Table 1. *Neutral 16-hydroxy steroids isolated from urine, and their precursors*

16-Hydroxy steroid	16-Deoxy precursor
Androst-5-ene-3β:16α:17β-triol Androst-5-ene-3β:16β:17β-triol 16α-Hydroxydehydro*epi*androsterone	Dehydro*epi*androsterone
5α-Androstane-3α:16α:17β-triol	3α-Hydroxy-5α-androstan-17-one (Androsterone)
5β-Androstane-3α:16α:17β-triol	3α-Hydroxy-5β-androstan-17-one (Etiocholanolone)
5α-Pregnane-3β:16α:20α-triol	3β-Hydroxy-5α-pregnan-20-one
Pregn-5-ene-3β:16α:20α-triol	3β-Hydroxypregn-5-en-20-one

Seven neutral steroids containing a hydroxyl group at position 16 have now been isolated from human urine. These are shown in Table 1 with the corresponding 16-deoxy precursors. 5α-Pregnane-3β:16α:20α-triol is possibly a metabolite of pregn-5-ene-3β:16α:20α-triol. The C_{19} 16-deoxy steroids shown in Table 1 are those which occur in the largest amount in urine and possibly therefore are present in relatively large amounts in the body. Since the amounts of the 16-hydroxysteroids found in the urine are small it would appear that this hydroxylation is one that does not proceed too readily. If this is the case also with the C_{21} 16-hydroxy steroids it would suggest that large amounts of pregnenolone (3β-hydroxypregn-5-en-20-one) or pregn-5-ene-3β:20α-diol are produced *in vivo*. Current views on the biosynthesis of the adrenocortical hormones (cf. Hechter & Pincus, 1954) suggest that pregnenolone is an intermediate in the conversion of cholesterol into progesterone (pregn-4-ene-3:20-dione), but pregnenolone has not so far been isolated from urine or from the adrenal. Whether the 16-hydroxy steroids have any unique biological function has yet to be determined.

SUMMARY

1. Pregn-5-ene-3β:16α:20α-triol has been isolated from the urine of normal males which has been boiled for 2 hr. without adjustment of the pH.

2. The 16-hydroxy steroids found in urine are considered and the possible significance of the present isolation is discussed.

I am grateful to Professor G. F. Marrian, F.R.S., for his interest in the work and to Dr H. Hirschmann, Department of Medicine, Western Reserve University, Cleveland, for a sample of 3β:16α:20α-triacetoxypregn-5-ene.

REFERENCES

Butenandt, A. & Dannenbaum, H. (1934). *Hoppe-Seyl. Z.* **229**, 192.
Fotherby, K. (1958). *Biochem. J.* **69**, 596.
Fotherby, K., Colas, A., Atherden, S. M. & Marrian, G. F. (1957). *Biochem. J.* **66**, 664.
Hechter, O. & Pincus, G. (1954). *Physiol. Rev.* **34**, 459.
Hirschmann, H. & Hirschmann, F. B. (1950*a*). *J. biol. Chem.* **184**, 259.
Hirschmann, H. & Hirschmann, F. B. (1950*b*). *J. biol. Chem.* **187**, 137.
Marrian, G. F. & Butler, G. C. (1944). *Biochem. J.* **38**, 322.
Mason, H. L. & Engstrom, W. W. (1950). *Physiol. Rev.* **30**, 355.
Munson, P. L., Jones, M. E., McCall, P. J. & Gallagher, T. F. (1948). *J. biol. Chem.* **176**, 73.

Nitrogenous Compounds and Nitrogen Metabolism in the Liliaceae

5. THE METABOLISM OF AZETIDINE-2-CARBOXYLIC ACID*

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Studies on the nitrogenous constituents of members of the Liliaceae family of plants have been reported in earlier papers of this series (Fowden & Steward, 1957*a*, *b*; Zacharius, Cathey & Steward, 1957; Fowden & Bryant, 1958). Particular emphasis was placed on the more recently discovered amino and imino acids, several of which are fairly widely distributed in species of the family. Azetidine-2-carboxylic acid (A-2-C), the lower imino acid analogue of proline, is practically confined to liliaceous plants; Fowden & Steward (1957*a*) showed it to be present in about one-quarter of the

* Part 4: Fowden & Bryant (1958).

species they examined. In some plants, e.g. *Convallaria majalis* (lily-of-the-valley) and *Polygonatum multiflorum* (a Solomon's seal), it is present in very high concentrations relative to those of other free amino acids.

