Pyrrolizidine Alkaloids. The Biosynthesis of Senecic Acid

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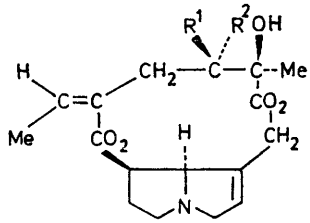
Feeding experiments have shown that two five-carbon units derived from isoleucine are specifically incorporated into senecic acid (IV) in *Senecio magnificus*. The specific incorporation of the methyl carbon atom of methionine into C-3 of serine in pea seedlings has been demonstrated. The significance of this result in relation to necic acid biosynthesis is discussed.

THE pyrrolizidine alkaloids characteristic of *Senecio* species (fam. Compositae) have structures which are variants of a basic type, exemplified by seneciphylline (I) and senecionine (II), in which a ten-carbon substi-

(I) R^1R^2=CH_2
(II) R^1= Me, R^2= H



tuted adipic acid is esterified with a tetrahydro-1-hydroxy-7-hydroxymethylpyrrolizine base. Although the corresponding seneciphyllic (III) and senecic (IV) acids have structures which can be formally derived from isoprene units, feeding experiments with labelled acetate and mevalonate have shown that these acids are not formed by the acetate–mevalonate pathway of terpene biosynthesis.[1,2] On the other hand, isoleucine (V) and its biological precursor threonine (VI) are efficient and specific precursors of seneciphyllic acid (III) in *Senecio douglasii* DC.[1] With uniformly labelled [^{14}C]-L-isoleucine as precursor, the five-carbon component comprising (C-4, -5, -6, -7, and -10) of seneciphyllic acid (III) was most heavily labelled, and it was suggested that isoleucine was incorporated according to Scheme 1(*a*). This conclusion was based on a consideration of the biosynthetic pathway leading to isoleucine, the salient features of which are summarised in Scheme 1. According to this pathway, uniformly labelled [^{14}C]threonine (VI) would be expected to label C-1, C-2, C-4, and C-5 of isoleucine equally. If, therefore, isoleucine were to be incorporated into seneciphyllic acid (III) in the manner shown in Scheme 1(*b*), [*u*-^{14}C]-L-threonine (VI) would be expected to produce equal labelling at C-6, C-7, and C-10 of the necic acid. However, it was observed [1] that the C-6,7 unit of seneciphyllic acid (III) derived from [*u*-^{14}C]-L-threonine contained more than four times as much activity as C-10. Accordingly, the route (*a*) in Scheme 1 was preferred to route (*b*). It was assumed that activity from [*u*-^{14}C]-L-threonine appeared in C-10 of seneciphyllic acid (III) by an indirect pathway.

There is now evidence to suggest that this analysis requires modification, since enzyme systems are known which may operate on uniformly labelled threonine (VI) *in vivo* to introduce an inequality in the labelling of its C-1,2 and C-3,4 components.

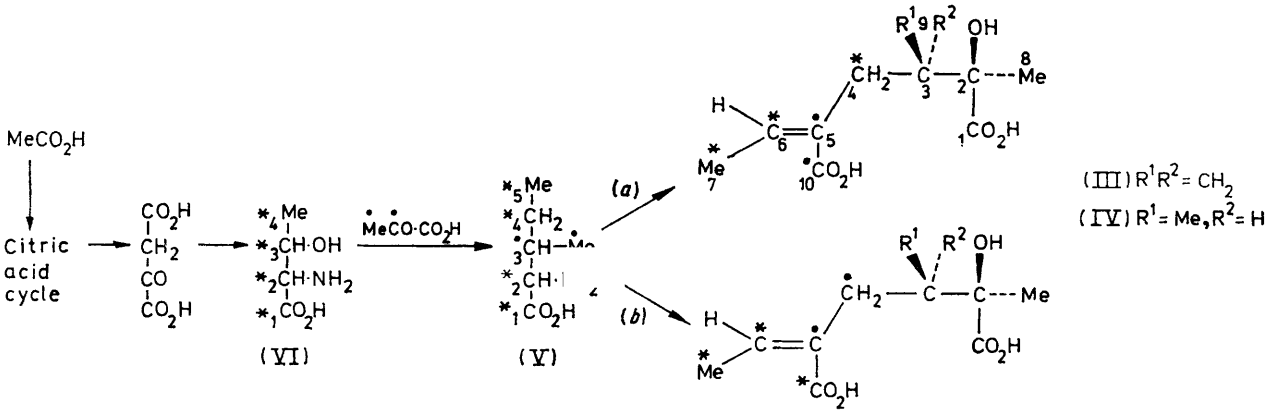
The essential chemical transformations mediated by

[1] D. H. G. Crout, M. H. Benn, H. Imaseki, and T. A. Geissman, *Phytochemistry*, 1966, **5**, 1.

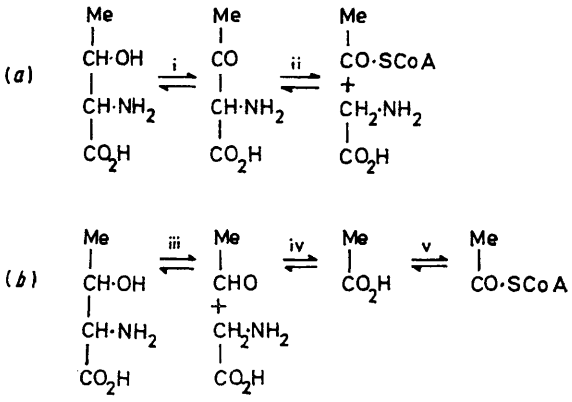
[2] C. G. Gordon-Gray and F. D. Schlosser, *J. South African Chem. Inst.*, 1970, **23**, 13.

the enzyme systems in question are summarised in Scheme 2(*a* and *b*). The operation of threonine dehydrogenase and α-amino-oxobutyrate–CoA ligase (aminoacetone synthetase) [3,4] results in the conversion of threonine into an equimolar mixture of acetyl CoA and glycine [Scheme 2(*a*)]. A similar result is obtained [5] by the sequential action of threonine aldolase, acetaldehyde dehydrogenase, and acetyl CoA synthetase [Scheme 2(*b*)]. If it is assumed that *in vivo* either or both of these systems are reversible to some degree, then the distribution of radioactivity in threonine (VI), initially uniformly labelled, will depend on the extent to which the system moves towards equilibrium and on the relative sizes of the existing pools of acetyl CoA and glycine. Thus the observed distribution of activity in seneciphyllic acid (III) derived from [*u*-^{14}C]-L-threonine might have resulted from dilution of the label in the C-1,2 portion of threonine by equilibration, *via* either of the two enzyme systems, with a pool of glycine which was considerably larger than the pool of acetyl CoA at the site of necic acid biosynthesis.

Threonine-cleaving enzyme systems have been studied almost exclusively in micro-organisms. The occurrence of threonine dehydrogenase in higher plants has not been recorded. However, it has been reported that threonine aldolase is widely distributed in higher plants [6] and recently *Senecio magnificus* F. Muell., the species used in the present studies, has been found to contain low but significant levels of threonine aldolase activity.[7] This observation increased the possibility that isoleucine might be incorporated into necic acids of the seneciphyllic (III) type by route (*b*) (Scheme 1) rather than by route (*a*). Incorporation experiments with specifically labelled isoleucine have been carried out in order to resolve this question.



