Metabolic Incorporation of L-[¹⁴C]Valine into Protein and Bound Non-Protein Forms in Italian Ryegrass

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Aqueous protein-free extracts of Italian ryegrass contain a complicated mixture of substances which on acid hydrolysis yield amino acids. About 5% of the nitrogen of these extracts is contributed by such bound amino acid residues. Compounds of this kind, apparently devoid of basic ionizing groups, have been studied by Synge & Wood (1958); Carnegie (1961*b*) has studied those possessing basic groups. No individual compounds, apart from pyrrolidone carboxylic acid, which is probably an artifact, have yet been identified in either of these classes.

It should be possible, after administering an isotopically labelled amino acid to the plant and measuring incorporation of its residues into compounds of these two classes as well as into protein, to obtain a view of the metabolic relationships of these non-protein compounds of amino acids. We were particularly concerned whether they could be intermediates in the anabolism or catabolism of protein. We used generally-labelled L-[¹⁴C]valine because valine residues are present in reasonable abundance in these non-protein compounds and because valine is not metabolized into other amino acids to any appreciable extent; this is the general experience in work with animals (e.g. Campbell & Work, 1952; Harris & Watts, 1958) and seems also to be true for plants. Miettinen (1959) has described a slight conversion (1–2%) of DL-[¹⁴C]-valine administered to pea plants through the roots into γ -aminobutyric acid and leucine-isoleucine in the course of 3 hr. It is also technically simple to isolate valine as its *N*-2:4-dinitrophenyl derivative for determination of its amount and specific activity.

We wished the physiological conditions to be close to those of active photosynthesis and growth. For this, injection into the stem of the intact growing plant [McConnell and colleagues, see McConnell (1959) and earlier papers in this series] or uptake through the root (Miettinen, 1959) seem the best means of introducing labelled non-volatile compounds. However, the former method might complicate the recovery of labelled material, if any had entered the root. Moreover, the previous chemical studies on which we based our analytical procedures had related only to the aerial parts of the plant.

Accordingly we severed the stalk near the ground and introduced amino acid of high specific activity in water by the transpiration stream across the cut surface (cf. McCalla & Neish, 1959). Metabolism was allowed to proceed in the open air on fairly bright days for rather short periods. We hoped that under these conditions there would be some net synthesis of protein and that the breakdown which occurs when severed leaves are kept in water for longer periods would be avoided (for a review see McKee, 1958). Although we did not measure changes in total protein, we found no evidence for net catabolism of protein, in that none of the fractions of non-protein nitrogen, expressed as a percentage of the total nitrogen of the plant, increased during our experiments above the values found on analysis of freshly severed material. Stephenson, Thimann & Zamecnik (1956) incubated tobacco-leaf disks over a liquid medium containing L-[¹⁴C]valine and observed vigorous incorporation of ¹⁴C into the protein, presumably as valine residues. Incorporation was stimulated by light. Arnstein & Clubb (1957), who studied the incorporation of [¹⁴C:¹⁵N]valine into mycelial protein during growth of *Penicillium chrysogenum*, observed good incorporation of the carbon skeleton, with substantial transfer of ¹⁵N to other amino acids, etc., by transamination or deamination.

MATERIALS

L-[¹⁴C]₂]Valine. This was purchased from The Radiochemical Centre, Amersham, Bucks. (CFB 21, batch 8, specific activity 68.5 μ C/mg.). Six months after its analysis at Amersham, during which time it had been stored as received (in a sealed ampoule in the dark at room temperature), 0.1 mc was accurately subdivided into portions bearing a known ratio to one another by evaporation of weighed portions (approx. 1 μ C and 10 μ C) of an aqueous solution in neutral-glass ampoules, which were sealed under a vacuum of 0.1 mm. Hg (after drying overnight over P₂O₅ at this pressure) and stored at room temperature in the dark until required. Two-dimensional filter-paper chromatograms showed, on radioautography (see below), besides the main valine spot a subsidiary radioactive spot having R_{val} 0.93 in phenol-NH₃ and R_{val} 1.41 in 'collidine', which we judged to have about 2% of the activity of the main spot. This component was not found on radioautographs of the diffusates or of hydrolysates of protein fractions from grass into which [¹⁴C]valine residues had been

incorporated. Tolbert *et al.* (1953) (cf. Lemmon, 1953) observed 1–2% of a non-volatile product of radiochemical decomposition on storage of $[\gamma\text{-}^{14}\text{C}]\text{valine}$ ($6.0\text{ }\mu\text{C}/\text{mg.}$) for 15 months. The experiments described here were completed within 1 year of the analytical checking of the material at The Radiochemical Centre.

