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DETERMINATION OF THE RELATIVE RATES OF INCORPORATION OF ARGININE AND ORNITHINE INTO RETRONECINE DURING PYRROLIZIDINE ALKALOID BIOSYNTHESIS

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Abstract—A double-isotope technique for simultaneously measuring the per cent incorporation of two precursors into a metabolite is described. The method has been used to show that ornithine is a more efficient precursor than arginine for the biosynthesis of retronecine, the necine base component of the pyrrolizidine alkaloid senecionine.

INTRODUCTION

Per cent incorporations of labelled compounds have frequently been used as criteria of the relative efficiencies of the compounds tested as biosynthetic precursors of a metabolite. The uncertainties inherent in this approach have been pointed out repeatedly [1–3]. Variations in the per cent incorporations of a precursor into a metabolite can vary markedly even in closely controlled duplicate experiments. The use of raw per cent incorporations are therefore often of little value as a measure of the relative biosynthetic efficiencies of two different precursors. However, this does not mean that incorporation values are never informative In our studies of pyrrolizidine alkaloid biosynthesis, for example, we have found that when an incorporation of $>0{\cdot}1\%$ has been observed the compound tested has almost invariably proved to be a specific precursor of either the necic acid or necine base moiety of the alkaloid. Under defined conditions, therefore, incorporation values by themselves *can* be informative. However, in general, some other criterion must be applied before a judgment can be made about the significance of relative per cent incorporations of two or more labelled compounds.

Spenser and his colleagues have recently demonstrated the application of a double-isotope technique for the direct and simultaneous determination of the relative incorporations of optical antipodes and have applied this technique to a demonstration of the roles of L- and D-lysine as precursors of various plant metabolites containing a piperidine nucleus [3]. In this procedure, the ^{3}H:^{14}C ratio of the metabolite was compared with that of a doubly-labelled substrate, one isomer of which was labelled with ^{14}C and the other doubly-labelled with ^{3}H and ^{14}C. It is essential for the success of this procedure that the ^{3}H label in the precursor should be retained during conversion into the product.

We have investigated a method for the direct, simultaneous measurement of the relative rates of incorporation of two precursors which is of general applicability and which does not depend on the use of precursors in which a tritium label is fully retained during conversion into the product.

If a doubly-labelled compound consisting of a mixture of ^{3}H- and ^{14}C-labelled species were administered to a series of plants, we considered that the absolute per cent incorporations of the

isotopes might vary considerably from plant to plant, as is frequently found in practice, but that the *relative* incorporations of the isotopes would be a function predominantly of the biosynthetic pathway and would not be expected to vary greatly from one plant to another and from one incorporation experiment to another. If this assumption were correct it would follow that the ^3H:^{14}C ratio in the metabolite would be related to the ^3H:^{14}C ratio in the precursor by some factor r to be determined experimentally, i.e.

$$\left(\frac{^3H}{^{14}C}\right)_p r = \left(\frac{^3H}{^{14}C}\right)_m \quad (1)$$

(p and m refer to precursor and metabolite respectively).

Further, if, in a given incorporation experiment, the ^3H activity fed were x disintegrations per minute (dpm) and the ^{14}C activity y dpm, and if in the isolated metabolite the corresponding activities were x' dpm and y' dpm respectively, then

$$\frac{^3H_p}{^{14}C_p} = \frac{x}{y} = r_1 \quad \text{and} \quad \frac{^3H_m}{^{14}C_m} = \frac{x'}{y'} = r_2$$

$$\therefore \quad ^3H_p = {}^{14}C_p . r_1 \quad \text{and} \quad ^3H_m = {}^{14}C_m . r_2$$

$$\therefore \quad \frac{^3H_m}{^3H_p} = \frac{^{14}C_m}{^{14}C_p} . \frac{r_2}{r_1}$$