SCHEME 1



SCHEME 2

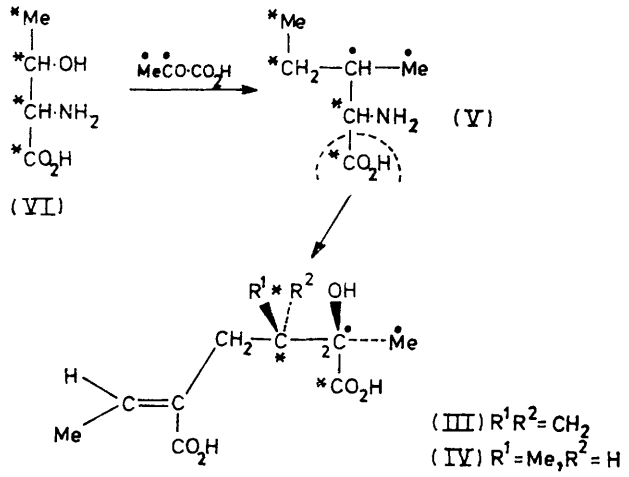
Reagents: i, threonine dehydrogenase; ii, α-amino-oxobutyrate–CoA ligase ('aminoacetone synthetase'); iii, threonine aldolase; iv, acetaldehyde dehydrogenase; v, acetyl CoA synthetase.

These experiments were designed at the same time to shed light on the origin of the five-carbon (C-1, -2, -3, -8, and -9) unit of the necic acids (*cf.* Scheme 1). Although isoleucine was incorporated into this portion of seneciphyllic acid (III) at only half the rate at which it was incorporated into the other component, it was observed that with [*u*-^{14}C]-L-isoleucine as precursor, C-1, C-8, and C-9 were nearly equally labelled.[1] It appeared possible therefore that the C-1, -2, -3, -8, and -9 unit of seneciphyllic acid (III) might also be derived from isoleucine (V) as indicated in Scheme 3.

There was an objection to this proposal in that C-2 of seneciphyllic acid (III) appeared to be labelled when [*u*-^{14}C]-L-threonine was administered to *Senecio douglasii*,[1] whereas this position should not be labelled if threonine (VI) is incorporated *via* isoleucine (V) (Scheme 3). However, the activity of C-2 was not measured directly but was obtained by difference from a number of independent activity determinations, a procedure recognised

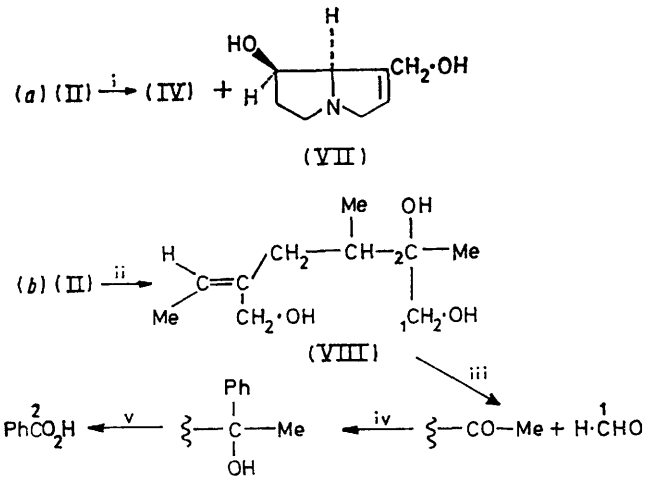
[3] W. G. Laver, A. Neuberger, and J. J. Scott, *J. Chem. Soc.*, 1959, 1483.
[4] (*a*) W. H. Elliot, *Biochim. Biophys. Acta*, 1958, **29**, 446; (*b*) A. Neuberger and G. H. Tait, *Biochem. J.*, 1962, **84**, 317; (*c*) G. Urata and S. Granick, *J. Biol. Chem.*, 1963, **238**, 811.
[5] J. G. Morris, *Biochem. J.*, 1969, **115**, 603.
[6] Tsung-ya Lo and Yu-Wei Tang, *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao*, 1964, **4**, 527 (*Chem. Abs.*, 1965, **62**, 15,004).
[7] Dr. J. M. Turner, personal communication.

as leading to an unreliable result.[1] This uncertainty has been resolved by administering [*u*-^{14}C]-L-threonine to *Senecio magnificus*, which produces senecionine (II) as



SCHEME 3

the major alkaloid.[8] The labelled senecionine was hydrolysed to senecic acid (IV) and retronecine (VII) [Scheme 4(*a*)]. As in the experiments with *S. douglasii*,[1]



SCHEME 4

Reagents: i, $Ba(OH)_2$; ii, $LiAlH_4$; iii, HIO_4; iv, PhMgBr; v, $KMnO_4$.

[*u*-^{14}C]-L-threonine was found to be efficiently and selectively incorporated into the necic acid component of the alkaloid (see Table 1). The C-2 atom of the necic acid was isolated as the carboxy-group of benzoic acid [see Scheme 4(*b*)], and was essentially inactive (see Table 2, experiment 1).

The labelled necic acid was further degraded by methods illustrated in Scheme 5. As in the corresponding experiment with seneciphyllic acid,[1] C-8 was essentially unlabelled, whereas C-1 was strongly labelled. On the other hand, the ratio of the activities of the C-6,7 unit and C-10 was relatively close to the expected value of 2 as compared with the value of 4·3 observed in the case of seneciphyllic acid (see Table 2).

With the removal of this objection to the proposition that the C-1, -2, -3, -8, and -9 unit of the necic acids is derived from isoleucine, the incorporation of [*u*-^{14}C]-L-isoleucine into senecionine (II) was examined. As expected, this precursor was incorporated efficiently and selectively into the necic acid component (Table 1, experiments 2 and 3). Degradation of the senecionine (Scheme 5) showed that C-1 and C-8 were nearly equally labelled, in confirmation of the result obtained with

TABLE 1

Incorporation of ^{14}C-labelled precursors into senecionine and distribution of the label between senecic acid and retronecine

Expt.	Precursor	% Incorporation *	% Activity in senecic acid (IV)	% Activity in retronecine (VII)
1	[*u*-^{14}C]-L-Threonine	0·25	98·5 ± 3·9	0·6 ± 0·06
2	[*u*-^{14}C]-L-Isoleucine	0·44	99·0 ± 3·6	0·98 ± 0·05
3	[*u*-^{14}C]-L-Isoleucine	0·48	96·4 ± 3·9	0·80 ± 0·08
4	[2-^{14}C]Isoleucine	0·27	95·2 ± 3·8	0·0
5	[2-^{14}C]Isoleucine	0·44	100 ± 3·8	1·6 ± 0·3
6	[5-^{14}C]Isoleucine	0·42	96·6 ± 3·5	2·0 ± 0·1
7	[6-^{14}C]Isoleucine	0·15	99·4 ± 4·0	3·9 ± 0·8
8	[*Me*-^{14}C]-L-Methionine	0·00015		

* Where mixtures of DL-isoleucine and DL-alloisoleucine were fed, incorporations were calculated on the assumption, now known to be valid (D. H. G. Crout and N. M. Davies, unpublished observations), that only L-isoleucine is an effective precursor of senecionine.

seneciphyllic acid in *S. douglasii*. [The lack of functionality at C-9 in senecic acid (IV) precluded direct determination of the activity at this position.]

