobutyric acid; ε-*N*-acetyl-lysine; ε-*N*-acetyl-*allo*-δ-hydroxy-lysine; γ-L-glutamyl-γ-aminobutyric acid.

Abstract—A study of the nitrogenous fraction, resulting from large-scale processing of sugar beet, has indicated that many amino acids occur in very minor quantities. γ-*N*-Lactyl-L-α,γ-diaminobutyric acid, ε-*N*-acetyl-L-lysine and ε-*N*-acetyl-*allo*-δ-hydroxy-L-lysine represent newly-recognized plant products, whilst L-azetidine-2-carboxylic acid, γ-L-glutamyl-γ-aminobutyric acid and γ-*N*-acetyl-L-α,γ-diaminobutyric acid are also shown as constituents of beet. The idea is advanced that particular genetic complexes necessary for the formation of individual secondary products may be more widely distributed in plants than often thought.

INTRODUCTION

ON SEVERAL occasions in the past 2 years,[1-3] I have written that the number of amino acids now fully characterized as plant products stands at about 200, whilst many further ninhydrin-reactive components of plants still await isolation and identification. Such statements naturally lead to the question how widely individual compounds may be distributed throughout the plant kingdom, and what their roles in plants may be. Answers given to both questions tend to be evasive, because the information available at present is either too uncertain or too slight to be dogmatic. Undoubtedly, many individual amino acids are accumulated by relatively few species, and the distribution patterns of such accumulated constituents within genera, tribes or families may have considerable relevance in plant chemotaxonomy. However, the possibility remains that many of these compounds may be synthesized by more types of plants, or even by all plants, but only in amounts that fall below those threshold concentrations that can be recognized in plant extracts without difficulty using routine analytical (normally chromatographic) procedures. If fractionation procedures are applied to extracts of much larger quantities of plant material, then many more compounds of any one type may be revealed, thereby establishing that particular compounds may have a wider distribution than was originally appreciated. A recent co-operation with an industrial processer has provided an opportunity to test this concept in a small way.

[1] L. FOWDEN, in *Progress in Phytochemistry* (edited by L. REINHOLD and Y. LIWSCHITZ), Vol. 2, pp. 203–266, Wiley, New York (1970).
[2] L. FOWDEN, J. W. ANDERSON and A. SMITH, *Phytochem.* **9**, 2349 (1970).
[3] P. J. LEA and L. FOWDEN, *Phytochem.* **11**, 2129 (1972).

The investigation described in this paper was initiated by a personal communication from Dr. H. Knobloch (Paris), in which he requested assistance in confirming the identity of a compound isolated from sugar-beet as azetidine-2-carboxylic acid: we had isolated this imino acid earlier from members of the Liliaceae[4] and from the legume, *Delonix regia*.[5] The compound had been obtained in kg quantities by separation (following traditional cation-exchange resin chromatographic procedures) of the soluble nitrogenous fraction arising as a by-product of sugar refining from beet. For this extended study, Dr. Knobloch kindly made available a viscous residue containing amino acids derived from mother liquors from fractions used for the crystallization of glutamic acid. Using this as source material, five amino acids or peptides were separated in pure form and fully characterized, whilst two other compounds await final assignment of structure. Three of the identifications represent amino acids described as plant products for the first time, whilst two other compounds (γ-*N*-acetyl-L-α,γ-diaminobutyric acid and γ-L-glutamyl-γ-aminobutyric acid) have been isolated previously on only one occasion.[6,7]

RESULTS AND DISCUSSION

During sugar refining from beet, it is essential to remove nitrogenous compounds from the aqueous extract before crystallization of sucrose is practicable. An early step of processing therefore involves the absorption of the nitrogenous fraction (betaine, amino acids and any small peptides) on to cation-exchange resin. Regeneration of the resin by passage of ammonia through the exchanger gives an ammoniacal eluate that contains the nitrogenous constituents of the original sugar-beet. The enormous scale of the sugar-refining industry makes available very large quantities of nitrogenous compounds; for example, one million tons (10^9 kg) of sugar-beet could yield about 500 tons (5×10^5 kg) of a mixture of free amino acids. The availability of starting material on this scale makes the isolation of azetidine-2-carboxylic acid (identity fully confirmed by comparison with authentic material using GLC, IR and NMR spectrometric techniques) practicable in kg amounts, although normal chromatographic procedures, applied directly to the original beet extract, are too insensitive to reveal its presence. In fact, azetidine-2-carboxylic acid is probably present in extracts at about one-fiftieth of the concentration of proline: pipecolic acid and baikiain (4,5-dehydropipecolic acid) have been identified as other amino acid constituents of the beet extract.*