This paper presents the results of a study of the mechanism whereby A-2-C is synthesized in *Convallaria* leaves. To determine whether the presence of A-2-C affects the basic reactions involved in amino acid synthesis, the pathway of photosynthetic assimilation of [^{14}C]carbon dioxide into the amino acids of *Convallaria* leaves has been compared with pathways previously demonstrated to exist in leaves of other species in which the imino acid is absent (see Racusen & Aronoff, 1954; Towers & Mortimer, 1956). Fowden (1956) suggested that the biosynthesis may follow the same pattern as that established for proline synthesis. In this scheme, aspartic acid, homoserine and $\alpha\gamma$-diaminobutyric acid are possible precursors. A further possibility is that γ-aminobutyric acid may undergo reductive cyclization. ^{14}C-Labelled amino acids and other substrates were therefore fed to tissues of *Convallaria* and to a lesser extent those of *Polygonatum* to test these ideas.

METHODS

Plant materials. Crowns of *Convallaria* were collected in March and stored at about 4°. They were planted as required and could be grown successfully until about September. Immature leaves (0·3–0·6 g. each) were used in most experiments, but apical portions of roots and disks of rhizome tissue were also used in feeding experiments with [^{14}C]aspartate.

Polygonatum rhizome and root tissue was collected from vigorously growing plants in April.

[^{14}C]*Carbon dioxide assimilation.* Immature *Convallaria* leaves were excised from plants and the leaf bases were kept in water. Several leaves were placed in a chamber, vol. 300 ml., filled with CO_2-free air and illuminated to give a surface light intensity of 600 ft.candles. $^{14}CO_2$ was then generated from $Ba^{14}CO_3$ (100 μc; 1·1 mg.) within the chamber to produce a CO_2 concentration of 0·04%. The leaves were allowed to assimilate the $^{14}CO_2$ for 7·5, 15, 30 and 60 min. After removal from the chamber they were (*a*) immediately killed by dropping into boiling 75% (v/v) ethanol, or (*b*) kept in the light, or (*c*) kept in complete darkness for further periods of time up to 24 hr. before being killed.

Feeding of ^{14}C-labelled organic substrates. Most of the experiments were performed with immature *Convallaria* leaves. The bases of the leaves were placed in a solution of the appropriate ^{14}C-labelled substance in front of a fan to give an increased transpiration rate: a leaf assimilated 0·05 ml. of solution in about 2 hr. Details of the amounts of each substance supplied are given when the experimental results are described. When uptake was complete the leaves were placed in water and kept in the light or dark for further periods before being killed with ethanol.

[^{14}C]Aspartic acid was supplied to root and rhizome tissue of *Convallaria* and *Polygonatum*. The tissue (approx. 0·2 g.) was shaken with 1 ml. of [^{14}C]aspartate solution in a Warburg flask at 23° for 4 hr. The tissue was then washed free from residual aspartate and transferred to sterile water and shaken for a further 20 hr.

Tissue extraction and radioactivity assays

All tissues were treated in a similar way and all values for radioactivity are recorded as counts/min. present in fractions representing 0·1 g. fresh wt. of material.

Extraction. The tissues were killed by dropping into hot 75% (v/v) ethanol (20 ml./g. fresh wt. of tissue) and were finely ground. Extraction was allowed to continue for 24 hr. at room temperature. Extract and residue were separated by centrifuging, after which the residue was twice re-extracted with further portions of aqueous ethanol. The combined extracts were evaporated to dryness in a current of warm air and the residues redissolved in water. Measured volumes were used for radioactivity assays.

Cationic fractions. These were prepared from the above extracts with small columns packed with Zeo-Karb 215 cation-exchange resin exactly as described by Fowden & Webb (1958). Measured volumes of the final fractions were used for radioactivity assays.

Separation of individual amino acids. Amino acids present in the cationic fractions were separated on two-dimensional paper chromatograms (final size 25 cm. × 20 cm.; Whatman no. 3MM chromatographic-grade filter paper) with phenol–aq. NH_3 soln. (Dent, 1947) as the first solvent followed by butan-1-ol–acetic acid–water (Partridge 1948) in the second direction. Radioautographs were prepared from each chromatogram to determine which amino acids had become radioactive.

Radioactivity in individual amino acids. The areas of the chromatograms containing radioactive amino acids were cut out and extracted by disintegrating in 10 ml. of hot water. The radioactivity was then determined in measured fractions of this extract, after concentration, by the 'infinitely thin' sample method of Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). The sample was pipetted on to a recessed aluminium planchette (2·1 cm. diam.) and covered with a circle of lens tissue paper. After drying, it was counted in a windowless gas-flow (methane) proportional chamber connected to a scaler. As samples possessed appreciable mass, correction was made for self-absorption of the radiation by the lens tissue and the sample itself. The background count was determined after the assay of each sample. The standard error of a determination was normally less than 10% of the recorded activity: the largest errors occurred during chromatography and subsequent elution of the radioactive amino acids from the filter paper.