$\therefore$ % Incorporation ^3H

$$= \%\text{Incorporation } ^{14}C \times \frac{r_2}{r_1}$$

$\therefore$ % Incorporation ^3H

$$= \% \text{ Incorporation } ^{14}C \times r \quad (2)$$

$$\left[\text{where } r = \frac{r_2}{r_1} = \frac{^3H:^{14}C \text{ ratio in metabolite}}{^3H\cdot^{14}C \text{ ratio in precursor}} \quad \text{(equation (1))}\right]$$

Thus, provided that the factor r could be shown to be constant for a given precursor, the predicted incorporation of the ^{14}C-labelled precursor could be calculated from the incorporation of the ^3H-labelled precursor alone. It is essential, of course, that the ^3H- and ^{14}C-labelled precursors used should be constant in a given series of experiments.

Further, if the relative incorporations of two different precursors were to be compared, the precursor for which the ratio r had been determined (say precursor A) could be fed in ^3H-labelled form and the other precursor, B, in ^{14}C-labelled form. The retention of ^3H relative to ^{14}C would then be given by

$$\frac{\% \text{ Incorporation } ^3H}{\% \text{ Incorporation } ^{14}C} = \frac{\dfrac{^3H}{^{14}C} \text{ Ratio in metabolite}}{\dfrac{^3H}{^{14}C} \text{ Ratio in precursors}}$$

$$= R$$

Since % incorporation of $[^3H]A$ = % incorporation of $[^{14}C]A \times r$ (equation 2),

$$\therefore \quad \frac{\% \text{ Incorporation } [^{14}C]A}{\% \text{ Incorporation } [^{14}C]B} = \frac{R}{r} . \quad (3)$$

We have tested the validity of this procedure in experiments designed to investigate the relative efficiencies of ornithine (**1**) and arginine (**2**) as precursors of the necine base retronecine (**3**) of the pyrrolizidine alkaloid senecionine (**4**) (Fig. 1). Ornithine (**1**) has been shown to be a specific precursor of retronecine (**3**) [4]. It is probable that ornithine is incorporated into retronecine after decarboxylation to putrescine (**5**) since labelled putrescine has also been shown to be specifically incorporated into retronecine [4c].

Arginine can be hydrolysed to ornithine by the enzyme arginase, which has been detected in a number of higher plants. However, putrescine can also be formed from arginine via agmatine (**6**) and *N*-carbamylputrescine (**7**). Arginine was shown to be the major precursor of putrescine in potassium-deficient barley [6]. Agmatine was accumulated and labelled agmatine was converted into *N*-carbamylputrescine and putrescine; ornithine was only slowly converted into putrescine. Arginine and agmatine were shown to be precursors of putrescine in the halophyte *Limonium vulgare* and the presence in the plant of *N*-carbamylputrescine was demonstrated [7]. Both arginine and ornithine were incorporated efficiently into homospermidine in sandalwood [8].

The pyrrolidine nucleus present in a variety of alkaloids has been shown to be derived from ornithine, but the possible role of arginine as a precursor has been little investigated. However, arginine, agmatine and *N*-carbamylputrescine were incorporated specifically into the pyrrolidine