However, whereas in the experiments with *S. douglasii* the labelling pattern indicated that the C-4, -5, -6, -7, and -10 unit of seneciphyllic acid (III) contained two-thirds of the total activity and the remaining C_5 unit one-third, in the experiments with *S. magnificus* the labelling pattern indicated that the activity was evenly distributed throughout the necic acid (Table 2, experiments 2 and 3).

We next carried out a series of incorporation experiments with specifically labelled isoleucine in order to determine the manner in which isoleucine is incorporated into the C-4, -5, -6, -7, and -10 component of senecic acid (IV) as discussed above, and to test the proposition that isoleucine is a specific precursor of the C-1, -2, -3, -8, and -9 component. [2-^{14}C]-, [5-^{14}C]-, and [6-^{14}C]-Isoleucine were synthesised, as described later, and administered to *S. magnificus* plants hydroponically. The labelled alkaloid was degraded by the methods shown in Scheme 5. (In order to optimise yields, more than one route to certain degradation products was developed. The choice of method for a particular degradation depended on the location of the label in the precursor.)

Each precursor was efficiently and selectively incorporated into the necic acid component of the alkaloid

[8] C. C. J. Culvenor, *Austral. J. Chem.*, 1962, **15**, 158.

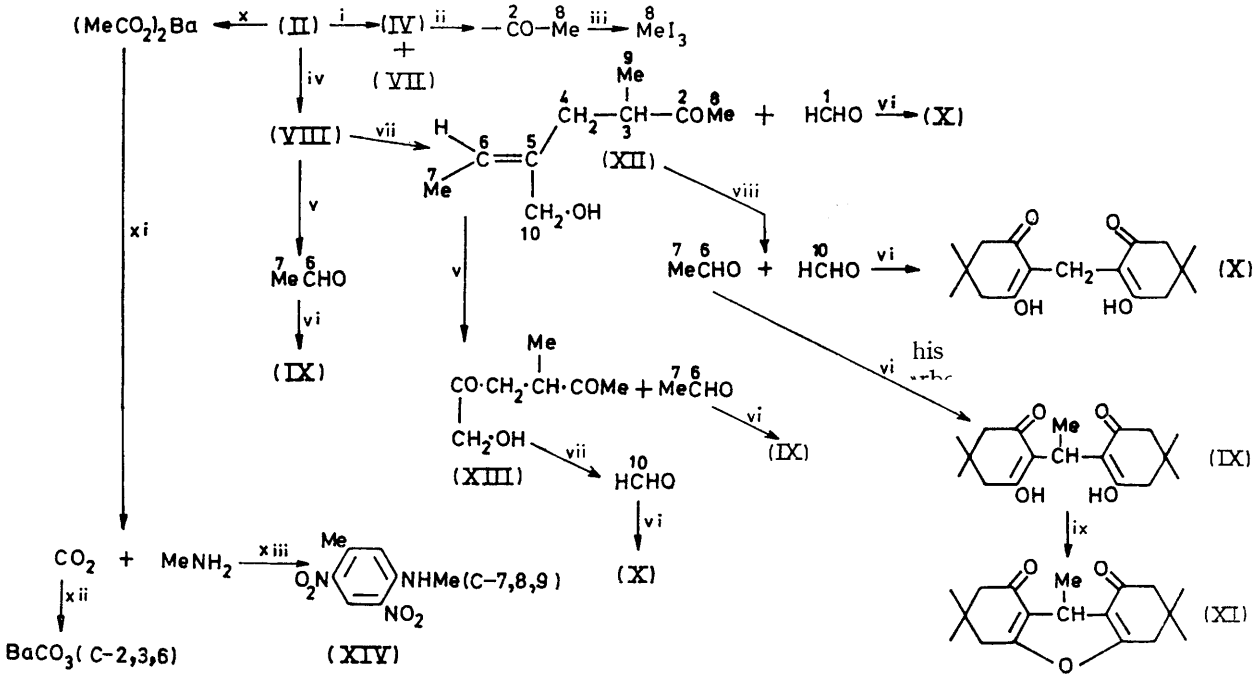
(Table 1, experiments 4—7). Moreover, [2-^{14}C]isoleucine labelled C-1 and C-10 of senecic acid (IV) equally and exclusively and the label from [5-^{14}C]isoleucine was located at C-9 and C-7, also equally and exclusively (Table 2, experiments 4—6). [6-^{14}C]Isoleucine gave senecic acid in which 58% of the label was at C-8; the as the activity of C-7. The activity of C-9 was obtained by subtracting the activity of the C-6,7 unit from the (statistically corrected) activity of the barium acetate (C-2, -3, -6, -7, -8, and -9) obtained by Kuhn–Roth oxidation of senecionine (II) (Scheme 5). The ratios of the activities of C-9 and C-1, and of C-7 and C-10 were

TABLE 2

Distribution of activity in senecic acid (IV) derived from ^{14}C-labelled precursors

Expt.	Precursor	C-1	C-2	C-6,7	C-7	C-8	C-9	C-10	C-2,3,6, 7,8,9	C-2,3,6	C-7,8,9
1	[*u*-^{14}C]-L-Threonine	15·4 ± 0·6 16·3 ± 0·6	0·59 ± 0·10	18·8 ± 0·7		0·10 ± 0·01		12·1 ± 0·5			
2	[*u*-^{14}C]-L-Isoleucine	9·8 ± 0·4				8·5 ± 0·4					
3	[*u*-^{14}C]-L-Isoleucine	11·6 ± 0·5		18·3 ± 0·6				10·0 ± 0·4			
4	[2-^{14}C]Isoleucine	54·5 ± 2·4						43·6 ± 2·2			
5	[2-^{14}C]Isoleucine	50·1 ± 2·0		2·95 ± 0·90		0·35 ± 0·24		49·0 ± 2·0			
6	[5-^{14}C]Isoleucine			49·7 ± 1·8	49·4 ± 1·8 *	0·51 ± 0·17	48·1 ± 3·9 †		98·0 ± 3·5	0·94 ± 0·09	98·0 ± 3·5
7	[6-^{14}C]Isoleucine					57·5 ± 3·3					

* Calculated on the assumption that the activity of the $BaCO_3$ (C-2,3,6) is equally distributed between C-2, C-3, and C-6. † By difference {[C-7,8,9] − [(C-7) + (C-8)]}.



SCHEME 5

Reagents: i, $Ba(OH)_2$; ii, $NaIO_4$; iii, NaIO; iv, $LiAlH_4$; v, O_3; vi, dimedone; vii, HIO_4; viii, OsO_4–$NaIO_4$; ix, HOAc; x, Kuhn–Roth oxidation; xi, HN_3; xii, $Ba(OH)_2$; xiii, 2,4,5-trinitrotoluene.

remainder was presumably at C-4. (The activity at this position could not be determined directly.)

