+137°. Compounds active at the muscarinic receptor sites appear to require a $(CH_3)_3N^+$ group and, in addition, an alkyl or NH_2 group at the position of the acetoxy methyl in acetylcholine.[18] For the nicotinic receptor sites, the groupings which appear to be essential for activity are a quaternary N^+ moiety and a hydrogen bond receptor located approximately 5.9 Å from the center of the positive charge.[17]

It may be instructive to examine whether there are geometrically similar groupings in the histrionicotoxins and cholinergic agonists and antagonists. In Figure 5, the geometry of isohistrionicotoxin is compared with muscarine, tropine, and the choline moiety in acetyl- or succinylcholine. Coordinates determined from crystal structure analyses[20,21] were used to execute precise drawings of the configurations by computer.[22] Similar geometric, although not necessarily chemical, moieties are outlined with heavy lines. Although there is a group of atoms in the histrionicotoxins with a geometry resembling that of choline, the histrionicotoxin structure does not possess the requirements as mentioned above either for muscarinic or nicotinic activity. For this potent inhibitor, the occurrence of a N^+ atom with a hydrogen bond receptor only 2.71 Å from the N^+ apparently is sufficient for activity.

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A General Method for the Determination of Precursor Configuration in Biosynthetic Precursor–Product Relationships. Derivation of Pipecolic Acid from D-Lysine, and of Piperidine Alkaloids from L-Lysine[1]

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Abstract: The configuration of a metabolic precursor is determined experimentally by comparison of the $^3H/^{14}C$ ratio of the metabolic product with that of a doubly labeled substrate. This new method for the determination of precursor stereochemistry in intact systems does not depend on a comparison of incorporation efficiencies. It is shown by this method that the alkaloids sedamine, *N*-methylpelletierine, and *N*-methylallosedridine from two *Sedum* species and anabasine from *Nicotiana glauca* are derived from L-lysine, whereas pipecolic acid, from each of these plants, is derived from D-lysine.

In biosynthetic tracer studies in which specifically ^{14}C-labeled amino acids are used as substrates, the DL racemates are most frequently employed since they are more readily available and generally less expensive than correspondingly labeled samples of the L and D enantiomers. If nonrandom incorporation of label from a ^{14}C-labeled DL-amino acid into a biosynthetic product is observed, the assumption tends to be made (in the absence of evidence to the contrary) that it is the L enantiomer which represents the normal metabolic substrate and serves as the actual precursor (*e.g.*, ref 2 and 3).

Tracer experiments intended to establish, in intact systems, which one of the two enantiomers of an amino acid served as the actual precursor have, in general, led to results which were indicative rather than conclusive, and have on occasion yielded contradictory data. Inferences were based:

(i) on a comparison of the efficiencies,[4] observed in parallel experiments, of incorporation of radioactivity from the labeled DL racemate and the labeled L and D isomers of the substrate into the biosynthetic product,[6]

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(4) Incorporation efficiencies have been expressed in several ways: *specific radiochemical yield* (100 × molar specific activity of product/molar specific activity of substrate) or its reciprocal, *dilution value* (molar specific activity of substrate/molar specific activity of product), are expressions of tracer concentration within the isolated product. Such values depend[5] not only on the rate of conversion of substrate to product, but also on the pool sizes, within the intact system, of the substrate and of the intermediates between substrate and product, as well as on the amount of product present in the system at the time of administration of tracer. Apart from demanding rigorous purification of end product, these values are independent of manipulative variables such as losses in the course of isolation of product. The *per cent incorporation* of activity into the product (100 × total activity recovered in product/total activity administered in substrate), on the other hand, depends on the chemical yield of product which, in turn, is a function of the skill of the experimenter.
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(7) H. R. V. Arnstein and P. T. Grant, *Biochem. J.*, **57**, 353 (1954).
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(ii) on competition experiments, involving a comparison of incorporation efficiencies of radioactivity from labeled DL racemate in the presence of excess inactive L or D enantiomer, or of activity from a labeled sample of one enantiomer in the presence of an unlabeled sample of the other,[21]

or (iii) on a comparison of the retention or loss, within the biosynthetic product, of α-tritium or α-^{15}N, relative to ^{14}C, observed in parallel experiments with multiply labeled samples of the L enantiomer, of the D enantiomer, and of the DL racemate of an amino acid substrate.[24]