N-2,4-Dinitrophenyl-L-valine. DL-Valine (Marvel & du Vigneaud, 1943; Marvel, 1955) was resolved to L-valine according to Fischer (1906). This gave a single spot on filter-paper chromatograms (as below); $[\alpha]_{\text{D}}^{20} + 28.6^\circ$ (6N-HCl, c, 4). From this, working always in subdued artificial light, the dinitrophenyl (DNP) derivative was prepared according to Rao & Sober (1954). When first prepared, yellow crystals were obtained, which softened at 129° before melting at 131° (uncorr.); $[\alpha]_{\text{D}}^{20} - 39^\circ$ (acetic acid, c, 1), with slow positive mutarotation. On all later occasions redder crystals were obtained, which melted sharply at 131° and gave $[\alpha]_{\text{D}}^{20} - 28^\circ$ (no mutarotation). Rao & Sober (1954) reported m.p. 132° ; $[\alpha]_{\text{D}}^{24-26} - 28^\circ$. Campbell & Work (1952) reported m.p. 129° . It seems possible that the crystals first obtained were of some metastable tautomeric form of the compound.

DNP-L- ^{14}C valine for use as a standard in comparisons of specific radioactivity was prepared as described above from L- ^{14}C valine diluted with a known weight of inactive L-valine.

METHODS

General. Unless otherwise stated, evaporations were done *in vacuo* below 40° . Optical rotations were determined in a 0.5 dm. tube. Dry matter, N and carboxyl N (ninhydrin CO_2) were determined on the non-radioactive controls as described by Syngé & Wood (1958). Two-dimensional paper chromatograms were also made by their procedure.

Results are reported on the basis of total dry matter or total N of the original grass, after making appropriate allowance for subdivisions, removal of samples etc.

Outline of fractionation procedures. These were based on the work of Syngé & Wood (1958) and Carnegie (1961*a, b*) and are shown in Fig. 1. The different categories of bound amino acids were isolated largely in fractions 5 and 8.

Cutting of grass and administration of L- ^{14}C valine. On a fairly bright and breezy day three stems of Italian ryegrass, growing on the Duthie Experimental Stock Farm, were cut just above a node and the bottom leaf was stripped back from the stem. The stems (wet wt. about 4 g.) were placed in an opened ampoule containing ^{14}C valine ($1\text{ }\mu\text{C}$) dissolved in $100\text{ }\mu\text{l.}$ of water. The stems imbibed this solution in a few minutes. Further portions ($50\text{ }\mu\text{l.}$) of water were then added as soon as each portion was nearly sucked up. This was done in the field for the time stated, after which the grass and tube were quickly taken to the laboratory. Control stems cut at the same time were supplied with water only.

Extraction and washing. The grass and tube were immediately placed in a large beaker covered with a clock glass and containing 200 ml. of briskly boiling water. After 10 min. boiling the beaker and contents were quickly cooled to room temperature and the aqueous extract was poured off the leaf residues. The residues were brought to the boil with two further portions of 200 ml. of water and these washings were added to the initial extract. The residues were further washed in the same way at the boil

with five 100 ml. portions of 0.05% commercial L-valine in aqueous 1.2% (v/v) acetic acid, followed by two 100 ml. portions of water. These washings were discarded. The residues were spread out and dried between filter papers for radioautography (fraction 1).

In expt. IV the grass was extracted thrice with water in the presence of excess of ether at room temperature (in all about 30 min.). The residues were then washed with boiling solutions as described above. With the non-radioactive controls, valine was omitted from the wash liquor and the washings were collected for analysis (fraction 2).