The direct incorporation of C-5 units derived from isoleucine without rearrangement or degradation was demonstrated by feeding isoleucine doubly labelled with ^{14}C at the 2- and 5-positions. The labelled senecionine (II) was degraded by methods given in Scheme 5. Since the activity of senecic acid (IV) derived from [5-^{14}C]-isoleucine was almost entirely at C-7 and C-9 (Table 2, experiment 6), the activity of the C-6,7 unit was taken the same, within experimental error, as the corresponding ratio in the administered doubly-labelled isoleucine (Table 3).

These experiments demonstrate conclusively that two C-5 units derived from isoleucine are incorporated into senecic acid (IV) in the manner indicated in Scheme 6. This pathway involves, at some stage, the loss of the carboxy-carbon atom from both participating isoleucine molecules, a result that had been suggested by earlier feeding experiments in *S. douglasii*.[1]

The conclusion that isoleucine can serve as a specific precursor of senecic acid introduced an apparent anomaly in that in experiments with *S. douglasii*, the label from [*Me*-^{14}C]-L-methionine was found to be incorporated

TABLE 3

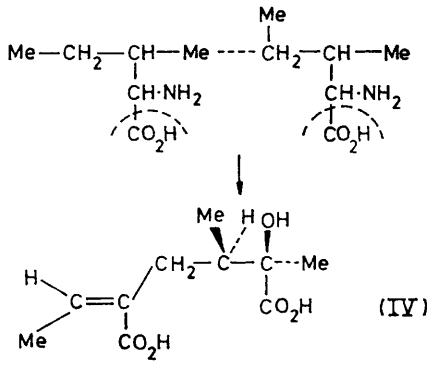
Percentage activities of degradation products of senecionine (II) derived from [2,5-$^{14}C_2$]isoleucine (V)

$$\left(\frac{\text{Activity of C-5}}{\text{Activity of C-2}} = 2{\cdot}52 \pm 0{\cdot}10\right)$$

Carbon atoms	Percentage activity
C-1	15·5 ± 0·6
C-10	14·0 ± 0·6
C-6,7	32·0 ± 1·2
C-2,3,6,7,8,9	70·8 ± 2·7
Ratio C-7/C-10	2·29 ± 0·13
Ratio C-9/C-1	2·50 ± 0·22

preferentially (73—83%) into the necic acid component of seneciphylline (I). In addition, 22—26% of the activity of the seneciphyllic acid (III) was found to be at C-8.[1]

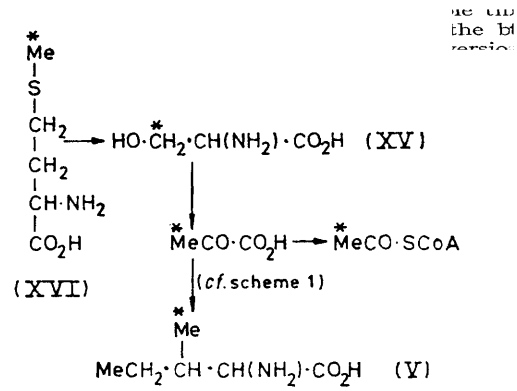
It therefore appears that a pathway exists in higher plants whereby the methyl group of methionine can



SCHEME 6

enter metabolic pathways which lead to isoleucine as an intermediary product. The C-8 atom of the necic acids (III) and (IV) is derived from C-6 of isoleucine (V), which in turn is derived from the methyl carbon atom of pyruvate (*cf.* Scheme 1). It follows that the methyl group of methionine can be converted in *Senecio* species into the methyl group of pyruvate.

Since it is well known that serine (XV) can be incorporated *via* pyruvate and acetyl CoA into metabolites (*e.g.* chloroplastidic terpenoids) directly derived from acetate,[9,10] it appeared possible that the methyl group of methionine (XVI) enters the biosynthetic pathway leading to isoleucine by conversion into the hydroxymethyl group of serine (XV) (see Scheme 7).* Such a conversion would constitute a reversal, or an effective reversal, of the tetrahydrofolate-mediated pathway of methionine biosynthesis. Splittstoesser and Mazelis[12] have shown that radioactivity from [*Me*-^{14}C]-L-methionine is effectively incorporated into serine in a number of higher plants. However, the distribution of radioactivity in the labelled serine was not determined. We have confirmed the observation that, in pea seedlings, the label from [Me-^{14}C]-L-methionine is incorporated



SCHEME 7

(0·14%) into serine. Degradation of the rigorously purified serine showed that the radioactivity was entirely located in the hydroxymethyl group (see Scheme 8).

SCHEME 8

Reagents: i, $NaIO_4$; ii, dimedone.

A similar selective incorporation of methionine methyl carbon atom into serine has been demonstrated in the rat.[13] These results lend support to the suggestion made above concerning the route whereby the methyl carbon atom of methionine is incorporated into seneciphyllic acid.

By comparison with the results obtained with *S. douglasii*,[1] methionine was found to be a very poor precursor of senecionine in *S. magnificus* (see Table 1). It therefore appears that there are large interspecies differences in the degree to which methionine participates in metabolic pathways having serine as an intermediate.

If it is assumed that acetate enters isoleucine and the necic acids by the pathway outlined in Scheme 1, the labelling pattern in necic acids derived from [^{14}C]acetate can be predicted. Although the incorporation of [1-^{14}C]acetate into seneciphyllic acid accords with that predicted,[1] the pattern of incorporation of activity from [2-^{14}C]acetate into seneciphyllic, senecic, and retronecic[1,2] acids shows that considerable randomisation of acetate

* For further examples of incorporation studies which reflect the probable operation of this pathway, see refs. 9 and 11, J. P. Kutney, J. F. Beck, V. R. Nelson, K. L. Stuart, and A. K. Bose, *J. Amer. Chem. Soc.*, 1970, **92**, 2174, and D. Groger, W. Maier, and P. Simchen, *Experientia*, 1970, **26**, 820.

[9] L. J. Rogers, S. P. J. Shah, and T. W. Goodwin, *Photosynthetica*, 1968, **2**, 184.

[10] S. P. J. Shah, L. J. Rogers, and T. W. Goodwin, *Biochem. J.*, 1967, **103**, 52P; 1967, **105**, 13P; 1968, **108**, 17P.

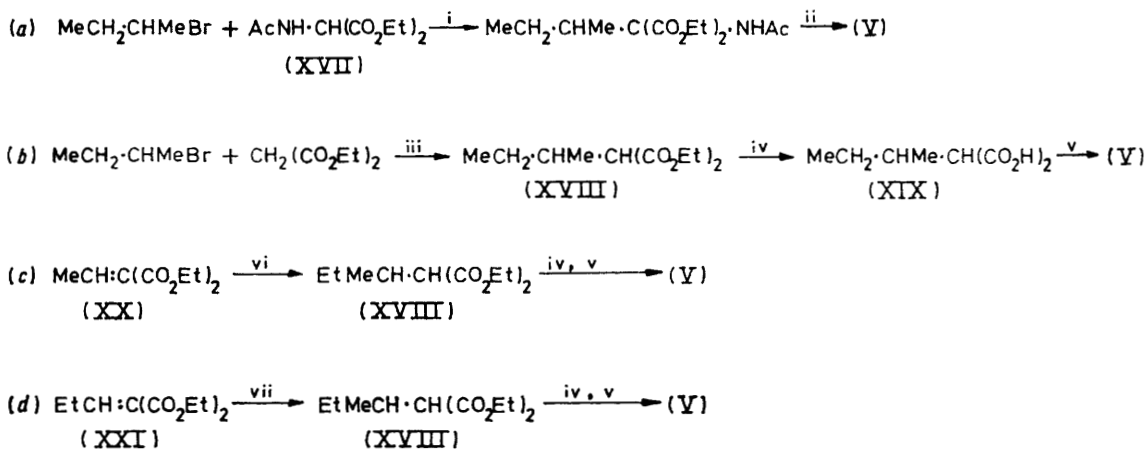
[11] S. P. J. Shah and L. J. Rogers, *Biochem. J.*, 1969, **114**, 395.