^{14}C-Labelled chemicals. $Ba^{14}CO_3$, biosynthetic L-[^{14}C]-aspartic acid, L-[^{14}C]glutamic acid and D-[^{14}C]glucose were obtained from The Radiochemical Centre, Amersham, Bucks. γ-Amino-[^{14}C]butyric acid was obtained from the L-[^{14}C]glutamic acid by decarboxylation with *Clostridium welchii* SR. 12 (NCTC 6784). L-$\alpha\gamma$-Diamino-[^{14}C]butyric acid was prepared from L-[^{14}C]glutamic acid by a micro-modification of the method of Adamson (1939). A portion of the L-$\alpha\gamma$-diamino-[^{14}C]butyric acid was converted into DL-[^{14}C]azetidine-2-carboxylic acid by the method of Fowden (1956). The chemically synthesized amino acids were chromatographed and their purity was checked by

radioautography. Phenol–aq. NH_3 soln., butan-1-ol–acetic acid–water and 70% (v/v) propan-1-ol were used as solvent systems.

RESULTS

Assimilation of [^{14}C]*carbon dioxide by* Convallaria *leaves.* Leaves used were harvested in April or May. All the [^{14}C]carbon dioxide supplied was assimilated during the first 30 min. of illumination. Approximately 50% was absorbed after 7·5 min. and 90% after 15 min. During the early phase of the reaction a higher proportion of the radioactivity was located in amino acids (the cationic fraction) than when the carbon dioxide uptake was complete. Usually, though not in every case, the proportion continued to decrease when the leaves were kept either in the light or dark for further periods of time up to 6 hr. (Table 1). After standing for 24 hr. in either light or dark, the total activity in amino acids was increased and accounted for a higher percentage of the activity of the ethanol-soluble fraction.

Table 2 shows the changing pattern with time of the distribution of radioactivity between individual amino acids of the leaves. Within the first 15-min. assimilation period the amino acids alanine and

Table 1. *Assimilation of radioactivity into* 75% *(v/v) ethanol-soluble and cationic substances after feeding* Convallaria *leaves with* [^{14}C]*carbon dioxide*

L, Leaves maintained in light after CO_2 fixation; D, leaves maintained in complete darkness after CO_2 fixation.

Period of $^{14}CO_2$ supply (min.)	Further period in light or dark (hr.)	Activity of 75% (v/v) ethanol fraction (10^{-3} counts/min./0·1 g. fresh wt. of leaf extract)	Activity of cationic fraction (10^{-3} counts/min./0·1 g. fresh wt. of leaf extract)	Percentage of activity of ethanol-soluble fraction in cationic fraction
Photosynthetic fixation:				
7·5	0	633	126·4	19·9
15	0	2049	294·6	14·3
30	0	2135	95·6	4·5
60	0	2176	129·2	5·9
30	3L	1661	67·8	4·1
30	3D	1835	107·6	5·8
60	6L	2363	61·9	2·8
60	6D	1940	67·8	3·7
60	12L	2015	57·1	2·8
60	12D	1950	87·3	4·5
60	24L	1110	105·0	9·4
60	24D	997	102·3	10·2
Dark-fixation of CO_2:	5 hr.	82·3	14·7	17·1
	20 hr.	—	11·6	—

Table 2. *Radioactivities of individual amino acids at different times after feeding* Convallaria *leaves with* [^{14}C]*carbon dioxide*

Activities of individual amino acids are expressed as percentages of the total activity present in all amino acids. –, No detectable radioactivity present. γ-AB, γ-Aminobutyric acid; L and D, see Table 1.

Period of $^{14}CO_2$ supply (min.)	Further period in light or dark (hr.)	Activities of individual amino acids: Alanine	Serine	Glutamic acid	Glutamine	A-2-C	Asparagine	γ-AB
Photosynthetic fixation:								
15	0	58·7	37·2	1·9	1·4	–	–	0·6
30	0	42·7	35·3	7·5	1·1	4·5	–	–
60	0	16·1	43·8	5·5	2·0	12·2	2·4	6·0
30	3L	5·6	7·0	11·8	9·4	37·7	–	6·9
30	3D	3·6	17·9	35·1	5·3	18·4	2·4	7·3
60	6L	3·5	2·4	3·5	18·1	51·9	7·3	3·9
60	6D	4·6	3·5	1·9	3·5	64·0	9·7	6·4
60	12L	4·7	1·5	2·0	12·8	63·9	–	3·3
60	12D	6·1	4·4	1·2	7·5	54·5	2·4	–
60	24L	8·2	1·3	0·6	17·5	53·5	–	18·5
60	24D	15·2	4·9	1·3	3·2	64·5	–	10·8
Dark-fixation of CO_2:	5 hr.	3·3	39·7	30·9	8·8	3·0	–	–
	20 hr.	1·5	31·6	12·7	10·2	12·3	15·6	–

serine became heavily labelled. The labelling of glutamic acid occurred more slowly and was heavier in leaves kept in the dark. The activity in A-2-C slowly increased until it was the major radioactive compound of the cationic fraction. γ-Aminobutyric acid and glutamine ultimately became appreciably labelled; labelling of glutamine was more extensive in leaves kept in the light, whereas dark conditions in the first 6 hr. favoured γ-aminobutyric acid marking. Aspartic acid was present only in very low concentrations in relation to the other free amino acids and never gained measurable radioactivity. Proline, threonine, glycine and the basic amino acids became weakly labelled later and radioautographs showed that the cationic fractions occasionally contained unidentified substances possessing low activity.