The results of such experiments do not permit an unequivocal choice to be made regarding the chirality, L or D, of the true precursor. Firstly, efficiency of incorporation of the label of the precursor into the product is the resultant of several factors,[28] and is, therefore, not a reliable criterion for a direct precursor–product relationship. Thus, the D and the L isomers of an amino acid may be taken up by the system at different rates, and, in short-term experiments, a higher recovery of label within the product derived from one of the enantiomers may simply reflect faster uptake of that enantiomer (*e.g.*, ref 9). Secondly, a labeled enantiomer may be converted into its antipode prior to incorporation into a product, either directly if the requisite racemase is present in the system, or indirectly *via* the corresponding α-keto acid, by transamination or by oxidative deamination followed by stereospecific reamination. The level within the product of ^{14}C from a singly labeled sample of substrate, or the loss, in the course of incorporation, of α-^{15}N or α-^{3}H, relative to ^{14}C, from a multiply labeled specimen of an amino acid enantiomer are then not direct indicators of the chirality of the precursor.

Since most chiral samples of specifically ^{14}C-labeled amino acids must be prepared by resolution of the DL racemates, a further methodological difficulty is related to the steric purity of the substrate. The lower limits in the detection of traces of the unwanted enantiomer in a resolved amino acid sample are $\pm 1\%$ by optical methods and $\pm 0.1\%$ by enzymic methods.[29-31] These limits are of a magnitude similar to the recovery of precursor activity in biosynthetic products generated in intact systems. Thus, about 1% of the label of the amino acid substrate is recovered within the product in tracer studies on the biosynthesis of antibiotic peptides.[32] In investigations of alkaloid biosynthesis in intact plants, the recovery of label within the product, from amino acid substrates shown by degradation studies to be incorporated specifically and nonrandomly, is usually less than 1%, often less than 0.1% and sometimes as low as 0.01% (*e.g.*, ref 33 and 34).

It is thus conceivable that in a given experiment with an intact system much or all of the activity which enters the product originates from an enantiomeric impurity present in the chiral substrate. Since chiral samples of tritium-labeled amino acids are prone to partial racemization,[35] and ^{14}C-labeled samples also deteriorate with time,[36] enantiomeric impurities are quite likely to be present in labeled samples of chiral amino acids, supplied commercially.

To achieve the limits of confidence in the determination of the optical purity of a labeled sample of an amino acid substrate which are required to exclude such a possibility, enzymic assays may have to be carried out on more labeled material than is available to the experimenter. A single tracer experiment generally requires no more than 100μ Ci of ^{14}C or 1 mCi of ^{3}H, and singly labeled compounds are now commercially available with nominal specific activities of 10 mCi of ^{14}C or 5 Ci of ^{3}H per mmole or higher. A radiochemical technique, reverse dilution analysis, is regarded as the most sensitive method available for determining impurities of one enantiomer in a radioactively labeled sample of its antipode.[37] The method depends on the determination of residual radioactivity in a sample obtained by repeated fractional crystallization after addition of an excess of radioinactive optically pure carrier of the same configuration as the chiral impurity. The shortcomings of this method have been repeatedly pointed out.[38-40] It is not generally practicable for the investigator himself to establish the degree of optical purity of his labeled substrate. It is also clearly unwise to accept suppliers' claims of optical purity in experiments in which optical purity is of the essence.

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(21) *E.g.*, incorporation of tryptophan into ergot alkaloids,[22] of valine into penicillin N and cephalosporin C,[13] and of proline, leucine, valine, ornithine, and phenylalanine into gramicidin S.[23]
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A further source of uncertainty in the interpretation of results obtained by a comparison of data from parallel experiments is particularly prevalent in work with intact plants, in which a small number of individuals are used in a given experiment. Individual variability becomes an important and unpredictable factor which is reflected in the level of incorporation of label. Many instances are recorded in which incorporation efficiencies of a given labeled precursor differed by as much as an order of magnitude in duplicate experiments carried out under apparently comparable conditions (*e.g.*, phenylalanine into sedamine[41]). Comparison of incorporation efficiencies of different substrates, whose rate of penetration across cell membranes can be quite different, is entirely unreliable as an interpretable parameter (*cf.* ref 42).

In connection with a problem related to current investigations on the biosynthesis of the piperidine nucleus,[43] it was essential to establish whether both L- and D-lysine, or only one of them, served as a precursor of the piperidine alkaloids. Since, in our opinion, separate experiments with L- and D-lysine could not yield a conclusive result, we had to design a method which did not depend on a comparison of incorporation efficiencies obtained in separate experiments.