During large-scale fractionation of the nitrogenous constituents of a beet extract, many very minor components can be detected at almost all points on the amino acid elution profile from the large cation-exchange resin columns. The identification of some components that elute with glutamic acid has been achieved by using the liquors remaining after crystallization of glutamic acid, i.e. a fraction much enriched in these minor constituents. The sample used contained about 10 g solids; glutamic acid represented about one-third of this mixture of amino acids, and a trace of aspartic acid was present. Two dimensional paper chromatography indicated the presence of eight unidentified ninhydrin-reactive compounds. The sample was separated into dicarboxylic and monocarboxylic amino acids using an anion-exchange resin column. Rather surprisingly, only one of the unidentified compounds was

* H. KNOBLOCH, personal communication.

[4] L. FOWDEN, *Biochem. J.* **64,** 323 (1956).
[5] M-L. SUNG and L. FOWDEN, *Phytochem.* **8,** 2095 (1969).
[6] I. LISS, *Phytochem.* **1,** 87 (1962).
[7] P. OLESEN-LARSEN, *Acta Chem. Scand.* **19,** 1071 (1965).

retained, together with glutamic and aspartic acids, on this column. The compound was identified as γ-L-glutamyl-γ-aminobutyric acid by comparison with authentic material provided by Olesen-Larsen, who isolated the γ-glutamyl derivative from *Lunaria annua* (Resedaceae), and successfully synthesized the compound.[7]

The mixture of amino acids not retained by Dowex-1 was fractionated further on a cation-exchange resin column (Dowex-50), and the individual amino acids were separated from the simpler mixtures so obtained by preparative paper chromatography. Six compounds were isolated finally as crystalline solids. All the compounds were labile during hydrolysis with 2 N HCl for 2 hr at 100°, and a diamino acid was always present in the hydrolysate. Eventually four of these compounds were identified as ω-*N*-acylated derivatives, i.e. γ-*N*-acetyl-L-α,γ-diaminobutyric acid, γ-*N*-lactyl-L-α,γ-diaminobutyric acid, ε-*N*-acetyl-L-lysine, and ε-*N*-acetyl-*allo*-δ-hydroxy-L-lysine. The two compounds still awaiting full characterization each gave α,γ-diaminobutyric acid after hydrolysis with 2 N HCl: they probably represent further examples of γ-*N*-acylated derivatives. Among these *N*-acyl derivatives, only γ-*N*-acetyldiaminobutyric acid has been identified previously as a plant constituent: it was isolated from *Euphorbia pulcherrima* (Euphorbiaceae) by Liss.[6] The existence of ε-*N*-acetyl-lysine in nature does not seem to have been reported, although synthesis of the compound from L-lysine and acetyl phosphate has been reported using a purified enzyme from beef liver.[8] A pure authentic sample of ε-*N*-acetyl-L-lysine, required for comparative purposes, was prepared by a simple chemical synthesis, described by Neuberger and Sanger.[9]

γ-*N*-Lactyl-L-α,γ-diaminobutyric acid is certainly a newly-characterized natural product, and also seems never to have been chemically synthesized. Lactic acid arising after hydrolysis of the γ-acyl compound was identified as its benzylthiouronium salt by IR spectroscopic comparison with authentic crystalline material, whilst L-α,γ-diaminobutyric acid was isolated by crystallization of the dihydrochloride from 80% (v/v) ethanol. The attachment of the lactyl residue at the γ-*N* atom was inferred by methods establishing that an unsubstituted α-amino group was present in the original compound, e.g. conversion of amino to hydroxyl with nitrous acid and the production after hydrolysis of γ-amino-α-hydroxybutyric acid. ε-*N*-Acetyl-*allo*-δ-hydroxy-L-lysine is also a newly-recognized natural product, that does not appear in the chemical literature. Wilding and Stahmann[10] claimed that δ-hydroxylysine is a minor constituent of roots of alfalfa (*Medicago sativa*). However, their evidence was inconclusive, being based only on comparative elution times from an amino acid autoanalyser, and no clear statement was made concerning the diastereoisomeric nature of the δ-hydroxylysine encountered. δ-Hydroxylysine, previously identified as a bound component in collagen and some other proteins, has the normal L-configuration.[11] This difference in diastereoisomeric configuration resembles that noted earlier for natural 4-hydroxyprolines: the material encountered in collagen is the normal (*trans*)-4-hydroxy-L-proline, whilst that occurring in an unbound form in the sandal tree (*Santalum album*) is allo(*cis*)-4-hydroxy-L-proline.[12] The acetyl moiety in the isolated material was established by synthesis as being on the ε-*N* atom, although it is possible that a δ-*O*-acetyl substituted compound may be the natural form within the plant: the alkaline conditions associated with steps in the fractionation procedure would cause an *O*- to *N*-acetyl group shift.