When leaves were exposed to [^{14}C]carbon dioxide in complete darkness for 5 and 20 hr., fixation slowly occurred. After 5 hr. 40% of the total activity of the cationic fraction was present in serine, 31% in glutamic acid and 3% in A-2-C. After 20 hr. serine remained the most heavily marked amino acid and accounted for 32% of the cationic activity. Glutamic acid, asparagine, glutamine and A-2-C each contained about 10–15% of the total activity.

Although the results in Tables 1 and 2 are those of individual experiments, repeat experiments on similar leaves led to the same general conclusions: exact duplication of the numerical figures was not obtained nor could such duplication be expected since the leaves were from plants grown under natural conditions of light and temperature. Normal daily variation in these environmental factors would be expected to influence the physiological status of leaves collected on different days. When leaves collected from plants grown later in the summer were used, the activity present in A-2-C 24 hr. after the supply of [^{14}C]carbon dioxide represented a smaller proportion (about 20%) of the total activity present in all amino acids. The early pattern of ^{14}C-labelling of the amino acids remained unchanged.

Experiments with ^{14}C-labelled organic substrates. Table 3 shows the results of experiments in which various ^{14}C-labelled organic substances were fed to *Convallaria* leaves. The specific activities of the γ-aminobutyric and αγ-diaminobutyric acids used were one-tenth of those of glutamic and aspartic acids. All the substances were metabolized and when ^{14}C-labelled amino acids were fed the cationic fractions contained higher proportions of the total ethanol-soluble activity than when [^{14}C]carbon dioxide was supplied. After feeding [^{14}C]-aspartic acid for 24 hr., no radioactivity remained in the amino acid: asparagine became the most heavily labelled amino acid in leaves kept in the dark, whereas activity was accumulated principally into glutamic acid and glutamine in the light. Glutamine was also labelled most actively among the amino acids when [^{14}C]glutamic acid was fed. When γ-amino-[^{14}C]butyric acid was supplied only 3% of the original activity remained in the ethanol-soluble fraction after 24 hr., and the low activity of the cationic fraction was largely accounted for by residual γ-amino-[^{14}C]butyric acid. Alanine, serine and glutamine gained the heaviest ^{14}C-labelling when [^{14}C]glucose was supplied, although many other amino acids also became radioactive. An unknown compound X, the main radioactive metabolite of αγ-diamino-[^{14}C]butyric acid, occupied a position close to A-2-C (Fig. 1*b*) on a two-dimensional chromatogram developed in phenol–aq. NH_3 soln. (Dent, 1947), followed by the butan-1-ol–acetic acid–water mixture of Partridge (1948). The radioactive spot did not correspond with any ninhydrin-reactive spot on the chromatogram. Smaller amounts of this substance were formed when [^{14}C]aspartic acid was fed.

A-2-C never became heavily labelled. No activity could be detected in the imino acid when

Table 3. *Distribution of radioactivity among various leaf fractions after feeding different ^{14}C-labelled organic substrates*

L, Leaves maintained in the light; D, leaves maintained in the dark; LD, leaves subjected to normal day and night conditions.

Substance supplied and metabolism time (hr.)	Quantity supplied/0·1 g. fresh wt. of leaf* (μc)	(μg.)	Activity of 75% (v/v) ethanol fraction (10^{-3} counts/min./0·1 g. fresh wt. of leaf extract)	Activity of cationic fraction (10^{-3} counts/min./0·1 g. fresh wt. of leaf extract)	Percentage of ethanol-soluble activity in cationic fraction
Aspartic acid (24 L)	1·25	25	346	49·4	14·3
Aspartic acid (24 D)	1·25	25	284	37·4	13·2
Glutamic acid (24 L)	2·5	50	940	235	25·0
γ-Aminobutyric acid (24 L)	0·62	85	14·6	8·2	56
αγ-Diaminobutyric acid (48 LD)	0·30	48	145	70·5	47·1
Glucose (24 D)	1·25	14	467	32·9	7·0

* 1·25 μc is equivalent to approx. 10^6 counts/min.

radioactive γ-aminobutyric acid, αγ-diaminobutyric acid or glucose was supplied. Weak labelling of A-2-C occurred in experiments in which [^{14}C]glutamic acid and [^{14}C]aspartic acid were fed, but the activity represented only a small percentage of the total present in amino acids. No labelling of homoserine was ever detected.