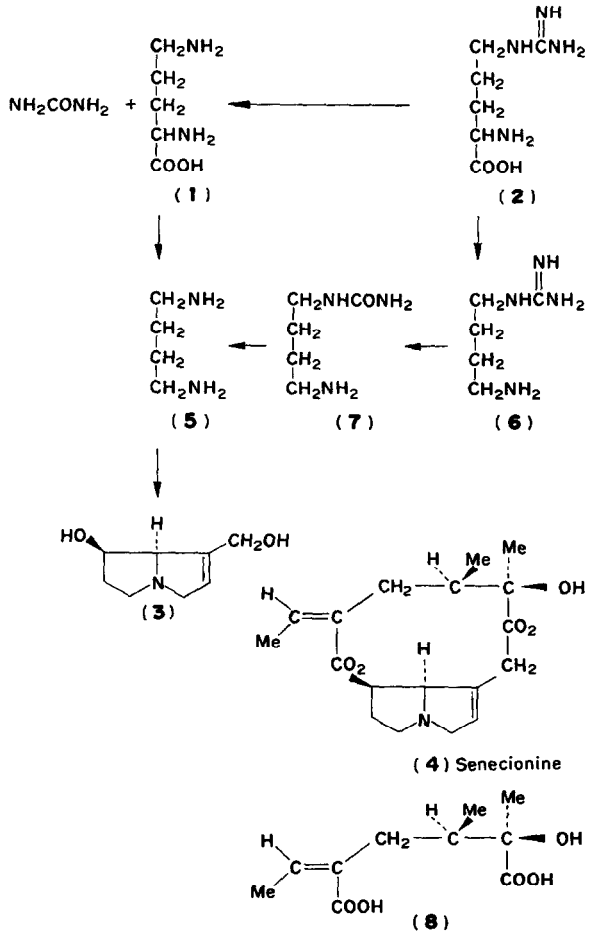

Fig 1

ring of nicotine [9]. Both arginine and ornithine were shown to be precursors of putrescine in tobacco plants; putrescine was probably derived from arginine via agmatine [10].

RESULTS

In the first series of experiments (Tables 1 and 2), a mixture of L-[U-^{14}C]arginine and L-[3-^{3}H(N)]arginine (^{3}H:^{14}C = 4·84:1) was fed to six *Senecio magnificus* plants growing in hydroponic solution. After five days, 95% of the ^{14}C had been taken up by the plants. The ^{3}H:^{14}C ratio in the isolated senecionine (**4**) was determined (Table 1). The incorporation of activity was very low and in one experiment (plant 3) it was essentially zero, However, the ratio r (equation 1) was fairly constant at 0·63 ± 0·09. Further, it can be seen (Table 1) that although the per cent incorporations of

Table 1 Relative retention of ^{3}H and ^{14}C on incorporation of L-[U-^{14}C]arginine and L-[3-^{3}H(N)]arginine into senecionine

Experiment	1	2	3	4	5	6
^{3}H/^{14}C ratio in arginine fed	4 84	4·84	4 84	4 84	4 84	4·84
^{3}H/^{14}C ratio in senecionine	3 44	3 43		3 30	2·61	2 38
r (ratio ^{3}H/^{14}C incorporated − ^{3}H/^{14}C fed)	0 71	0 70		0 68	0·53	0 49
% Incorporation of ^{14}C	0 009	0 009	0 0	0 007	0 001	0 002

Mean ratio (r) = 0 63 ± 0 09
Mean ^{14}C incorporation = 0·005%

^{14}C varied by a factor of nine (neglecting the single zero value), the ratio r only varied by a factor of 1·4. We next fed a mixture of L-[U-^{14}C]ornithine and L-[3-^{3}H(N)]arginine to six *S. magnificus* plants (Table 2). The ratio of the amount of ornithine to the amount of arginine fed was 10 : 1, 1 : 1 and 1 : 10 for each of two pairs of plants respectively. The ^{3}H:^{14}C ratio in the isolated senecionine was determined and the ratio *per cent incorporation* L-[*U*-^{14}*C*]*arginine* (*calculated*): *per cent incorporation* L-[*U*-^{14}*C*]*ornithine* (*observed*) was determined using equation 3 (Table 2). The ratios thus obtained were multiplied by a factor of 6/5 to correct for the probable incorporation of only four of the six carbon atoms of arginine and four of the five carbon atoms of

Table 2 Relative retention of ^{3}H and ^{14}C on incorporation of L-[U-^{14}C]ornithine and L-[3-^{3}H(N)]arginine into senecionine