The method here described yields data which, for a given experiment, are self-consistent and interpretable independently of the results of other experiments. By application of this method, it is demonstrated that whereas in several systems pipecolic acid is derived from D-lysine, other piperidine derivatives found in these systems originate from L-lysine.

Experimental Section

Radioactive Compounds. DL-[2-^{3}H]Lysine[43] (nominal specific activity 35 mCi/mmol, Commissariat à l'Énergie Atomique, France), DL-[4,5-$^3H_2$]lysine (nominal specific activity 5.4 Ci/mmol, New England Nuclear), L-[4,5-$^3H_2$]lysine (nominal specific activity 0.25 Ci/mmol, Radiochemical Centre), and DL-[6-^{14}C]lysine (nominal specific activity 48 mCi/mmol, Commissariat à l'Énergie Atomique, France) were commercial products. D-[6-^{14}C]Lysine was prepared from the DL-[6-^{14}C]lysine as described below.

The intermolecularly doubly labeled samples of lysine which were used in experiments 1–6 were prepared by mixing suitable quantities of these tracers. The ^{3}H/^{14}C ratios of the doubly labeled samples, determined by liquid scintillation counting, are given in Tables I–III. Nominal total activities with respect to ^{14}C which were used were 0.1 mCi (expt 3 and 4), 0.05 mCi (expt 5 and 6), and 0.03 mCi (expt 1 and 2), respectively.

D-[6-^{14}C]Lysine. L-Lysine decarboxylase ("type II," ex *Bacillus cadaveris*, Sigma Chemical Co.) (10 mg) was dissolved in phosphate buffer (0.2 *M*, pH 6.0, 3.5 ml) and mixed with a solution of DL-[6-^{14}C]lysine (as dihydrochloride in 0.4 ml of water, nominal total activity 0.1 mCi) in phosphate buffer (0.2 *M*, pH 6.0, 1.5 ml). The mixture was agitated gently at 37°. Samples of unlabeled L-lysine monohydrochloride were added after 15 min (2 mg) and again after 35 min (3 mg), and incubation was continued.

The progress of the reaction was monitored by chromatography and chromatoscanning (Model 7201, Radiochromatography Scanner, Packard Instrument Co.). The scan of a paper chromatogram (Whatman No. 1, 2-propanol–0.880 *M* ammonia–water (8:1:1)) of a sample of the original solution revealed a single radioactive peak corresponding to lysine (R_f 0.1). After 1 min a second peak, corresponding to cadaverine (R_f 0.5), had appeared; after 15 min equal peak areas associated with the lysine and cadaverine peaks indicated complete destruction of L-lysine. Complete destruction of the labeled L-lysine was ensured further by continuation of the incubation in the presence of unlabeled L-lysine.

After 7 hr the incubation mixture was heated 10 min on the steam bath, the precipitate was removed by centrifugation, the pellet was resuspended in hot water, and centrifugation was repeated. The combined supernatant solutions were applied to a column (0.5 × 10 cm) of Dowex 50W-X4 (mesh 50–100, H^+ form), and lysine eluted with ammonia (1 *M*, *ca.* 50 ml). The eluate was evaporated, and the residue was chromatographed on paper as described above. The radioactive area, R_f 0.1, due to D-[6-^{14}C]-lysine (25 μCi), was eluted with water. The stereochemical purity of the product was confirmed by incubation of a small sample (approximately 5000 dpm) with L-lysine decarboxylase. A chromatoscan (*vide supra*) failed to reveal activity at R_f 0.5.

Determination of the Enantiomeric Composition of a Commercial Sample of [4,5-$^3H_2$]Lysine. A portion (approximately 1 μCi) of the sample of [4,5-$^3H_2$]lysine (nominal specific activity 18.8 Ci/mmol, nominal total activity 1 mCi, in sterile aqueous solution, Radiochemical Centre) was mixed with a sample of DL-[6-^{14}C]lysine (approximately 0.1 μCi) (nominal specific activity 48 mCi/mmol, Commissariat à l'Énergie Atomique, France), inactive DL-lysine (5 mg) was added as carrier and the mixture was dissolved in phosphate buffer (0.2 *M*, pH 6.0, 1.5 ml) (^{3}H/^{14}C ratio 8.3 ± 0.2). L-Lysine decarboxylase ("type II," ex *Bacillus cadaveris*, Sigma Chemical Co.) (10 mg) was added, and the mixture was incubated at 37°.