A small amount of substance X was eluted from paper chromatograms and subjected to hydrogenation at 4 atm. H_2 pressure with Adams platinum oxide catalyst, when it was converted into a second radioactive compound Y. This latter substance was not separable from added L-A-2-C on a two-dimensional paper chromatogram run in phenol–aq. NH_3 soln. for 24 hr. and in butan-1-ol–acetic acid–water mixture for 7 days, although the radioactive and ninhydrin-reactive spots were not entirely coincident (see Fig. 1c).

When [^{14}C]A-2-C was supplied to the leaves it was only slowly degraded; 48 hr. after feeding a high percentage of the activity remained in A-2-C, but threonine, glutamine and two unidentified substances had become weakly labelled.

Only [^{14}C]aspartic acid has been supplied to root and rhizome tissue. The behaviour of corresponding tissues from *Convallaria* and *Polygonatum* was generally similar (Table 4). For all tissues a fairly constant and small percentage of the radioactivity supplied appeared in the respiratory carbon dioxide during 24 hr. The total activity present in substances of rhizome tissues soluble in 75% (v/v) ethanol was appreciably higher than that of roots. Activity present in cationic substances of *Polygonatum* rhizome was far higher than for any other tissue, and was due largely to the ^{14}C-labelling of glutamine. This amide also accounted for a high percentage of the activity of the cationic fraction of *Convallaria* rhizome. In root tissues of both plants lysine gained the heaviest ^{14}C-marking among the cationic substances. A-2-C was labelled more heavily in root than in rhizome tissues. The degree of labelling of the imino acid observed in these root tissues was considerably larger, in relation to the other amino acids, than that obtained in the earlier experiments in which *Convallaria* leaves were fed with [^{14}C]-aspartic acid.

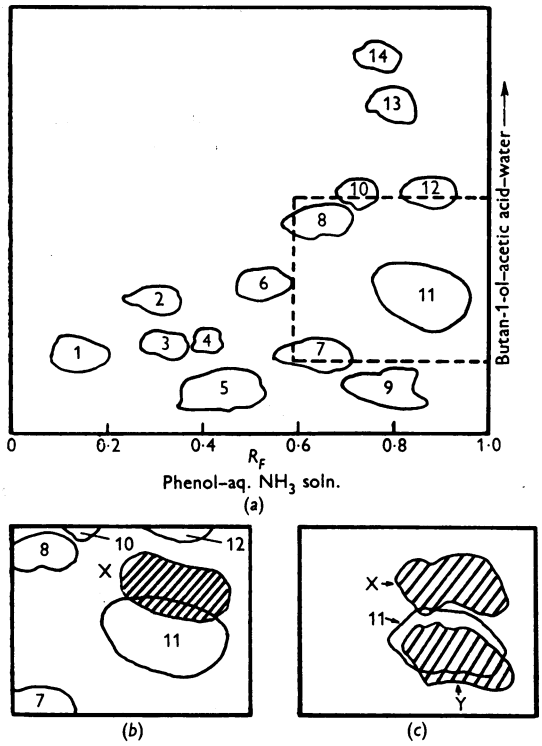

Fig. 1. (*a*) Diagram of a two-dimensional chromatogram of the ninhydrin-reactive substances present in an extract of *Convallaria* leaves. The spots shown are: 1, aspartic acid; 2, glutamic acid; 3, serine; 4, glycine; 5, asparagine; 6, threonine; 7, glutamine; 8, alanine; 9, probably lysine; 10, β-alanine; 11, azetidine-2-carboxylic acid; 12, proline; 13, γ-aminobutyric acid; 14, valine. (*b*) Inset area of (*a*) with the superimposed spot (hatched) of radioactive substance X formed after feeding αγ-diamino-[^{14}C]butyric acid to *Convallaria* leaves. (*c*) Diagram obtained by superimposing a radioautograph showing radioactive substance Y formed by catalytic hydrogenation of radioactive compound X upon the two-dimensional chromatogram from which it was prepared and to which L-azetidine-2-carboxylic acid had been added.

DISCUSSION

During the photosynthetic assimilation of [^{14}C]-carbon dioxide by *Convallaria* leaves, alanine was the first amino acid to attain its maximum degree of labelling. Subsequently serine and then glutamic acid reached their own maxima. This succession is similar to that reported for soya-bean leaves (Racusen & Aronoff, 1954), sugar-beet leaves (Towers & Mortimer, 1956) and groundnut leaves (Fowden & Webb, 1958). In some of these leaves aspartic acid became labelled in the early stages of [^{14}C]carbon dioxide fixation, but in *Convallaria* leaves no appreciable incorporation of radioactivity into this amino acid could be detected. This finding was probably due to the unusually low concentrations of free aspartic acid present in the leaves.