[12] W. E. Splittstoesser and M. Mazelis, *Phytochemistry*, 1967, **6**, 39.

[13] R. L. Kisliuk, W. Sakami, and M. V. Patwardhan, *J. Biol. Chem.*, 1956, **221**, 885.

methyl carbon atom takes place. The main cause of randomisation is probably the obligatory passage of acetate through the citric acid cycle (Scheme 1) and the involvement of acetate in associated anabolic and anaplerotic pathways. Compartmentation of intermediary metabolites, for which there is now compelling evidence,[9,14] is also probably involved.

Equilibration of [2-^{14}C]acetate in the citric acid cycle leads to distribution of activity over all four carbon atoms of the C_4 intermediates, with the terminal positions each carrying one sixth of the activity and the central atoms one-third. The label from [1-^{14}C]acetate, however, is distributed only in the terminal positions of the C_4 intermediates and is completely eliminated, as CO_2, after two turns of the cycle. The opportunity for randomisation of acetate carboxy-carbon atom is therefore much less than for the methyl carbon atom. These considerations afford a possible explanation for the difference in the specificity of the incorporation of the acetate carboxy- and acetate methyl carbon atom into senecic acid and related metabolites. The specific and random incorporations of [1-^{14}C]- and [2-^{14}C]-acetate, respectively, have recently been observed in the course of investigations into the biosynthesis of coniine.[15]



SCHEME 9

Reagents: i, KOBu^t–dimethyl sulphoxide; ii, HCl, iii, NaOEt; iv, KOH; v, HN_3; vi, EtMgI; vii, MeMgI.

Synthesis of Specifically Labelled Isoleucine.—[2-^{14}C]-Isoleucine was prepared by the two routes (*a*) and (*b*) illustrated in Scheme 9. Condensation of diethyl acetamidomalonate (XVII) with 2-bromobutane in the presence of base gave only poor yields of isoleucine (V); the product invariably contained a large excess of glycine. In ethanol with sodium ethoxide, yields of isoleucine in the range 1—6% were obtained. The synthesis was improved to give a yield of 9% with potassium t-butoxide in dimethyl sulphoxide. A radiochemical synthesis under the latter conditions gave a 5% radiochemical yield of [2-^{14}C]isoleucine from diethyl acetamido[2-^{14}C]malonate. The product was shown by amino-acid analysis to consist of DL-isoleucine (60 ± 3%) and DL-alloisoleucine (40 ± 3%). This material was used for feeding in experiment 4 (see Tables 1 and 2).

Method (*b*) (Scheme 9), although longer than method (*a*) gave a much improved yield of isoleucine (22%). In the radiochemical synthesis, a 17·5% radiochemical yield of [2-^{14}C]isoleucine was obtained from diethyl [2-^{14}C]malonate. This material consisted (amino-acid analysis), of equal amounts, within experimental error, of DL-isoleucine and DL-alloisoleucine. The final Schmidt reaction [Scheme 9(*b*)], which differed from the published procedure [16] in that it was carried out under homogeneous conditions, therefore proceeded without an appreciable degree of asymmetric induction. The [2-^{14}C]isoleucine obtained by this method was used in experiment 5 (Tables 1 and 2).

[5-^{14}C]- and [6-^{14}C]-Isoleucine were prepared by conjugate addition of [2-^{14}C]ethylmagnesium iodide and [^{14}C]methylmagnesium iodide respectively to the appropriate diethyl alkylidenemalonate [Scheme 9(*c*) and (*d*)], followed by hydrolysis and Schmidt degradation of the resulting substituted malonic acid. A 30—33% chemical yield of isoleucine was obtained by both methods. The radiochemical yield of [6-^{14}C]isoleucine was lower as the labelled methyl iodide was used in excess.*

EXPERIMENTAL

All m.p.s are corrected. Radioactivity was measured in a Packard 2000 series Tri Carb liquid scintillation spectrometer. Samples were counted in dioxan-based scintillation solutions NE 220 and NE 250 (Nuclear Enterprises Ltd.) and in B.D.H. dioxan scintillation solution. Coloured samples

* A five-step procedure designed to allow for the preparation of isoleucine labelled in the 6-position has been described but does not appear to have been put into effect (G. B. Ceresia, G. L. Jenkins, and E. F. Degering, *J. Amer. Pharm. Assoc.*, 1951, **40**, 341).

[14] A. Oaks and R. G. S. Bidwell, *Ann. Rev. Plant Physiol.*, 1970, **21**, 43.
[15] E. Leete, *J. Amer. Chem. Soc.*, 1970, **92**, 3835.
[16] S. Takagi and K. Hayashi, *Chem. and Pharm. Bull.* (*Japan*), 1959, **7**, 96, 183.

were oxidised to CO_2 by the Van Slyke method.[17] The CO_2 was absorbed in a solution of 2-aminoethanol in 2-methoxyethanol (1 : 11 v/v; 6 cm³). For counting, a 5 cm³ aliquot portion was added to a solution (7 cm³) of 2,5-diphenyloxazole (PPO) (8·25 g) in scintillation-grade toluene (1 dm³). Barium acetate and amino-acids were also counted in the latter system. Alternatively, amino-acids were dissolved in hyamine hydroxide (1 mol dm^{-3}) in methanol (1 cm³) and the resulting solution was added to toluene–PPO scintillator (11 cm³). Radioactive samples were normally recrystallised to constant activity and counted in duplicate. In a few cases, where only limited amounts of material were available, samples were rigorously purified by methods which were known to give radiochemically pure material and were then counted in duplicate. For radioautography, Kodirex *X*-ray film was used. Alternatively, paper chromatograms were scanned by cutting the paper into narrow strips, moistening each strip with NE 220 scintillator (1 cm³) and counting the strips in the scintillation counter. For paper chromatography of the amino-acids the systems butanol–acetic acid–water (37 : 9 : 25) and butanol–pyridine–water (1 : 1 : 1) were used. For preparative t.l.c. of amino-acids, Kieselgel PF_{254} (Merck) was used. Electrophoresis was carried out at 100 V cm^{-1} for 1 h at pH 1·9 in acetic acid–formic acid–water (6 : 2 : 40). For the preparation of 5,5-dimethylcyclohexane-1,3-dione (dimedone) derivatives of aldehydes, a 0·4% solution of dimedone was used. All radiochemicals were purchased from the Radiochemical Centre, Amersham, Bucks. The quoted errors in percentage activities are overall standard errors computed from the calculated statistical errors and weighing errors in the usual way.