Dialysis. The extract was evaporated to small volume and dialysed in cellophane against approx. 500 ml. of water saturated with chloroform for several days at $0-1^\circ$, with stirring at intervals. After measuring volumes of diffusate (fraction 3) and bag contents, the latter were discarded (in the non-radioactive experiments their N and dry-matter were determined, so that total initial N and dry matter of the grass could be calculated). In calculating results on fractions derived from the diffusate, it was assumed that its components had been present in equal concentration in the bag contents.

Countercurrent distribution in phenol-water. The diffusate was evaporated to small volume, adjusted to pH 2 by addition of 2N- H_2SO_4 and transferred with water (final vol. 20 ml.) to the first of a train of five separating funnels. We here followed the procedure of Syngé & Wood (1958), 20 ml. charges of each phase being used; the first eight charges of phenol-rich phase to leave the train were pooled and evaporated to small volume with water as described by them (fraction 4). The residues in the train were discarded.

Ionophoresis in three-compartment diaphragm cell. Fraction 4 was transferred in about 15 ml. of aqueous 10% (v/v) acetic acid to the middle compartment of the cell (Syngé, 1951; Syngé & Wood, 1958). The catholyte was aqueous 1% (w/v) NH_3 and the anolyte was 0.1N- H_2SO_4 . After 6 hr. running the catholyte was removed and its compartment was recharged with fresh 1% NH_3 solution. After 24 hr. the middle-compartment contents were collected and evaporated to dryness (fraction 5).

The 6 hr. catholyte was evaporated to dryness and run again for 6 hr. in a fresh cell as described above. The final catholyte (fraction 6) was collected and evaporated to dryness, finally in a high vacuum over H_2SO_4 and soda lime at room temperature to remove the bulk of the ammonium acetate present.

Fractionation on Dowex 1-X4. The product was chromatographed in ammonium acetate buffer (pH 8.69) on a column (1 cm. $\times$ 6 cm.) of Dowex 1-X4 by elution-development with 0.05M-buffer as described by Carnegie (1961*a, b*). After 25 ml. of effluent had been collected (fraction 7), the column was developed with N-HCl and effluent was collected until breakthrough of Cl^- (fraction 8). These fractions were evaporated as described above to minimize the content of ammonium acetate.

Acid hydrolysis. Fractions 1 (after radioautography), 5 and 8 were hydrolysed by refluxing for 24 hr. in excess of 6N-HCl. The hydrolysates were evaporated to dryness *in vacuo* up to 100° .

Isolation and determination of valine as its dinitrophenyl derivative. This was done on suitable portions of hydrolysates of fractions 1, 5 and 8 and of fractions 7 (unhydrolysed). The general procedure of Perrone (1951) and Krol (1952) was followed (cf. Campbell & Work, 1952; Muir,

Fig. 1. Scheme for fractionation of grass.

Neuberger & Perrone, 1952). Dinitrophenylation and all subsequent operations were done in subdued artificial light. Dinitrophenyl (DNP)-valine was isolated by chromatography with ether on a column of Hyflo-Supercel (Johns-Manville Co. Ltd., London) charged with 0.25M-sodium phosphate buffer, pH 6.7, followed by rechromatography on a similar column in 2% (v/v) butanol in chloroform. The effluent (30 ml.) was extracted with 10 ml. of 0.05M-sodium phosphate buffer, pH 11.3, and *E* at 410 m μ was determined on this solution in a Unicam SP. 1400 spectrophotometer with a 1 cm. cell, reading against a blank of the same solvent. DNP-L-valine under these conditions gave a linear calibration curve, corresponding to *E* 0.274 for 100 μ g. Control recoveries of valine from a made-up mixture of amino acids simulating a leaf-protein hydrolysate averaged 110%, probably due to contamination by other DNP derivatives. No correction has been made for this.

Determination of radioactivity. All counting was done on DNP-valine samples under 'infinite thinness' conditions. The aqueous phase (recovered quantitatively with water from the spectrophotometer cell) was acidified with HCl and swirled in a separating funnel with ether (5 ml.). The ether phase was washed thrice with 15 ml. portions of 0.1N-HCl and evaporated to dryness. The residue was transferred quantitatively in acetone to an aluminium planchet (AERE Harwell Drg. no. A. 36688; 2.25 cm. diam.) on which a circle of 105 lens tissue (J. Green, Maidstone) had been fixed by dropping on to it 3 drops of a 0.25% (w/v) solution of Perspex in chloroform. When dry, the sample was counted with a Geiger-Müller end-window counter. Corrections were made for background and coincidence. Provided that less than 100 μ g. of DNP-

valine was plated, counts/min. were directly proportional to the radioactivity present, and were unchanged after storage of the planchets for 6 months at room temperature. Counting efficiency was approx. 5%. For calculating the recoveries in Table 3, reference was to the standard DNP-L-[¹⁴C]valine spread for counting in the same manner.