A-2-C was labelled only weakly in the first hour after the supply of [^{14}C]carbon dioxide, but it continued to gain activity slowly for about 12 hr. and then remained at approximately the same absolute activity for a further 12 hr. When expressed as a percentage of the total cationic activity, the increase in the activity present in the

imino acid was more marked and was especially noticeable between the first and sixth hours after supply of [^{14}C]carbon dioxide, when the activities of alanine and serine were decreasing rapidly. Synthesis of A-2-C then would seem to be a process only distantly related to the primary products of photosynthesis and to involve reactions that are additional to and probably much slower than the intermediary processes of metabolism leading to the synthesis of the common amino acids of proteins. Although A-2-C eventually contained more activity than any other amino acid, its specific activity remained relatively low; analyses have shown that more than 70% of the total free α-amino- and imino-nitrogen of *Convallaria* leaves may be accounted for by the imino acid (Fowden, 1956). The relatively slow rate of synthesis and the large accumulations in which it is found in some plants are reminiscent of alkaloid physiology. The apparent similarity with alkaloids can be seen also in the slow rate at which A-2-C was degraded by *Convallaria* leaves and by the results of the [^{14}C]-aspartic acid-feeding experiments with the different tissues, which suggest that roots may be the most effective organ for imino acid synthesis (compare nicotine synthesis, which is primarily a function of the roots in the tobacco plant).

No definite conclusions about the manner in which A-2-C is synthesized are possible from the experiments in which ^{14}C-labelled organic substrates were fed to *Convallaria* leaves. A-2-C was weakly labelled after the supply of [^{14}C]glutamic acid and [^{14}C]aspartic acid, but its activity after 24 hr. was less than 1% of the total present in all the free amino acids (compare with the corresponding figure of 60% obtained in the main [^{14}C]carbon dioxide feeding experiments). According to Linko (1958) A-2-C was not labelled 12 hr. after feeding [^{14}C]aspartic acid to *Convallaria* leaves. [^{14}C]Glucose might be expected to produce by the glycolytic pathway the same ^{14}C-labelled intermediates that are found among the early products of photosynthetic [^{14}C]carbon dioxide assimilation. The failure to obtain labelling of A-2-C with [^{14}C]glucose was therefore unexpected and no convincing explanation can be advanced at present to account for this result.

The main [^{14}C]carbon dioxide experiments were performed on leaves from plants stored for only a short period at 4° and grown in April and May, whereas those used in the ^{14}C-labelled amino acid-feeding experiments were from plants grown later in the year after considerably longer storage of the crowns at 4°. Prolonged cold storage may have limited the ability of excised leaves from such plants to synthesize A-2-C. However, the maximum labelling of A-2-C resulting after [^{14}C]amino acid feeding was still only about one-twentieth of that obtained when [^{14}C]carbon dioxide was supplied to similar late-season leaves. Translocation

Table 4. *Distribution of radioactivity between various fractions and in individual amino acids after feeding* [^{14}C]*aspartic acid to* Convallaria *and* Polygonatum *root and rhizome tissues*

Figures in parentheses represent the activities of individual amino acids expressed as percentages of the total activity of the cationic fraction.

	Activities of fractions and amino acids (10^{-3} counts/min./0·1 g. fresh wt. of tissue)			
	Convallaria		*Polygonatum*	
	Rhizome	Root	Rhizome	Root
Aspartic acid supplied	4000*	6400*	4000*	6400*
Respiratory CO_2	474	647	310	400
Fraction soluble in 75% (v/v) ethanol	1469	949	1810	632
Cationic fraction	251	273	710	53·8
Aspartic acid	7·5 (3·0)	11·6 (4·2)	15·1 (2·1)	2·9 (5·5)
Glutamic acid	17·2 (6·9)	12·3 (4·5)	80 (11·3)	1·8 (3·3)
Serine	5·3 (2·1)	7·4 (2·7)	16·9 (2·4)	2·3 (4·2)
Threonine	3·2 (1·3)	—	10·5 (1·5)	—
Alanine	3·1 (1·2)	3·7 (1·4)	21·1 (3·0)	—
Glutamine	96 (38·2)	36 (13·2)	454 (63·9)	1·5 (2·7)
Arginine	6·5 (2·6)	6·2 (2·3)	0·5 (0·1)	—
Lysine	4·3 (1·7)	41 (15·0)	4·5 (0·6)	20·3 (37·7)
Proline	1·6 (0·6)	9·4 (3·4)	7·0 (1·0)	1·2 (2·2)
γ-Aminobutyric acid	0·5 (0·2)	2·3 (0·8)	1·0 (0·1)	—
A-2-C	1·9 (0·8)	29 (10·7)	2·0 (0·3)	2·5 (4·7)
Valine	—	—	5·9 (0·8)	—
Asparagine	36 (14·3)	26·2 (9·6)	—	1·8 (3·4)
Total in determined amino acids	183 (73·0)	186 (68·9)	618 (87·1)	34·3 (63·7)