The reaction mixture was monitored and protein was removed as described above. The supernatant solutions were combined and applied to Whatman No. 1 paper; the chromatogram was developed with 2-propanol–0.880 ammonia–water (8:1:1), and cadaverine (R_f 0.5) was eluted with methanol (^{3}H/^{14}C ratio 8.1 ± 0.1).

Plant Material, and Methods of Infusing Labeled Compounds. *Nicotiana glauca* R. Grah. plants were grown from seed. *Sedum acre L.* and *S. sarmentosum* Bunge, perennials, were propagated from plant material collected in the Royal Botanical Gardens, Hamilton.

In each of the two experiments with *S. acre* (expt 1 and 2), cuttings (8–10-cm long) (total fresh weight 40–50 g) of the aerial shoots of the plant were placed, cut surface downwards, into five 30-ml beakers. The tracer solution was distributed among the beakers[44] and kept in contact with the plant material for 24 hr. The experiments with *S. sarmentosum* (expt 3 and 6) were carried out similarly, except that cuttings (12–15-cm long) (total fresh weight 90 g) were placed in ten 30-ml beakers.

In each of the two experiments with *N. glauca* (expt 4 and 5), tracer was applied over a period of 24 hr to 12 plants (total fresh weight, including roots, 160–170 g), approximately 4 months old, by the wick technique.

Isolation of Pipecolic Acid. After 24 hr in contact with tracer solution, the plant material was homogenized and the pH was adjusted to 11 by addition of aqueous ammonia. The homogenate was transferred to a glass column (40 × 5 cm) and percolated first with ether, saturated with water (500 ml), and then with ether (U.S.P) until 1 l. of eluate had been collected. In the experiments with *S. acre*, (±)-sedamine (100 mg) was added to the ether (U.S.P) as a carrier. The ether layer of the eluate was separated from the aqueous layer, washed with water, and extracted with hydrochloric acid (5%, 4 × 20 ml). Alkaloids were isolated from this acid extract (*vide infra*). The column was further percolated with hot water (1 l.) containing carrier DL-pipecolic acid (100 mg). The combined aqueous eluates and washings were evaporated to dryness on a steam bath. The residue was suspended in methanol (30 ml); the suspension was kept 24 hr at room temperature with occasional stirring, a little charcoal was added, and the mixture was filtered through cotton wool. The methanol extract was evaporated, and the residue, dissolved in water, was applied to a column (1.5 × 30 cm) of Dowex 50W-X4 (H^+ form), which was washed with water. Amino acids were eluted from the washed column with 1 *M* ammonia. Eluate was collected until no further radioactivity emerged. The eluate was evaporated to dryness under reduced pressure, and the residue was dissolved in hydrochloric acid (6 *M*, 3 ml). Sodium nitrite (0.5 g, dissolved in a little water) was added in small portions and the mixture was allowed to stand for 15 min, when a sample no longer gave a purple color with ninhydrin. The solution was extracted with ether (3 × 10 ml) and the ether extract was dried (Na_2SO_4) and evaporated. The yellow oily residue was

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Table I. Experiments with L-[4,5-$^3H_2$]/DL-[6-^{14}C]Lysine

Expt no.		Specific activity (^{14}C),[a] dpm/mmol	$^3H/^{14}C$ ratio	% of product derived from L-Lys[b]
1	*Sedum acre*			
	Feeding solution		6.8 ± 0.3	
	Sedamine	2.52 ± 0.15 × 10^4	14.0 ± 0.2	103 ± 5
	Pipecolic acid	2.41 ± 0.03 × 10^6	0.8 ± 0.03	6 ± 1
4	*Nicotiana glauca*			
	Feeding solution		11.3 ± 0.4	
	Anabasine	1.17 ± 0.01 × 10^6	21.4 ± 0.2	95 ± 4
	Pipecolic acid	5.86 ± 0.06 × 10^6	0.1 ± 0.03	0.4 ± 0.1
3	*Sedum sarmentosum*			
	Feeding solution		9.1 ± 0.1	
	N-Methylpelletierine	Not determined	18.4 ± 0.4	101 ± 3
	N-Methylallosedridine	Not determined	18.3 ± 0.3	101 ± 2
	Pipecolic acid	1.24 ± 0.01 × 10^7	0.4 ± 0.01	2 ± 0.1

[a] After dilution with inactive carrier. [b] *Cf.* Figure 1.

dissolved in concentrated hydrochloric acid (2 ml) and heated on a steam bath for 10 min, when the solution had evaporated to dryness. The residue was dissolved in a little methanol. Addition of ether gave a crystalline precipitate. This was dissolved in water and the solution was applied to a column (10 × 1 cm) of Dowex 50W-X4 (H^+ form). The column was washed with water containing methanol (10% v/v), until the eluate was neutral, and was then eluted with 1 *M* ammonia. Eluate was collected until no further radioactivity emerged. The ammoniacal eluate was evaporated to dryness and the residue was sublimed at 180–190° and 1 × 10^{-3} mm. The sublimate was dissolved in methanol (1 ml). Anhydrous ether was added to induce crystallization of pipecolic acid, melting at 280–281°.