Radioautography. Paper chromatograms were dried at 105° for 1½ hr. before radioautography. The chromatogram or pressed-grass specimen was opposed to Ilfex safety-base X-ray film (Ilford Ltd., Ilford, Essex); after a suitable exposure the film was developed as recommended for X-ray work.

RESULTS

Control expt. (C) and corrections used in calculations. Instead of the introduction of [¹⁴C]valine under conditions favouring its metabolism, freshly cut grass was placed immediately in boiling water containing the [¹⁴C]valine. On preparation, no radioactivity was detected in the protein valine (i.e. < 0.1% of the total radioactivity initially added). This grass was cut on 8 April 1958 at 10.00 hr. and consisted entirely of short tender leaves.

The free valine present in this grass specimen was calculated by isotope dilution to be 0.13% of the total N of the grass specimen (Table 2). On this basis, 32% of the free valine was recovered in fraction 7. Most of the loss was presumably into the aqueous residue from the countercurrent distribution. In expts. I-IV the percentages of free

valine and of radioactivity in free valine (Tables 2 and 3) have been calculated from results on fraction 7, assuming the same recovery.

If the radioactivity of valine in the hydrolysates of fractions 5 and 8 in expt. C was assumed to be due to contamination of these fractions by the free valine present in the cell catholyte, put on to the Dowex 1-X4 column and recovered in fraction 7, the contaminations amounted respectively to 0.046 and 0.30 % of this free valine. In calculating the results for expts. I-IV, corresponding percentages of the radioactivity of valine in fraction 7 have been subtracted from the radioactivity of the valine in hydrolysates of fractions 5 and 8; the amounts subtracted in no case exceeded 30 % of the radioactivity found.

Expts. I-IV. Expts. I (1 hr. transpiration) and II (2 hr. transpiration) were done on 4 June 1958 between 10.30 and 12.30 hr. (temp. 16-18°). The grass was just about to flower (Fig. 2a), and was selected from a well-fertilized patch where growth and depth of green were above average.

Expts. III and IV (2 hr. transpiration) were done on 12 August 1958 between the same hours (temp. 19-23°). The grass was at the flowering stage (Fig. 2b). As before, the tallest and greenest grass was selected. Stems were assigned to each ampoule in pairs whose members resembled each other as closely as possible; III was then prepared with boiling water as usual and IV by the ether-water procedure.

Incorporation of valine into grass protein. Radioautography of the boiled-out residues (fraction 1) showed that in grass not yet flowering the radioactivity was mainly fixed in the lower part of the stem near the point of entry (Fig. 2a), whereas at the flowering stage there was widespread fixation mainly in leaves and flower, with much less in the stem (Fig. 2b).

The two-dimensional chromatograms of hydrolysates of fraction 1 showed a single radioactive spot in the valine position. A partial hydrolysate (10N-HCl for 96 hr. at 37°) showed numerous faint radioactive spots, a majority of which ran fast in the phenol phase. These observations, taken with our inability to detect radioactivity in residues (fraction 1) of leaves boiled with [^{14}C]valine (expt. C) leave little doubt that the [^{14}C]valine was incorporated into protein by a metabolic process.

Analytical results on grass specimens. These are given in Tables 1 and 2. The high protein content of C, as well as the high proportion of non-protein to total nitrogen, reflect the immaturity of the specimen.

Distribution of radioactivity in grass fractions. Table 3 shows the recoveries (expressed as percentages of the radioactivity initially administered) of radioactive valine from the different fractions

studied, and also total recovery of radioactivity in the form of valine residues. The amount by which this falls short of 100 % gives an idea of the extent of metabolism of the valine administered into other chemical compounds during each experiment.