Experiment	1	2	3	4	5	6
Arginine fed (mg per plant)	2 5	2 5	0 25	0 25	0·025	0 025
Ornithine fed (mg per plant)	0 25	0·25	0 25	0 25	0 25	0·25
^{3}H/^{14}C ratio fed	3 62	3 62	3 01	3 01	3 21	3 21
^{3}H/^{14}C ratio in senecionine	2 01	2 48	1·56	1 59	1 77	1 67
R (ratio ^{3}H/^{14}C incorporated ÷ ^{3}H/^{14}C fed)	0 56	0 69	0·52	0 53	0 55	0 52
% Incorporation of ^{14}C	0 03	0 25	0 13	0·02	0 01	0 01
R/r [cf equation (3) with r = 0 63 (Table 1)]	0 89	1 10	0·83	0·84	0 87	0 83
R/r × 6/5	1 07	1·32	1 00	1 01	1·04	1 00

Mean ^{14}C incorporation = 0 07%.
Mean relative incorporation = 1 07 ± 0 13

ornithine (Fig. 1). The final relative per cent incorporations of the precursors are given in Table 2, from which it can be seen that arginine and ornithine were incorporated with nearly identical efficiencies.

The results just described were based on very low incorporations in the calibration experiment which were due in part to infection by a species of *Botrytis*, which developed shortly after the plants were placed in hydroponic solution. The experiments were therefore repeated with plants which had been treated with a systemic fungicide. The fungicide was also added to the nutrient solution. This treatment resulted in a considerable increase in the per cent incorporation of the labelled precursors.

The results of the second series of experiments are given in Tables 3 and 4. Uptake of the labelled amino acids in this series was determined by dilution analysis of the residual nutrient solutions and was found to be >99% after 5 days. (Much of the tritium label appeared as $[^3H]H_2O$, which made it impossible to determine the uptake of the [^{3}H]arginine from the gross ^{3}H activity in the nutrient solution). The ratio r determined with doubly-labelled arginine in experiments with six individual plants was very uniform at $0{\cdot}90 \pm 0{\cdot}01$ (Table 3). The batches of labelled arginine used in the first and second series of experiments were different which may account for the difference in the values of r for the two series.

The incorporation experiments with [^{3}H]arginine and [^{14}C]ornithine were carried out as before except that, in each individual experiment, two plants were used. The results given in Tables 3 and 4 reveal some interesting features. In support of the initial premise, it can be seen that although the ^{3}H:^{14}C ratio remained very constant at $0{\cdot}90 \pm 0{\cdot}01$, the per cent incorporations of [^{14}C]arginine varied over an order of magnitude. The constancy of the isotope ratio r indicated that it could be used with confidence in calculating predicted values for the incorporation of [^{14}C]arginine from the observed incorporation of [^{3}H]arginine.

* These figures are derived by dividing the mean per cent incorporation of [^{14}C]ornithine (0·07, Table 2) by the mean per cent incorporation of [^{14}C]arginine (0·005, Table 1) and multiplying by 5/6, and by treating the corresponding results from the second series (Tables 3 and 4) in the same way.

Table 3. Relative retention of ^{3}H and ^{14}C on incorporation of L-[U-^{14}C]arginine and L-[3-^{3}H(N)]arginine into senecionine

Experiment	1	2	3	4	5	6
$^3H/^{14}C$ ratio in arginine fed	3·00	2·99	2·99	3·00	3·03	3·06
$^3H/^{14}C$ ratio in senecionine	2·76	2·69	2·62	2·69	2·68	2·73
r (ratio $^3H/^{14}C$ incorporated ÷ $^3H/^{14}C$ fed)	0·92	0·90	0·89	0·90	0·88	0·89
% Incorporation of ^{14}C	0·02	0·10	0·18	0·22	0·02	0·03

Mean ratio (r) = 0·90 ± 0·01.
Mean ^{14}C incorporation = 0·09%.