Feeding Methods.—*Senecio magnificus* plants were grown from seed in a standard compost. Immediately before the feeding experiments, 3—6 plants, (2—6 months old) were removed from the compost and their roots were washed first with tap water and then with deionised water. The plants were placed in nutrient solution [Phostrogen (Phostrogen Ltd.) (0·6 g dm^{-3})] and the solutions were continuously aerated for the duration of the experiment. Radioactive precursors, dissolved in deionised water, were added directly to the nutrient solution. After 8—10 days, the plants were removed from the solution and their roots were washed with deionised water. The plants were repeatedly macerated with methanol in a Waring blendor until the filtered methanolic extract was colourless. In a typical work-up, the combined methanolic extracts were evaporated, the residue was taken up in H_2SO_4 (1 mol dm^{-3}, 30 cm³), and the acidic solution was washed with chloroform (6 × 30 cm³). The acidic solution was stirred with zinc dust for 90 min (to reduce *N*-oxides) and filtered. The filtrate was washed with chloroform (3 × 30 cm³), made strongly alkaline with conc. ammonia, and extracted with chloroform (6 × 30 cm³). The combined extracts were dried (Na_2SO_4) and evaporated to give the crude alkaloid. This was purified by dissolving it in H_2SO_4 (1 mol dm^{-3}, 30 cm³), washing the acidic solution with chloroform (4 × 30 cm³), making the residual solution strongly basic with conc. ammonia, and again extracting with chloroform (6 × 30 cm³). The extracts were dried (Na_2SO_4) and evaporated to give substantially pure senecionine (II) (0·2—0·5% based on the dry weight of the plant material). The labelled senecionine was diluted with inactive senecionine (200 mg) and applied to a column of acid-washed, activated alumina (12 g). Senecionine was eluted with chloroform and recrystallised (chloroform–methanol) to constant activity. Uptake of radioactivity by the plants was usually greater than 95% after 8—10 days. In the experiment with doubly labelled isoleucine (Table 3), in which the precursor had a relatively low specific activity, the uptake was 87%.

Hydrolysis of Senecionine.—In a typical experiment, senecionine (II) (200 mg, 0·6 mmol) in water (4 cm³) was boiled under reflux with barium hydroxide octahydrate (190 mg, 0·6 mmol) for 2·5 h. The solution was allowed to cool and was treated with solid CO_2. The resulting slurry was filtered; the filtrate was acidified (Congo Red) with dilute HCl and extracted continuously with ether for 48 h. The ether extract was dried (Na_2SO_4) and evaporated. The residue was recrystallised [ethyl acetate–light petroleum (b.p. 60—80°)] to give senecic acid (IV) (78 mg), m.p. 142—144°. The residual acidic solution was passed through a column of Dowex 1-X8 ion-exchange resin (OH^-, 10 g) and the eluate was collected until it was no longer alkaline (litmus) and evaporated to dryness. The residue was extracted thrice with boiling acetone. The extracts were filtered and evaporated. The residue was neutralised with dil. HCl, filtered to remove polypyrrolic material, and evaporated. The residue in methanol (3 cm³) was boiled briefly with activated charcoal and the solution was filtered and evaporated. The residue was crystallised (acetone) to give retronecine (VII) hydrochloride (41 mg), m.p. 164°.

Degradation for C-1 *of Senecic Acid* (IV).—Senecionine (II) (250 mg) in tetrahydrofuran (150 cm³) was heated under reflux with lithium aluminium hydride (800 mg) for 14 h. Water (50 cm³) was cautiously added to the mixture and the tetrahydrofuran was removed under reduced pressure. The residual slurry was filtered (Kieselguhr) and the filtrate was passed through a column of Dowex 50W-X8 ion-exchange resin (H^+; 20 g). The eluate was evaporated to give 5-hydroxymethyl-2,3-dimethylhept-5-ene-1,2-diol (VIII) as an oil (130 mg). This was dissolved in 0·05M-periodic acid (50 cm³) and left in the dark for 2 h. The solution was passed through a column of Dowex 1-X8 ion-exchange resin (HCO_3^-; 10 g) and the eluate was added to dimedone solution (100 cm³). After 24 h, the derivative (X) (120 mg) of formaldehyde was filtered off and purified by preparative t.l.c. [Kieselgel GF_{254}; benzene–methanol (9 : 1)]. The derivative was extracted with chloroform and recrystallised (ethanol–water) to give needles, m.p. 193°.

Degradation for C-8 *of Senecic Acid* (IV).—Senecic acid (IV) (108 mg, 0·5 mmol) in water (15 cm³) was treated with sodium periodate (300 mg, 1·4 mmol) and the solution was set aside in the dark for 48 h. Sulphur dioxide was passed into the solution until the iodine colour was just discharged. NaOH (1 mol dm^{-3}, 20 cm³) was added, followed by iodine–potassium iodide [I_2 (5 g) and potassium iodide (10 g) in water (50 cm³)] dropwise until the yellow colour persisted for >1 min. The mixture was kept overnight; the precipitate was filtered off and recrystallised (methanol–water) to give iodoform (110 mg), m.p. 119°.

Degradation for C-6,7 *and* C-10 *of Senecic Acid* (IV).—The filtrate after removal of the dimedone derivative of formaldehyde obtained by the periodate oxidation of the triol (VIII) (180 mg) as before, was steam-distilled until the distillate no longer gave any turbidity when tested with 2,4-dinitrophenylhydrazine (12·6 mmol dm^{-3}) in H_2SO_4 (1 mol dm^{-3}). The distillate was continuously extracted with

[17] D. D. Van Slyke, J. Plazin, and J. R. Weisiger, *J. Biol. Chem.*, 1951, **191**, 299; D. D. Van Slyke, R. Steele, and J. Plazin, *ibid.*, 1951, **192**, 769.

ether for 24 h; the extract was dried ($MgSO_4$) and evaporated to give 5-hydroxymethyl-3-methylhept-5-en-2-one (XII) (88 mg, 0·56 mmol) as an oil. This was stirred with ether–water (1 : 1 v/v; 30 cm^3). Sodium periodate (250 mg, 1·17 mmol) was added, followed by osmic acid solution (4 cm^3; osmium tetroxide 4 mg cm^{-3}) and stirring was continued for 4 h. The ether was removed under reduced pressure and the aqueous residue was passed through a column of Dowex 1-X8 ion-exchange resin (HCO_3^-; 10 g). The eluate and washings (45 cm^3) were treated with acetate buffer (pH 4·6, 20 cm^3) followed by dimedone solution (35 cm^3). After 3 days the precipitate was filtered off to give the mixture of dimedone derivatives of acetaldehyde and formaldehyde (121 mg). The mixture in acetic acid (2 cm^3) was heated on a steam-bath under reflux for 6 h. The product was poured into water (12 cm^3), left overnight, and filtered. The solid was washed with water (5 cm^3) and NaOH (1 mol dm^{-3}, 10 cm^3) in small portions. The insoluble residue was washed with water and dried to give the anhydro-derivative of acetaldehyde dimedone (21 mg). The alkaline washings were neutralised with acetic acid, treated with acetate buffer (pH 4·6, 25 cm^3), and left overnight, to give the dimedone derivative of formaldehyde (79 mg). Both derivatives were purified by preparative t.l.c. as described before to give the anhydro-derivative of acetaldehyde (XI) (11 mg), m.p. 177—178° (ethanol–water), and the dimedone derivative of formaldehye (X) (53 mg), m.p. 193—194° (ethanol–water).