[8] W. K. Paik and S. Kim, *Arch. Biochem. Biophys.* **108**, 221 (1964).
[9] A. Neuberger and F. Sanger, *Biochem. J.* **35**, 515 (1943).
[10] M. D. Wilding and M. A. Stahmann, *Phytochem.* **1**, 263 (1962).
[11] J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, Vol. 3, Wiley, New York (1961).
[12] A. N. Radhakrishnan and K. V. Giri, *Biochem. J.* **58**, 57 (1954).

Bulk processing could mean that compounds are exposed for long periods of time to conditions that might produce artefacts. This problem has been considered, especially in the more likely instances of the ω-*N*-acylated amino acids. The sugar refining process involves first a brief extraction of sliced beet with hot alkali (calcium hydroxide); calcium is next precipitated as carbonate, and the extract then goes to the cation-exchange resin to remove nitrogenous constituents, which are recovered by displacement by strong ammonia solution. These steps are essentially rapid, i.e. comparable in time to laboratory procedures that are not complicated by the production of artefacts. Factors further discounting the idea that the *N*-acylated compounds might be chemical artefacts are, (i) the higher reactivity to acylating agents of α-amino, rather than ω-amino groups of diamino acids (with the exception of α,β-diaminopropionic acid), and (ii) the non-occurrence of appropriate acylating agents (e.g. acid anhydrides or acid chlorides) in extracts of biological materials, and the extreme unlikelihood that acetate or lactate could act as acylating agents in dilute alkaline solution.

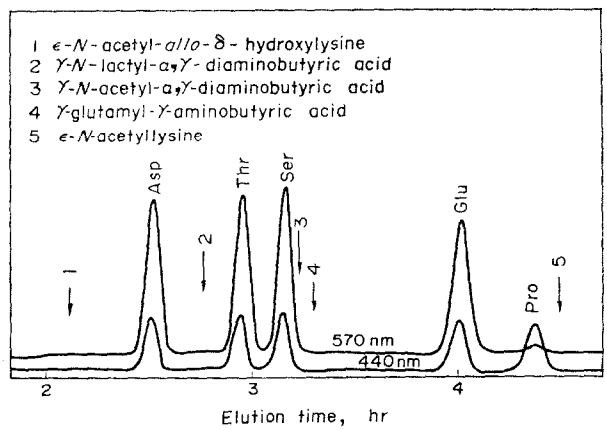

FIG. 1. ILLUSTRATES THE ELUTION PROFILE OF THE SUGAR-BEET CONSTITUENTS FROM THE 150 cm COLUMN (Chromobead A resin) OF THE TECHNICON AMINO ACID AUTOANALYSER.
The position of certain reference amino acids are shown as peaks, whilst arrows indicate the positions of the compounds isolated from beet.

These last considerations are particularly important because the ammoniacal eluate from the cation exchanger may be stored for considerable periods before amino acid fractionation is commenced. High concentrations of free ammonia normally prevent microbial contamination, and so the ω-*N*-acylated compounds are not thought to be products of microbial metabolism. Evidence available in the literature also suggests that bacteria and fungi synthesize α-, rather than ω-*N*-acylated derivatives of diamino acids.

Figure 1 shows the sequence of elution of these acylated compounds from the standard 150 cm column of a Technicon amino acid analyser. The compounds straddle the early portion of the elution profile containing the peaks of aspartic and glutamic acids, serine, threonine and proline.