Isolation of Radioactive Alkaloids. Sedamine and Methylpelletierine. Sedamine was isolated from the ether extract (*vide supra*) of *Sedum acre* (expt 1 and 2), and *N*-methylpelletierine from that of *S. sarmentosum* (expt 3 and 6), and purified to constant radioactivity, as described in earlier papers (ref 44 and 45, respectively). Sedamine was purified to constant activity by sublimation and crystallization from hexane, mp 88–89°.[41] *N*-Methylpelletierine hydrochloride was purified by sublimation and crystallization from methanol–ether, mp 156–157°.[45]

Anabasine. The acid extract from *Nicotiana glauca* (*vide supra*) was washed with ether until colorless, basified with sodium hydroxide (5 *N*, 10 ml), and extracted with chloroform (3 × 20 ml). The chloroform layer was dried (anhydrous sodium sulfate) and evaporated. A small portion of the residue was subjected to glc (*vide infra*) and the anabasine fraction collected and its $^3H/^{14}C$ ratio assayed.

Inactive (−)-anabasine (100 mg) (Fluka A.G.) was added to the major portion of the residue, the mixture was dissolved in a little methanol, and perchloric acid (95%, 0.5 ml) was added. Anabasine diperchlorate was precipitated by addition of ether and on recrystallization from methanol–ether was obtained as colorless needles, mp 168–170° (reported[46] mp 154–155°).

Anal. Calcd for $C_{10}H_{14}N_2(HClO_4)_2$: C, 33.07; H, 4.44; Cl, 19.53; N, 7.72. Found: C, 32.92; H, 4.45; Cl, 19.23; N, 7.70.

Isolation of Alkaloids by Gas Chromatography. The basic fraction obtained from the plant extract was chromatographed (Varian Aerograph 1840-1, equipped with a stream splitter which channeled 10% of column effluent into a flame ionization detector, allowing 90% to emerge through the collecting outlet) on a column of 20% Carbowax 20M on Chromosorb HMDS, coated with 5% KOH (mesh 60–70; 4 ft × 0.125 in., stainless steel, helium, 40 ml/min, inlet and detector temp 220°, initial column temp 60°, increasing (after 3 min) to 220° at 6°/min). The following retention times, identical with those of authentic specimens, were observed: *N*-methylpelletierine 18.3 min, *N*-methylallosedridine[47] 19.8 min, anabasine 23.5 min, sedamine 31 min.

Determination of Radioactivity. The radioactivity of all samples was determined by liquid scintillation counting (Mark 1, liquid scintillation computer, Model 6860, Nuclear-Chicago). Activity due to ^3H and ^{14}C was determined simultaneously, by external standardization counting, with ^{133}Ba. Samples, dissolved in water (lysine, pipecolic acid), were dispersed with the aid of methanol in a solution of Liquifluor (Nuclear-Chicago) diluted 25 times with toluene. The alkaloids were dissolved in the diluted Liquifluor solution. Duplicate samples of each compound were counted under comparable conditions of quenching. Confidence limits shown in the results are standard deviation of the mean. For highly quenched samples the confidence limits of the quench correction curves were ±5%.

Results

The $^3H/^{14}C$ ratios of the samples of lysine administered in each of the tracer experiments, and those of the products isolated from these experiments, are shown in Tables I–III. When L-[4,5-$^3H_2$]/DL-[6-^{14}C]lysine was the substrate (Table I), the three samples of pipecolic acid which were isolated from different tissues were rich in ^{14}C but contained little tritium. The alkaloids obtained from these sources, on the other hand, contained twice as much tritium, relative to ^{14}C, as the substrate. When DL-[4,5-$^3H_2$]/D-[6-^{14}C]lysine served as the substrate (Table II), the samples of pipecolic acid contained half as much tritium, relative to ^{14}C, as the administered lysine, whereas the alkaloids were rich in tritium, but contained little ^{14}C.