Alternative Degradation of 5-Hydroxymethyl-3-methylhept-5-en-2-one (XII).—The filtrate from the removal of the dimedone–formaldehyde derivative (*cf.* preceding section) was continuously extracted with ether for 18 h. The extract was dried ($MgSO_4$) and evaporated, and the residue was triturated with acetone and left at 3° for 1·5 h. The mixture was filtered and the filtrate was evaporated to give the ketol (XII) (182 mg from 497 mg senecionine). The ketol was ozonised in 50% aqueous acetic acid until no more ozone was consumed. The solution was stirred with zinc dust for 0·5 h and steam distilled until the distillate no longer gave a positive test with 2,4-dinitrophenylhydrazine reagent. The distillate was brought to pH 4·5 by the addition of sodium hydrogen carbonate and was treated with dimedone solution (50 cm^3). After 2 days, the solution was filtered to give the dimedone derivative of acetaldehyde (16 mg), m.p. 145—147° (homogeneous by t.l.c. [benzene–methanol (95 : 5)]. The derivative was diluted with inactive material (32 mg) and purified by preparative t.l.c. [Kieselgel GF_{254}, benzene–methanol (95 : 5)] to give acetaldehyde dimedone (IX) (32 mg), m.p. 142—143°. The residue from the steam distillation was filtered and extracted with ether for 17 h. The extract was dried ($MgSO_4$) and evaporated to leave an oil (165 mg) containing 1-hydroxy-4-methylhexane-2,5-dione (XIII), which was oxidised with periodic acid (0·05 mol dm^{-3}; 50 cm^3) as before to give, after purification by preparative t.l.c. and recrystallisation as before, the dimedone derivative of formaldehyde (24 mg), m.p. 192—193°.

Degradation for C-2 of Senecic Acid.—Magnesium turnings (70 mg) were stirred with bromobenzene (370 mg) in ether (2 cm^3) until the Grignard reagent was formed. The ketol (XII) (80 mg) [obtained by steam distillation of the product from the periodate oxidation of the triol (VIII), as described before] in ether (30 cm^3) was added to the solution of the Grignard reagent and the solution was stirred under nitrogen for 2 days. Hydrochloric acid (5%; 30 cm^3) was added and the mixture was extracted with ether (5 × 50 cm^3). The extracts were dried ($MgSO_4$) and evaporated. The residual oil was dissolved in ether (30 cm^3) and the solution was washed with sodium hydroxide solution (0·25 mol dm^{-3}; 4 × 10 cm^3) and water (2 × 10 cm^3). The ethereal solution was dried ($MgSO_4$) and evaporated to give an oil (120 mg). This was boiled under reflux with potassium permanganate (600 mg) in water (60 cm^3) for 4 h. The solution was cooled and ethanol (15 cm^3) was added to decompose the excess of permanganate. The mixture was filtered (Kieselguhr) and the filtrate was acidified (dil. HCl) and extracted with ether (6 × 60 cm^3). The extracts were dried ($MgSO_4$) and evaporated to give a white solid. This was dissolved in ether (25 cm^3) and the solution was extracted with sodium hydroxide solution (1 mol dm^{-3}; 4 × 2 cm^3). The alkaline extracts were acidified (conc. HCl) and extracted with ether (6 × 60 cm^3). The ethereal extracts were dried ($MgSO_4$) and evaporated, and the residual solid was purified by preparative t.l.c. [Kieselgel GF_{254}; benzene–methanol–acetic acid (45 : 8 : 4)] to give benzoic acid (16 mg), m.p. 120°.

Ozonolysis of 5-Hydroxymethyl-2,3-dimethylhept-5-ene-1,2-diol (VIII).—The triol (193 mg), obtained as before, was ozonised and the product acetaldehyde was similarly isolated as the dimedone derivative. The product was purified by preparative t.l.c. and recrystallisation to give the derivative (IX) (61 mg), m.p. 141—142°.

Kuhn–Roth Oxidation of Senecionine.—Senecionine (II) was oxidised by the standard procedure.[18] The acetic acid produced (2·6—2·8 mol. equiv.) was titrated with barium hydroxide. The solution of the barium salt was evaporated to dryness and the residue was recrystallised (water–ethanol) to give barium acetate monohydrate as silky needles (36—38% yield).

Schmidt Degradation of Barium Acetate.—This was carried out as previously described.[1] Methylamine was isolated as 5-methylamino-2,4-dinitrotoluene (XIV). The derivative was purified by preparative t.l.c. [Kieselgel GF_{254}; benzene–methanol (95 : 5)] and crystallisation (ethanol). From barium acetate (30 mg) there was obtained the derivative (XIV) (15 mg), m.p. 170—172°. The evolved CO_2 was collected as barium carbonate.

Feeding Experiments with Pea Seedlings.—The roots were removed from 24 ten-day-old pea seedlings and the seedlings were placed in tubes each containing [*Me*-^{14}C]-L-methionine (1 cm^3; 0·00416 mCi). After 24 h the seedlings were washed with deionised water and the amino-acids were extracted by maceration in a Waring blendor with 75% aqueous ethanol (94% of the administered radioactivity was absorbed by the seedlings). The extraction procedure was repeated twice. The filtered extracts were applied to a column of Dowex 50W-X8 ion-exchange resin (H^+; 15 g), the column was washed with deionised water and then with ammonia solution (1·5 mol dm^{-3}) to elute the amino-acids. The ammoniacal solution was evaporated to dryness under reduced pressure; the residue was dissolved in water and applied to a column of Amberlite CG-4B ion-exchange resin (OH^-; 15 g). The neutral and basic amino-acids were eluted with water and the acidic amino-acids were eluted with dilute HCl (1 mol dm^{-3}).[19] The eluate containing the neutral and basic amino-acids was concentrated and

[18] F. Wild, 'Estimation of Organic Compounds,' Cambridge University Press, Cambridge, 1953, p. 220.

[19] R. Consden, A. H. Gordon, and A. J. P. Martin, *Biochem. J.*, 1948, **42**, 443.

chromatographed on a column of Amberlite CG-120 ion-exchange resin (140 cm).[20] The amino-acids were eluted with pyridine–formic acid buffer (pH 3·1); the eluate was collected in 10 cm³ fractions. Serine (9 mg) was eluted in fractions 54—75 together with two other amino-acids, one of which was indicated by amino-acid analysis to be homoserine.[21] The amino-acids were isolated by lyophilisation; the mixture was diluted with inactive serine (540 mg) and recrystallised to constant activity.

Periodate Oxidation of Serine (XV).—Serine (XV) (76 mg) in water (57 cm³) containing sodium periodate (620 mg) was set aside for 17 h in the dark. Sodium arsenite was added, followed by dimedone solution (77 cm³). The precipitate was recrystallised (ethanol–water) to give the dimedone derivative of formaldehyde (172 mg) which was recrystallised to constant activity.

Synthesis of Specifically Labelled Isoleucine.—The radiochemical syntheses were carried out without purification of intermediates, following trial experiments in which intermediates were satisfactorily characterised by spectroscopic methods.