In Table 4 the specific radioactivity of the valine isolated from each fraction is expressed as a percentage of the specific activity of the free valine present in the grass at the end of the experiment.

Radioautography of two-dimensional chromatograms of diffusates in expts. III and IV showed only free valine, with a diffusely blurred background, presumably due to the spreading of the small amount of other radioactivity over a large number of components.

DISCUSSION

It is clear that substantial incorporation of free valine into protein was taking place under the conditions of these experiments. This is a prerequisite for a metabolic experiment aimed at searching for possible intermediates in the biosynthesis of protein.

Our interpretation of the present results is that the specific activities of the bound non-protein valine in acidic and neutral compounds (fractions 5) and in amphoteric compounds (fractions 8) are not sufficiently close to that of the free valine for any substantial proportion of such bound valine to have been serving as an intermediate stage in the incorporation of free valine into protein. We regard it as specially significant that the amounts and specific activities of the valine in these non-protein compounds were highest in expt. I, which of all the experiments shows the least incorporation of valine into protein. In this experiment, also, the total recovery of radioactivity in valine residues was smallest, which suggests that the entire metabolism of valine was for some reason diverted away from protein synthesis. These compounds, on the other hand, had specific activities too high to arise by indiscriminate catabolism of pre-existing proteins. It seems most reasonable to regard them as being metabolites of free valine unconnected with protein synthesis. The amphoteric fractions 8 in each experiment showed higher specific activity than the neutral and acidic fractions 5, and may therefore possibly have been precursors of them.

It remains a possibility that some minor components of fractions 5 or 8 contained valine residues having specific activity approaching that of the free valine, such as would be possessed by an intermediate of protein synthesis present in such small quantity. To test this would require more elaborate fractionation. It would also be desirable to try to make a more detailed study of the kinetics of labelling of the valine residues in such a fraction

Fig. 2. Radioautographs of extracted residues (fraction 1) of Italian ryegrass after administration of L-[^{14}C]valine (see text): (a) preflowering stage; (b) flowering stage. ($\times 0.41$.)

under conditions of rapid increase and decrease of the specific activity of the free valine.

The action of ether-water at room temperature resulted in a doubling or trebling of the amphoteric forms of bound amino acids and bound valine in fractions 6 and 8 when compared with the action of

boiling water (expts. III and IV). Since there was no parallel increase in the percentage of radioactivity entering the valine of fraction 8 it is reasonable to conclude that autolysis of pre-existing protein during the ether-water treatment caused the increase in this peptide-like

Table 1. *Distribution of dry matter and nitrogen in fractions from grass*

Expt.	...	...	...	Dry matter				Nitrogen							
				(% of total dry matter of grass)				(% of dry matter of fraction)				(% of total N of grass)			
				C	II	III	IV	C	II	III	IV	C	II	III	IV
Fraction															
Original grass				100	100	100	100	5.0	2.8	3.2	2.9	100	100	100	100
Residues (1)				62.6	58.4	68.3	63.1	5.8	4.1	4.0	3.8	71.4	85.9	85.4	82.4
Acetic acid washings (2)				3.1	5.2	4.5	17.9	1.9	0.8	2.2	1.5	1.2	1.5	3.1	9.0
Diffusate (3)				32.2	31.6	24.1	17.3	3.8	1.0	1.4	1.3	24.0	11.4	10.5	7.8
Phenol extract (4)				8.0	3.3	3.1	1.6	3.1	1.6	1.6	3.6	5.0	1.9	1.6	2.0
Middle compartment (5)				2.7	1.2	1.4	0.5	1.7	2.8	0.7	1.3	0.89	0.92	0.33	0.23

Table 2. *Distribution of carboxyl nitrogen and valine nitrogen in fractions from grass*

Expt.	...	...	...	...	...	...	Nitrogen (% of total N of grass)				
							C	I	II	III	IV
Valine N of hydrolysed residues (1)							2.8	3.5	4.9	4.8	4.2
Carboxyl N after hydrolysis of fraction 5							0.15	—	0.083	0.095	0.060
Valine N after hydrolysis of fraction 5							0.00096	0.0103	0.0036	0.0017	0.0012
Carboxyl N before hydrolysis of fraction 6							1.36	—	0.35	0.21	0.22
Carboxyl N after hydrolysis of fraction 6							1.75	—	0.40	0.35	0.50
Carboxyl N of bound amino acids of fraction 6 (by diff.)							0.39	—	0.05	0.14	0.28
Free valine N of fraction 7*							0.130†	0.130*	0.105*	0.044*	0.038*
Bound valine N after hydrolysis of fraction 8							0.0043	0.0154	0.0054	0.0074	0.0270

* Corrected values (see text).