With DL-[2-^{3}H]/DL-[6-^{14}C]lysine as the substrate (Table III), the alkaloids, *N*-methylpelletierine and sedamine, maintained the $^3H/^{14}C$ ratio of the precursor, whereas pipecolic acid had lost most of the tritium, relative to ^{14}C. With DL-[6-^{3}H]/DL-[6-^{14}C]-lysine, on the other hand, the alkaloids as well as the pipecolic acid maintained the $^3H/^{14}C$ ratio of the precursor.

Discussion

Argument was marshaled in the introduction to show that conclusive evidence concerning the stereochemistry of a biosynthetic precursor cannot be obtained from a comparison of efficiencies of incorporation of radioactivity, from chiral ^{14}C- or ^3H-labeled substrates, into biosynthetic products isolated from separate tracer experiments. It follows that if conclusive evidence is to be generated by tracer methods, it can only come from measurements which do not depend on a comparison of the results of parallel experiments, but are independently self-consistent. To obtain such self-consistent measurements in a given experiment, an

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(46) E. Leete, *J. Amer. Chem. Soc.*, 78, 3520 (1956).
(47) We thank Dr. L. Maat, Technische Hogeschool, Delft, Netherlands, for synthetic samples of (±)-*N*-methylsedridine and (±)-*N*-methylallosedridine.

Table II. Experiments with DL-[4,5-$^3H_2$]/D-[6-^{14}C]Lysine

Expt no.		Specific activity (^{14}C),[a] dpm/mmol	$^3H/^{14}C$ ratio	% of product derived from D-Lys[b]
2	*Sedum acre*			
	Feeding solution		9.2 ± 0.2	
	Sedamine		293 ± 21	2 ± 0.1
	Pipecolic acid	1.03 ± 0.01 × 10^6	5.3 ± 0.5	87 ± 8
5	*Nicotiana glauca*			
	Feeding solution		13.7 ± 0.2	
	Anabasine		665 ± 44	1 ± 0.1
	Pipecolic acid	1.20 ± 0.01 × 10^6	6.6 ± 0.1	104 ± 8

[a] After dilution with inactive carrier. [b] *Cf.* Figure 2.

Table III. Experiments with DL-[2-^{3}H]/DL-[6-^{14}C]Lysine, DL-[6-^{3}H]/DL-[6-^{14}C]Lysine, and DL-[4,5-$^3H_2$]/DL-[6-^{14}C]Lysine

	Substrate					
	DL-[2-^{3}H]/DL-[6-^{14}C]lysine		DL-[6-^{3}H]/DL-[6-^{14}C]lysine		DL-[4,5-$^3H_2$]/DL-[6-^{14}C]lysine	
	$^3H/^{14}C$ ratio	% retention of ^{3}H relative to ^{14}C	$^3H/^{14}C$ ratio	% retention of ^{3}H relative to ^{14}C	$^3H/^{14}C$ ratio	% retention of ^{3}H relative to ^{14}C
Sedum sarmentosum						
Expt no. or ref	Expt 6		Ref 45		Ref 45	
Substrate	13.4 ± 0.2	100 ± 2	18.3 ± 0.4	100 ± 2	7.4 ± 0.1	100 ± 1
N-Methylpelletierine	13.3 ± 0.1	99 ± 1	18.5 ± 0.2	101 ± 2	7.5 ± 0.2	101 ± 3
Pipecolic acid	0.3 ± 0.01	2 ± 0.1	18.3 ± 0.2	100 ± 1		101 ± 3[a]
Sedum acre						
Reference	Ref 43		Ref 44		Ref 44	
Substrate	13.2 ± 0.4	100 ± 3	15.6 ± 0.3	100 ± 2	9.4 ± 0.3	100 ± 3
Sedamine	12.6 ± 0.1	95 ± 1	15.5 ± 0.1	99 ± 1	9.5 ± 0.1	100 ± 1
Pipecolic acid	0.6 ± 0.01	5 ± 0.1	15.6 ± 0.1	100 ± 1		

[a] Unpublished result.

internal standard is mandatory. Such an internal standard is *potentially* present, if a multiply labeled substrate is employed, *e.g.*, a sample of an amino acid containing ^{14}C- as well as ^{3}H-labeled material.

Provided a number of conditions are fulfilled, investigations employing such multiply labeled substrates can provide conclusive evidence on the stereochemistry of a precursor–product relationship.