The validity of the double-labelling procedure is clearly illustrated by comparing the results of the two series of experiments. If the relative incorporations of arginine and ornithine were based on raw ^{14}C-per cent incorporation values it would be concluded from the first series (Tables 1 and 2) that ornithine was used, on average, nearly 12 times more efficiently than arginine for retronecine biosynthesis, whereas the results of the second series indicate that arginine was incorporated nearly seven times more efficiently than ornithine.* The results of the two series of experiments therefore differ by a factor of ~75! However, if the results of the double-labelling procedure are compared it can be seen that they are far more consistent, the relative mean efficiencies of arginine and ornithine incorporation in the two series being 1·07 ± 0·13 and 0·44 ± 0·18 respectively. Of these the former result is less reliable

Table 4. Relative retention of ^{3}H and ^{14}C on incorporation of L-[U-^{14}C]ornithine and L-[3-^{3}H(N)]arginine into senecionine

Experiment	1	2	3	4	5	6
Arginine fed (mg per plant)	2·5	2·5	0·25	0·25	0·025	0·025
Ornithine fed (mg per plant)	0·25	0·25	0·25	0·25	0·25	0·25
$^3H/^{14}C$ ratio fed	11·5	11·5	12·3	12·3	11·5	11·5
$^3H/^{14}C$ ratio in senecionine	5·43	5·57	4·73	4·01	1·64	2·18
R (ratio $^3H/^{14}C$ incorporated ÷ $^3H/^{14}C$ fed)	0·47	0·48	0·38	0·32	0·14	0·19
% Incorporation of ^{14}C	0·11	0·18	0·022	0·022	0·017	0·007
R/r [cf. equation (3) with r = 0·90 (Table 3)]	0·52	0·53	0·42	0·36	0·16	0·22
$R r \times 6/5$	0·62	0·64	0·50	0·43	0·19	0·26

Mean ^{14}C incorporation = 0·016%.
Mean relative incorporation = 0·44 ± 0·18.

than the latter as it is based on calibration experiments in which only low absolute incorporations were obtained. It was therefore concluded that arginine is a slightly less efficient precursor of retronecine than ornithine. Further investigations will be necessary to determine whether arginine is incorporated after hydrolysis to ornithine, by the agmatine–*N*-carbamylputrescine pathway, or by both routes simultaneously.

In the second series of experiments the incorporation of [^{14}C]arginine relative to [^{14}C]ornithine increased with the absolute amount of arginine fed to each plant (Table 4). (A similar effect has been noted in other incorporation studies [11]). It is noteworthy that this trend was evident even though the incorporation of ornithine itself increased in the same direction (compare lines 6 and 8, Table 4) A similar trend, although less marked, was noted in the experiments with the older plants used for the first series (Table 2).

In the foregoing discussion it was assumed that arginine, like ornithine, was specifically incorporated into the retronecine component of senecionine. This was confirmed by hydrolysis of senecionine from an incorporation experiment with doubly-labelled arginine The activity of the alkaloid (both isotopes) was found to be located almost exclusively in the necine base (Table 5).

The double labelling technique described here provides information only on the incorporation of those atoms of the precursors which are labelled with the isotope used for the comparative incorporation experiments. Interpretation of the results obtained depends on a knowledge of the manner in which the atoms of the precursor are incorporated into the metabolite; this information must be provided by incorporation experiments with specifically-labelled precursors in the usual way. However, it is not necessary for the isotope used for the calibration (^{3}H in the above experiments) to be located at any specific point or points in the precursor or even that the location of the isotope should be precisely known. All that is necessary is that some of the calibrating isotope should be retained during conversion into the metabolite and that the ratio r should be shown by experiment to be constant.

The technique described here eliminates many of the uncertainties that tend to invalidate the use of per cent incorporation values in assessing the relative efficiencies with which two or more precursors are used in a biosynthetic pathway. In particular, it circumvents the problem of isolating the labelled metabolite quantitatively, since the final relative incorporations depend only on measurements of ^{3}H:^{14}C ratios However, the method does not eliminate uncertainties due to differences in the modes of absorption and transport between different precursors; these factors remain the major source of uncertainty in the interpretation of relative incorporations in biosynthetic investigations.