† By isotope dilution (see text).

Table 3. *Recovery of radioactivity in valine residues of fractions from grass as a percentage of that initially administered as [¹⁴C]valine*

Expt.	...	...	...	...	...	...	Recovery (%)			
							I	II	III	IV
Free valine (fraction 7)							38.7	53.5	25.9	15.4
Protein (fraction 1)							13.0	21.0	54.1	46.8
Bound valine (fraction 5) (acidic and neutral)							0.29	0.018	0.013	0.010
Bound valine (fraction 8) (amphoteric)							0.77	0.088	0.32	0.37
Total recovery							52.8	74.6	80.3	62.6

Table 4. *Specific radioactivity of valine residues of fractions from grass as a percentage of the specific radioactivity of the free valine of the grass at the end of the experiment*

Expt.	...	...	...	...	...	...	Specific radioactivity (%)			
							I	II	III	IV
Protein (fraction 1)							1.2	0.8	1.9	2.7
Acidic and neutral bound amino acids (fraction 5)							9.5	1.0	1.3	2.0
Amphoteric bound amino acids (fraction 8)							16.8	3.2	7.3	3.4

material. The neutral and acidic bound amino acids in fraction 5 did not increase. The implications of these observations for the studies of Carnegie (1961*b*) are discussed by him in that paper.

The concentration of free valine found in the grass samples agrees fairly well with that found by Kemble & Macpherson (1954*b*). Neither the free valine nor the total free amino acids of fraction 6 seemed to increase as a result of the ether-water

treatment. The valine content of the grass protein was also close to the values found by Kemble & Macpherson (1954*a*).

Miettinen (1959) has studied the metabolism by pea plants of DL-[carboxy-¹⁴C]alanine. He usually administered this through the roots, and found some incorporation of [¹⁴C]alanine into fractions probably similar to our fractions 5 and 8. Incorporation of ¹⁴C into the neutral and anionic

fraction was particularly extensive; however, residues other than alanine, such as simple sugars, accounted for some of this. Nevertheless, much of the activity (in roots only) was present as alanine residues in compound 'A'. A somewhat similar compound was formed in roots from DL-[¹⁴C]valine. Dr J. K. Miettinen (personal communication) informs us that substance 'A' results from administration of DL-[¹⁴C]alanine but not of L-[¹⁴C]alanine.

Linko (1958) found rapid labelling of a number of compounds, some of which were of low molecular weight and contained bound amino acids, on infiltrating leaves of *Phlox decussata* and *Asplenium septentrionale* with [¹⁴C]pyruvate and [¹⁴C]glutamate.

The present experiments demonstrate that L-[¹⁴C]valine of high specific activity introduced in the transpiration stream can be fairly rapidly and extensively incorporated into protein distributed right through the severed portion of the plant, perhaps without much disturbance of the normal metabolic processes. We hope that these observations will prove useful for planning future studies of the biosynthesis of proteins in plants.

SUMMARY

1. Uniformly labelled L-[¹⁴C]valine of high specific activity was administered to severed shoots of Italian ryegrass in the transpiration stream and extensive incorporation of labelled valine residues into protein resulted. The distribution of labelled protein in the plant was observed by radioautography.

2. The specific activities acquired under these conditions by valine residues in bound non-protein form suggest that most of such compounds are not directly concerned in the biosynthesis or the catabolism of protein.

3. Extraction with ether-water resulted in some catabolism of protein to peptides not present when the tissue was extracted by boiling water.

4. A non-volatile radioactive impurity, presumably a product of radiochemical decomposition, was present in the [¹⁴C]valine as purchased.

5. Evidence was obtained for the occurrence of a metastable tautomeric form of *N*-2:4-dinitrophenyl-L-valine exhibiting mutarotation in acetic acid.

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