One of these conditions is that incorporation of an intact multicarbon fragment of the substrate in question into the product under investigation has been established, *i.e.*, that it has been shown by unequivocal chemical degradation of the product that radioactivity from one or more specifically ^{14}C-labeled radiomers of the DL-amino acid substrate enters predictable carbon atoms of the product nonrandomly. Incorporation of activity from generally labeled samples of substrate, *i.e.*, multiply labeled samples whose distribution of activity has not been established, does not fulfill this condition. A second condition is the demonstration that the $^3H/^{14}C$ ratio from a doubly labeled sample of DL-amino acid substrate is preserved within the product. If the sites of labeling within the product are established by degradation to correspond to prediction and the ratio of labeling (*e.g.*, $^3H/^{14}C$) within the product is identical with that of the substrate, it can be concluded that the multicarbon chain of the substrate (or at least a segment of it) has been incorporated into the product as a unit. This conclusion rests on the conviction that the chances of a fortuitous preservation of the ratio of labels, after fragmentation and recombination, is statistically infinitesimal (but see ref 48). Conclusions based on preservation of tracer ratio without establishing the site of ^{14}C labeling can be misleading (*e.g.*, ref 49).

Whereas preservation of the tracer ratio is, in general, interpretable without difficulty as indicating intact incorporation of substrate, a change in the ratio can be interpreted unequivocally only if it is known with certainty that loss of one tracer with respect to the other is the consequence of the process under investigation and none other.

Thus, the success in the exploitation of substrates multiply labeled with tritium and ^{14}C for studies of terpene and steroid biosynthesis and in the elucidation of the mechanism of cyclizations and rearrangements within these series, on the basis of the interpretation of changes in $^3H/^{14}C$ ratios,[50,51] rests on the understanding of the initial steps of the biochemical route emanating from mevalonic acid.[52]

Unless it is known that the loss of one label (*e.g.*, ^{3}H) relative to the other (*e.g.*, ^{14}C) is the consequence of a transformation which is directly relevant to the reaction sequence under investigation and not the result of some other process which happens to take place within the experimental system, a change in the tracer ratio cannot be conclusively interpreted. Thus, even though activity from D-tryptophan enters pyrrolnitrin more readily than activity from L-tryptophan, α-tritium from doubly

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labeled L-[α-^{3}H,β-^{14}C]tryptophan is retained within the product to a much larger extent, relative to ^{14}C, than α-tritium from DL-[α^3H,β-^{14}C]- or D-[α-^{3}H,β^{14}C]tryptophan (71, 26, and 3% retention, respectively).[27] Taken at face value, the results of these experiments appear to contradict one another, and evidently do not permit an unequivocal identification of the chirality of the biosynthetic precursor. Since transaminases are widely distributed and transamination is a reversible process, the α hydrogen of most amino acids is labile in a biochemical environment. α-Tritiated amino acids are, therefore, unsuitable for the desired tracer determination of the stereochemistry of an amino acid precursor.

To be of general utility a method is required which employs as the tracer a radiomer of the precursor labeled with tritium and ^{14}C in such a manner that incorporation of its racemate into the product takes place without change of the ^{3}H/^{14}C ratio, and which does not depend on the presence of tritium at the center whose chirality is to be determined.

Maintenance of the ^{3}H/^{14}C ratio of a doubly labeled sample of the DL racemate of a specific precursor within the biosynthetic product signifies that either the L isomer or the D isomer, or both, serves as a substrate.

In principle, an experiment might be performed in which DL racemate was administered as the substrate such that one of the enantiomers, the L isomer, say, was labeled with tritium and the other isomer, the D, with ^{14}C. Maintenance of the ^{3}H/^{14}C ratio of the precursor within the product would show that under the conditions of the experiment the two enantiomers are absorbed, metabolized, and incorporated into the product in identical fashion. A change in the ^{3}H/^{14}C ratio in such an experiment cannot be interpreted unequivocally, since the experiment is subject to several of the weaknesses which were discussed in the introduction in relation to the current approaches for determining precursor chirality (i and ii). Thus, a decreased ^{3}H/^{14}C ratio within the product does not necessarily mean that product is formed preferentially from the D isomer. It may simply indicate that under the experimental conditions the D isomer is taken up into the cell faster than the L isomer. Having entered the cell, the D isomer may have isomerized prior to incorporation. A study of the variation with time of the ^{3}H/^{14}C ratio within the product would be required in this case.