* 10^6 Counts/min. were equivalent to approx. 1·25 μc and 25 μg. of [^{14}C]aspartic acid.

from root or rhizome could conceivably be mainly responsible for the normal accumulations of the imino acid within these leaves. This possibility finds support in the observation that, when [^{14}C]aspartic acid was used, the most effective labelling of A-2-C occurred in root tissues, both in *Convallaria* and *Polygonatum*. The relatively heavy labelling of lysine in roots supports the idea that the biosynthesis of the amino acid involves a condensation utilizing aspartic acid as in *Escherichia coli* (Roberts, Abelson, Cowie, Bolton & Britten, 1955).

The nature of the main radioactive product X of feeding with $\alpha\gamma$-diaminobutyric acid is unknown. The similarity between its chromatographic position and that of A-2-C suggests that it might be the closely related $\Delta^{1:2}$-dehydroazetidine-2-carboxylic acid, which could be formed from diaminobutyric acid via γ-amino-α-oxobutyric acid as an intermediate. Catalytic hydrogenation of the unsaturated compound would lead to DL-A-2-C. This may be identical with substance Y formed from X by hydrogenation. Although slight separation was observed on paper chromatograms between the radioactive spot of Y and added L-A-2-C the resolution obtained was not greater than that previously reported for the stereoisomers of certain other amino acids (Lederer & Lederer, 1957). If X is $\Delta^{1:2}$-dehydroazetidine-2-carboxylic acid, the final reduction step to A-2-C must have been blocked within the leaf under the experimental conditions used, but no satisfactory reason for this can be advanced at present.

Since [^{14}C]carbon dioxide gave more effective labelling of A-2-C than any other substance fed to *Convallaria* leaves, it was possible that fixation of carbon dioxide by a three-carbon atom compound led directly to the imino acid or to a closely related four-carbon atom precursor. However, this idea requires that the period of most intensive A-2-C labelling should coincide with the time of maximum [^{14}C]carbon dioxide absorption by the leaf. But absorption was complete in 30 min., whereas the activity of A-2-C increased (both as absolute counts/min. and when expressed as a percentage of the total activity of the cationic fraction) most markedly between the first and sixth hours. Since it is most unlikely that carbon dioxide was simply absorbed by the leaf and remained in this form for long periods before assimilation, the idea of a fixation process in A-2-C biosynthesis finds no support.

The fact that the mechanism whereby A-2-C is synthesized remains unsolved can be attributed largely to the sluggishness of the metabolic processes involved: higher plant tissues used have proved unsuitable for this reason. Although germinating seeds often prove to be metabolically more active than maturing tissues it has not been possible to use them in the present studies because the germination of liliaceous seeds is notoriously sporadic. If a micro-organism capable of rapidly synthetisizing A-2-C were found there would be no difficulty in ascertaining the site of imino acid synthesis, and the processes involved would proceed at rates more suitable for biochemical analysis.

SUMMARY

1. The synthesis of azetidine-2-carboxylic acid in *Convallaria* leaves (lily-of-the-valley) has been studied by feeding ^{14}C-labelled carbon dioxide, glucose, aspartic acid, glutamic acid, γ-aminobutyric acid and $\alpha\gamma$-diaminobutyric acid. Appreciable labelling of azetidine-2-carboxylic acid occurred after the supply of [^{14}C]carbon dioxide, but the imino acid became radioactive more slowly than alanine, serine and glutamic acid. Weak labelling occurred after aspartic and glutamic acid feeding. No activity could be detected in azetidine-2-carboxylic acid after supplying ^{14}C-labelled glucose, γ-aminobutyric acid and $\alpha\gamma$-diaminobutyric acid.

2. [^{14}C]Azetidine-2-carboxylic acid was only slowly degraded by *Convallaria* leaves.

3. The main product X of feeding with $\alpha\gamma$-diamino-[^{14}C]butyric acid was catalytically hydrogenated to give a substance Y that overlapped L-azetidine-2-carboxylic acid on two-dimensional paper chromatograms. X may be $\Delta^{1:2}$-dehydroazetidine-2-carboxylic acid; Y would then be DL-azetidine-2-carboxylic acid.

4. [^{14}C]Aspartic acid was supplied to roots and disks of rhizome tissue of *Convallaria* and *Polygonatum*. More radioactivity was incorporated into azetidine-2-carboxylic acid by roots than by rhizomes or leaves.

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REFERENCES

Adamson, D. W. (1939). *J. chem. Soc.* p. 1564.

Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. F. (1949). *Isotopic Carbon*, p. 107. New York: John Wiley and Sons.

Dent, C. E. (1947). *Biochem. J.* **41**, 327.

Fowden, L. (1956). *Biochem. J.* **64**, 323.