The isolation of these compounds was possible only because extremely large quantities of plant material were initially processed in an industrial plant. As a consequence of this approach, a number of compounds are now recognized as constituents of the sugar beet plant, although it is certain that they would have remained uncharacterized if only conventional laboratory procedures had been employed. These observations have a bearing

upon chemotaxonomic work. It is often held that underlying genetic factors endow plants with different enzymic complements and that these differences are reflected, in part, in the production of a range of secondary products characteristic of particular species, or groups of species. Undoubtedly, a chemical approach to taxonomic problems has had numerous practical successes, and the non-protein amino acids feature among the types of compounds successfully utilized. The present findings do not lessen the importance or impact of this practical approach, where emphasis is placed upon differences in the distribution pattern of the major (i.e. *accumulated*) constituents of any one chemical type. However, the present study brings into question the concept that many plants totally fail to synthesize particular compounds because they completely lack the genetic complement necessary for the synthesis of the appropriate biosynthetic enzymes. The genetic potential of plants as related to secondary product synthesis, may be more uniform than has been suspected—different patterns of product accumulation (from the trace amounts undetectable by routine analysis to the massive accumulations associated with some species) may reflect differences in the degree to which particular genes are 'switched on'.

EXPERIMENTAL

A concentrate of the residual liquors obtained after large-scale crystallization of glutamic acid was used as starting material. The sample (containing about 10 g mixed amino acids) was diluted to 250 ml and adjusted to pH 7·0 by addition of NH_3. This solution was applied to a column (100 × 2·5 cm) of Dowex-1 (×10) to retain the acidic amino acid fraction. Thorough washing at this point gave an eluate containing the various *N*-acylated amino acids described below. The column was next eluted with 0·5 N acetic acid. Material identified as γ-glutamyl-γ-aminobutyric acid was present in fractions eluting just prior to glutamic acid: a little aspartic acid was eluted in later fractions.

γ-L-Glutamyl-γ-aminobutyric acid (152 mg) was obtained after evaporation *in vacuo* of combined fractions from the Dowex-1 column; this residue was recrystallized from EtOH–H_2O (Found: C, 46·7; H, 7·1; N, 11·9. Calc. for $C_9H_{16}N_2O_5$: C, 46·5; H, 7·0; N, 12·1%). $[\alpha]_D^{20}$ +4·5° (*c*, 2 in H_2O), literature value,[7] + 5·2°

Hydrolysis with 2 N HCl for 2 hr at 100° gave equimolar quantities of glutamic acid and γ-aminobutyric acid (determined by the Cd–ninhydrin method of Atfield and Morris[13] after separation by PC). A sample of glutamic acid, separated using a small Dowex-1 column, was shown to have the L-configuration $[\alpha]_D^{20}$ +30° (*c*, in 5 N HCl), literature value,[11] +31·8°.

The isolated material gave an IR spectrum identical with that produced from authentic material supplied by Olesen-Larsen, and the two materials eluted as one peak from a Technicon autoanalyser operating under standard conditions with a 150 cm Chromobead A column.[14]

N-*Acylated compounds*. The eluate containing amino acids not retained by Dowex-1 was first fractionated on a cationic Dowex-50 (×8) column (100×2 cm). This procedure did not effect complete separation of amino acids, but gave fractions enriched in particular compounds. Subsequent purification of each *N*-acyl derivative described below was achieved by preparative PC using either *n*-BuOH–HOAc–H_2O (90:10:29) or *t*-AmOH–HOAc–H_2O (20:1:20, upper phase), followed by crystallization from EtOH–H_2O mixtures. (a) γ-*N*-Acetyl-L-α,γ-diaminobutyric acid (103 mg). (Found: C, 45·2; H, 7·6; N, 17·2. Calc. for $C_6H_{12}N_2O_3$: C, 45·0; H, 7·5; N, 17·5%). $[\alpha]_D^{20}$ +43° (*c*, 1 in 5 N HCl), literature value,[15] +42·8°. The IR spectrum (Nujol) of the isolate was identical with authentic material supplied by Professor Benoiton (Ottawa). Hydrolysis with 2 N HCl (100°, 3 hr) gave L-α,γ-diaminobutyric acid dihydrochloride (recrystallized from aqueous ethanol), $[\alpha]_D^{20}$ +13·9° (*c*, 5 in H_2O); Adamson[16] reports a value of +14·6°. Acetic acid produced during hydrolysis was converted to the benzylthiouronium salt (see Ref. 17), which was compared with authentic material by IR spectroscopy. (b) γ-*N*-Lactyl-L-α,γ-diaminobutyric acid (0·98 g). (Found: C, 44·0; H, 7·6; N, 14·3. Calc. for $C_7H_{14}N_2O_4$: C, 44·2; H, 7·4; N, 14·4%). $[\alpha]_D^{20}$ +12·0° (*c*, 5 in H_2O). The presence of a lactyl residue was strongly indicated by the NMR spectrum. Treatment with N NaOH (100°, 24 hr) produced complete hydrolysis. L-α,γ-Diaminobutyric acid was removed on a cation-exchange resin, and