Table 5 Distribution of activity in senecionine* after administration of L-[3-^{3}H(N)]arginine and L-[U-^{14}C]arginine to *S magnificus*

Isotope	% Activity in Retronecine (3)	Senecic acid (8)
^{3}H	94	0
^{14}C	99	1

* The labelled senecionine used was that isolated from expt 4, Table 4

EXPERIMENTAL

General Radiochemicals were purchased from the New England Nuclear Corp Radioactive samples were counted in a Packard 2000 Series Liquid Scintillation Counter in Dioxan Liquid Scintillation Solution (BDH) Sufficient counts were accumulated to give a standard error of $<1\%$ for each determination

Feeding methods Senecio magnificus plants were grown from seed and then cultured in hydroponic soln as previously described [12] The nutrient soln contained Phostrogen (0 6 g dm^{-3} for the first series of expts, increased to 2 1 g dm^{-3} for the 2nd series) For the 2nd series of expts, the plants were treated with Benlate (Dupont) at the seedling stage Benlate (25 mg dm^{-3}) was also added to the hydroponic soln 4-Month-old and 3-month-old plants were used for the 1st and 2nd series of feeding expts respectively

Determination of precursor uptake by dilution analysis A soln of L-arginine (10 mg) and L-ornithine (10 mg) was added to the residual nutrient soln which was filtered and passed down a column of Dowex 50W-X8 cation exchange resin (H^+, 10 g) The column was washed with deionized H_2O, the amino acids were eluted with NH_4OH (2 mol dm^{-3}, 200 cm^3) and counted Residual activity (both isotopes) was always $<0\,3\%$ of the initial activity

Isolation and purification of secenionine (4) Senecionine (4) was isolated as previously described [12] The yield of senecionine varied between 0 5 and 1 5% based on the dry wt of the plant material After diln with inactive senecionine purification was effected by chromatography on neutral alumina with $CHCl_3$ as eluant, and recrystallization from MeOH to constant activity

Hydrolysis of senecionine (4) Senecionine (4) was hydrolysed

to senecic acid (**8**) and retronecine (**3**) (isolated as the hydrochloride) as previously described [12].

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REFERENCES

1. Swain, T. (1965) *Biosynthetic Pathways in Higher Plants* p. 9. (J. B. Pridham and T. Swain, eds) Academic Press, London.
2. Brown, S. A. (1972) *Biosynthesis* (Specialist Reports of the Chemical Society), **1**, 1.
3. Leistner, E. Gupta, R. N. and Spenser, I. D. (1973) *J. Am. Chem. Soc.* **95**, 4040.
4. (a) Nowacki E. and Byerrum, R. U. (1962) *Life Sciences* **5**, 157; (b) Hughes, C. A. Letcher, R. and Warren, F. L. (1964) *J. Chem. Soc.* 4974; (c) Bottomley W. and Geissman, T. A. (1964) *Phytochemistry* **3**, 357.
5. Schwerdtfeger, E. (1971) *Qual. Plant Mater. Veg.* **20**, 347.
6. Smith, T. A. (1972) *Endeavour* **31**, 22.
7. Lahrer, F. (1974) *Compt. Rend. Acad. Sci. Ser. D.* **279**, 271.
8. Kuttan, R. and Rhadhakrishnan, A. N. (1972) *Biochem. J.* **127**, 61.
9. Yoshida, D. and Mitake, T. (1966) *Plant Cell Physiol.* **7**, 301.
10. Yoshida, D. (1969) *Plant Cell Physiol.* **10**, 393.
11. Watkins, J. E. and Neish, A. C. (1960) *Can. J. Biochem. Physiol.* **38**, 559.
12. Crout, D. H. G., Davies, N. M., Smith, E. H. and Whitehouse, D. (1972) *J. Chem. Soc. Perkin Trans. I*, 671.