Synthesis of [2-^{14}C]*Isoleucine.*—(*a*). Diethyl acetamido-[2-^{14}C]malonate (XVII) (0·2 mCi; 6·16 mCi mmol^{-1}) in dimethyl sulphoxide (5 cm³) was added to a solution of inactive diethyl acetamidomalonate (0·5 g) in dimethyl sulphoxide (15 cm³). The resulting solution was added to potassium t-butoxide prepared from potassium (0·25 g) and t-butyl alcohol. 2-Bromobutane (1·5 g) was added and the mixture was heated at 60° for 14 h. Water (80 cm³) was added and the solution was extracted with ether (5 × 50 cm³). The extracts were dried (Na_2SO_4) and evaporated. The residue was boiled with conc. HCl (20 cm³) for 4 h and the solution was evaporated to dryness. The residue was dissolved in water (20 cm³) and again evaporated to dryness. The residue was dissolved in water (10 cm³) and applied to a column of Dowex 50W-X8 ion-exchange resin (H^+; 10 g). The column was washed with water (100 cm³) and the amino-acids were eluted with ammonia solution (0·5 mol dm^{-3}; 150 cm³). The eluate was evaporated to dryness and the product was purified by preparative t.l.c. [butanol–acetic acid–water (4 : 1 : 1)]. The isoleucine band was extracted with water and purified by passage through Dowex 50W-X8, as before. Evaporation of the ammoniacal eluate gave the product as a white solid (30 mg) in 5% radiochemical yield. Amino-acid analysis showed that the mixture consisted of DL-isoleucine (60 ± 3%) and DL-alloisoleucine (40 ± 3%). A radiochemical and chemical purity of 100% was indicated by autoradiography and paper chromatography.

(*b*) To a warm solution of sodium (33·6 mg, 1·46 mmol) in ethanol (1·5 cm³) was added with stirring diethyl[2-^{14}C]-malonate (0·1 mCi, 5 mCi mmol^{-1}) diluted with inactive material (232 mg, 1·45 mmol), followed after 5 min by 2-bromobutane (200 mg, 1·46 mmol). The mixture was stirred and heated under reflux for 26 h and was then left at room temperature for 17 h. Water (20 cm³) was added and the solution was extracted with ether (4 × 8·5 cm³). The extracts were dried ($MgSO_4$), filtered, and evaporated to give crude diethyl s-butyl[2-^{14}C]malonate (XVIII) as a pale yellow liquid (218 mg). The crude ester was heated with potassium hydroxide (218 mg) in water (2 cm³) for 5 h. The product was cooled and acidified (Congo Red) with conc. HCl, care being taken to ensure that the temperature never rose above 10° during acidification. The acidic solution was extracted with ether (4 × 8 cm³); the extracts were dried ($MgSO_4$) and evaporated to give the crude 2-butylmalonic acid (XIX) as a viscous oil (121 mg). The crude acid was dissolved in conc. H_2SO_4 (2 cm³) with cooling, the mixture was allowed to warm to room temperature, and sodium azide (70 mg, 1·08 mmol) was added. The mixture was warmed rapidly to 60° and maintained at that temperature for 3 h. Three portions of sodium azide (each 30·5 mg, 0·47 mmol) were added during this time, one after each hour. The mixture was maintained at 60° for 1 h after the addition of the last portion of sodium azide, and the mixture was cooled and poured into ice–water (15 cm³). The solution was washed with ether (3 × 5 cm³) and was brought to pH 3 with barium acetate. The solution was filtered (Kieselguhr), the precipitate was washed with water (20 cm³), and the combined filtrate and washings were concentrated to 15 cm³ under reduced pressure. The amino-acids were purified by passage through a column of Dowex 50W-X8 as described before, followed by preparative t.l.c. [chloroform–methanol–ammonia (40 : 40 : 20)] and a second passage through Dowex 50W-X8, to give [2-^{14}C]isoleucine (41 mg, 17·5% radiochemical yield). Amino-acid analysis indicated that the product contained DL-isoleucine (53 ± 3%) and DL-alloisoleucine (47 ± 3%). A radiochemical purity of >98% was indicated by radioautography of paper chromatograms and electrophoretograms and by paper chromatography with liquid scintillation scanning.

[5-^{14}C]*Isoleucine.*—To [2-^{14}C]ethyl iodide (0·5 mCi; 4·28 mCi mmol^{-1}) cooled in liquid nitrogen was added a solution of inactive ethyl iodide (404 mg, 2·58 mmol) in ether (3 cm³). The mixture was added to magnesium turnings (75 mg, 3·1 mmol). When the reaction had begun, ether (5 cm³) was added and the mixture was stirred for 20 min, after which time nearly all of the magnesium had reacted. Diethyl ethylidenemalonate (XX) (500 mg, 2·69 mmol) in ether (5 cm³) was added over 15 min; the mixture was stirred for 1 h and acidified with HCl (1 mmol dm^{-3}), and the ethereal layer was separated. The aqueous layer was extracted with ether (2 × 10 cm³). The combined extracts were dried ($MgSO_4$) and evaporated to give the crude [2-^{14}C]diethyl s-butylmalonate (XVIII) as an oil (550 mg). The ester was converted into isoleucine by method (*b*) for the preparation of [2-^{14}C]isoleucine. [5-^{14}C]Isoleucine was obtained in 22·6% radiochemical yield. A radiochemical purity of 100% was indicated by radioautography and of >99% by dilution analysis.

[6-^{14}C]*Isoleucine.*—Inactive methyl iodide (800 mg, 5·6 mmol), in ether (0·5 cm³) was added to [^{14}C]methyl iodide (0·05 mCi, 51·5 mCi mmol^{-1}). The solution was added to magnesium turnings (60 mg, 2·5 mmol), reaction was initiated by the addition of a crystal of iodine, and ether (2 cm³) was added. The mixture was stirred until all the magnesium had been consumed and diethyl propylidenemalonate (XXI) (500 mg, 2·5 mmol) in ether (2 cm³) was added dropwise. The solution was stirred for 1 h; water (20 cm³) was added, followed by conc. HCl (3 cm³), and the mixture was extracted with ether (5 × 40 cm³). The extracts were dried ($MgSO_4$) and evaporated to give the crude ester (XVIII) as a red oil (450 mg). This was converted into isoleucine as before. The amino-acid was purified by passage through Dowex 50W-X8 ion-exchange resin, preparative

[20] J. Liebster, M. Dobiasova, J. Kopoldova, and J. Ekl, *Coll. Czech. Chem. Comm.*, 1961, **26**, 1700.

[21] A. I. Virtanen, A. Berg, and S. Kari, *Acta Chem. Scand.*, 1953, **7**, 1423; L. A. Larson and H. Beevers, *Plant Physiol.*, 1965, **40**, 424; J. M. Lawrence and D. R. Grant, *ibid.*, 1955, **38**, 561.

t.l.c. [butanol–acetic acid–water (4 : 1 : 1)], and a second passage through Dowex 50W-X8 to give [6-^{14}C]isoleucine (21 mg) in 6% radiochemical yield. A radiochemical purity of 100% was indicated by dilution analysis and of >99% by radioautography and paper chromatography with liquid scintillation scanning.

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