[13] G. N. Atfield and C. J. O. R. Morris, *Biochem. J.* **81**, 606 (1961).
[14] *Assembly and Operating Instructions: Amino Acid Analyser*, Instruction manual AAA-1, Technicon Corporation, New York (1967).
[15] L. Benoiton and J. Leclerc, *Can. J. Chem.* **43**, 991 (1965).
[16] D. W. Adamson, *J. Chem. Soc.* 1564 (1939).
[17] R. Gmelin, A. Kjaer and P. O. Larsen, *Phytochem.* **1**, 233 (1962).

converted to the dihydrochloride before crystallization; $[\alpha]_D^{20}$ +13·9° (*c*, 5 in H_2O). Lactic acid was crystallized as the benzylthiouronium salt, and characterized by IR comparison with authentic material prepared from DL-lactic acid. Hydrolysis with N NaOH would have produced racemic lactic acid even if the lactyl moiety of the original *N*-acyl compound was optically active. When the original compound was boiled with $CuCO_3$, copper was chelated with an associated loss of ninhydrin reactivity: this behaviour is normally considered to establish the presence of an α-amino group. The compound was also treated with nitrous acid, which preferentially attacks α-amino groups replacing them with hydroxyl. Unchanged material was absorbed on to cationic Dowex-50 resin, and the product (presumed to be γ-acetamido-α-hydroxybutyric acid) was hydrolyzed with 2 N HCl for 2 hr at 100°. The formation of γ-amino-α-hydroxybutyric acid was confirmed by paper chromatography in several solvents, and by Technicon autoanalyser, in comparison with synthetic material.[18] (c) ε-*N*-Acetyl-L-lysine (34 mg). (Found: C, 49·9; H, 8·5; N, 14·7. Calc. for $C_8H_{16}N_2O_3$: C, 51·1; H, 8·5; N, 14·9%). $[\alpha]_D^{20}$ +4° (*c*, 5 in H_2O), literature value,[11] +3·6°. Identity with synthetic material[9] established by IR spectroscopy. Hydrolysis with 2 N HCl (100, 4 hr) gave L-lysine, $[\alpha]_D^{20}$ +25° (*c*, 2 in 5 N HCl), literature value,[11] +22·5°, and acetic acid identified by IR spectroscopy of benzylthiouronium salt. (d) ε-*N*-acetyl-*allo*-δ-hydroxy-L-lysine (0·59 g). (Found: C, 47·4; H, 7·9; N, 13·5. Calc. for $C_8H_{16}N_2O_4$: C, 47·1; H, 7·8; N, 13·7%). $[\alpha]_D^{20}$ +10·4° (*c*, 5 in H_2O). Hydrolysis with 2 N HCl (100°, 2 hr) gave *allo*-δ-hydroxy-L-lysine (containing a trace of *n*-δ-hydroxylysine), $[\alpha]_D^{20}$ +29° (*c* = 4 in 5 N HCl), literature value,[11] +32·1°. *allo*-δ-Hydroxylysine was identified by comparison with authentic material using the Technicon amino acid autoanalyser. The material was epimerized by heating with 5 N HCl (100°, 96 hr) to give an equilibrium mixture containing 47·6% of *n*-isomer and 52·4% *allo*-isomer: Hamilton and Anderson[19] reported values of 46 and 54%, respectively. Acetic acid formed after 2 N HCl hydrolysis was characterized as above. A small sample of ε-*N*-acetyl-*allo*-δ-hydroxylysine was synthesized by treating the Cu complex of *allo*-δ-hydroxylysine with acetic anhydride. The synthetic material eluted with the natural isolate as a single peak from the Technicon autoanalyser.

Acknowledgements—Thanks beyond those already expressed in the text are due to Mr. A. Smith and Miss C. M. MacGibbon for technical assistance, and to the Agricultural Research Council for their grant to purchase the amino acid autoanalyser.

[18] P. M. DUNNILL and L. FOWDEN, *Phytochem.* **4**, 445 (1965).
[19] P. B. HAMILTON and R. A. ANDERSON, *J. Biol. Chem.* **213**, 249 (1955)