Fowden, L. & Bryant, M. (1958). *Biochem. J.* **70**, 626.

Fowden, L. & Steward, F. C. (1957*a*). *Ann. Bot., N.S.*, **21**, 53.

Fowden, L. & Steward, F. C. (1957*b*). *Ann. Bot., N.S.*, **21**, 69.

Fowden, L. & Webb, J. W. (1958). *Ann. Bot., N.S.*, **22**, 73.
Lederer, E. & Lederer, M. (1957). *Chromatography*, 2nd. ed., p. 424. Amsterdam: Elsevier.
Linko, P. (1958). *Acta chem. scand.* **12**, 101.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Racusen, D. W. & Aronoff, S. (1954). *Arch. Biochem. Biophys.* **51**, 68.
Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Studies in Biosynthesis in* Escherichia coli. Washington D.C.: Carnegie Institution of Washington Publication, no. 607.
Towers, G. H. N. & Mortimer, D. C. (1956). *Canad. J. Biochem. Physiol.*, **34**, 511.
Zacharius, R. M., Cathey, H. M. & Steward, F. C. (1957). *Ann. Bot., N.S.*, **21**, 193.

Effect of pH on Fluorescence of Tyrosine, Tryptophan and Related Compounds

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Unlike the absorption spectrum, the fluorescence spectrum of proteins is not the simple sum of the contributions of the aromatic amino acids in neutral water solutions (Teale & Weber, 1956). A study of the behaviour of substituted aromatic amino acids and simple peptides was undertaken in an attempt to elucidate phenomena contributing to the fluorescence of proteins. Ultraviolet excitation was used to reach the lowest excited singlet state of the compounds and interest was concentrated in energy loss by processes competing with fluorescence, i.e. a reduction in the quantum yield of fluorescence. The changes in fluorescence yield with hydrogen-ion concentration were studied.

EXPERIMENTAL

Methods

The fluorescence-excitation spectra were determined by means of the apparatus described by Teale & Weber (1956). Briefly, the principle was to irradiate a cell containing the test solution with monochromatic light and to view the light emitted at right-angles to the incident light by means of a photomultiplier. A filter to block any scattered exciting light was interposed between the cell and the photomultiplier. The photomultiplier was an EMI 6255 or 27M3 Mazda. A Perspex filter absorbing light of wavelengths less than 300 mμ was used to separate exciting light and fluorescence.

Cells of black mat glass with crystalline-quartz windows attached by a non-fluorescent cement were used to reduce to a negligible value cell fluorescence with irradiating light of wavelengths 230–260 mμ (Weber & Teale, 1958).

To eliminate the effect of pH on absorption of the tyrosine derivatives the illuminating wavelength was chosen at the isosbestic point (Fig. 1).

Hydrochloric acid was used to adjust the pH of the aqueous solutions on the acid side of neutrality and sodium hydroxide solution for the alkaline pH values. No buffer solutions were used. The instrument used to measure the pH values was a Cambridge Pye pH meter with a glass and a standard calomel electrode. The pH scale was standardized according to the B.S. specification.

Materials

The methyl esters of tyrosine and tryptophan were prepared by the following modification of the method of Brenner & Huber (1953). A mixture of thionyl chloride, methanol and tyrosine (in the molecular proportions 1:8:1) was allowed to stand for 12 days at room temperature. The methanol was removed under reduced pressure, the residue dissolved in water, and adjusted to pH 6 and extracted with ether. A white crystalline material was obtained (m.p. 134–138° uncorr.), which on a paper chromatogram with butanol–acetic acid–water solvent mixture (4:1:5) had R_F 0·585 compared with 0·3 for tyrosine. In the preparation of the tryptophan methyl ester the thionyl chloride, methanol, tryptophan mixture (in the molecular proportions 1:8:1 as described above) was maintained at 0° for some hours until a heavy salmon-pink precipitate was formed from the dark-red solution. The methanol was removed as described above and an ether extraction performed. Upon crystallization from chloroform white crystals which became yellow on standing were obtained (m.p. 70° uncorr.) *N*-Acetyltyrosine was prepared from L-tyrosine by the method of du Vigneaud & Meyer (1932). *N*-Acetyltryptophan was prepared from DL-tryptophan by the method of du Vigneaud & Sealock (1932). Polytyrosine (average chain length 45) was a gift from Dr E. Katchalski. Glycyltyrosylglycine was a gift from Dr R. Pitt-Rivers. Tyrosine *O*-phosphate was a gift from Dr T. Hofman. The glycyl derivatives were commercially available, glycyltyrosine from Hoffman–La-Roche and Co. and glycyltryptophan from Roche Products Ltd.

RESULTS AND DISCUSSION

Fluorescence-excitation spectra

The fractional absorption spectra at neutrality were found to correspond well with the fluorescence-excitation spectra both at neutrality and in