These limitations do not arise if the doubly labeled substrate consists of one of the enantiomers labeled with tritium in admixture with DL racemate labeled with ^{14}C, or, alternatively, one of the enantiomers labeled with ^{14}C in admixture with DL racemate labeled with tritium. In effect such a mixture represents a doubly labeled sample of one enantiomer (whose ^{3}H/^{14}C ratio may be set at will) in admixture with a singly labeled sample of the other.[53]

As before, maintenance of the ^{3}H/^{14}C ratio of the substrate within the product shows that the two enantiomers are absorbed, metabolized, and incorporated into the product with equal efficiency. Any change in the ^{3}H/^{14}C ratio, on the other hand, must mean preferential absorption and/or incorporation into the product of one of the two enantiomers. From the value of the ^{3}H/^{14}C ratio of the product compared to that of the substrate, the contribution which each of the enantiomers of the labeled substrate makes to the product may be deduced.[56]

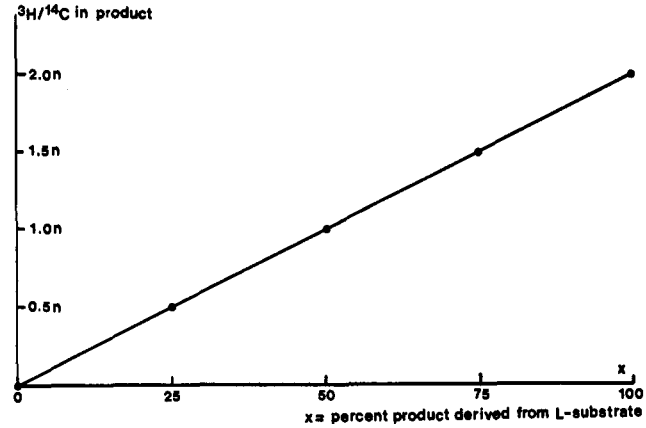

Figure 1. Variation of the ^{3}H/^{14}C ratio in a biosynthetic product as a function of the fraction of the product derived from the L enantiomer of a substrate, when L-[^{3}H]/DL-[^{14}C] labeled substance serves as the tracer (^{3}H/^{14}C in tracer = n; % of product derived from L substrate = x; ^{3}H/^{14}C in product = $nx/50$).

Figure 1 illustrates the predicted variation of the ^{3}H/^{14}C ratio ($nx/50$) of a product as a function of the fraction of the product ($x/100$) which is derived from the L enantiomer of the substrate, when L-[^{3}H]/DL-[^{14}C] labeled substance (^{3}H/^{14}C ratio = n) serves as the tracer, and enantiomeric purity of the L-[^{3}H] labeled substrate is assumed.

Similarly, the predicted variation of the ^{3}H/^{14}C ratio ($50n/y$) of the product as a function of the fraction of the product ($y/100$) which is derived from the D enantiomer of the substrate, when DL-[^{3}H]/D-[^{14}C] labeled substance (^{3}H/^{14}C ratio = n) serves as the tracer, is shown in Figure 2.

Thus, assuming enantiomeric purity of the resolved labeled substance (*i.e.*, of the L-[^{3}H] compound in the former case and the D-[^{14}C] compound in the latter), the fraction of the product which is derived from each of the enantiomers of a substrate can be deduced, if the ^{3}H/^{14}C ratio of the doubly labeled substrate is known and the ^{3}H/^{14}C ratio of the product has been determined.

Alternatively, assuming stereospecificity of a given biochemical process, the per cent enantiomeric impurity within a resolved labeled substrate can be determined.

The application of the method for each of these purposes will be illustrated in the sequel.

(53) Attention is drawn to two recent investigations[54,55] in which results based on experiments with multiply labeled substrates of this type are reported.

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(56) Even though the contributions made to the product by each of the L and D enantiomer of the administered labeled substrate are thus established, it does not necessarily follow that these contributions represent the enantiomeric state of the actual endogenous precursor from which the product is formed. This would be true only if the system did not contain a mechanism for interconverting the enantiomers in question. If such a mechanism were available, and if the rate of interconversion of the enantiomers were fast relative to the rate of incorporation of precursor into the product, the result would be a measure of the relative rates of absorption of the L and D enantiomers of the administered labeled substrate into the system, and not of the relative contribution of L and D enantiomers of the endogenous precursor into the product. Precursor chirality could then be established by the present method only if interconversion of the enantiomers were inhibited, *e.g.*, by the use of antimetabolites.[57]

(57) D. L. Miller and V. W. Rodwell, *J. Biol. Chem.*, 246, 2758 (1971).

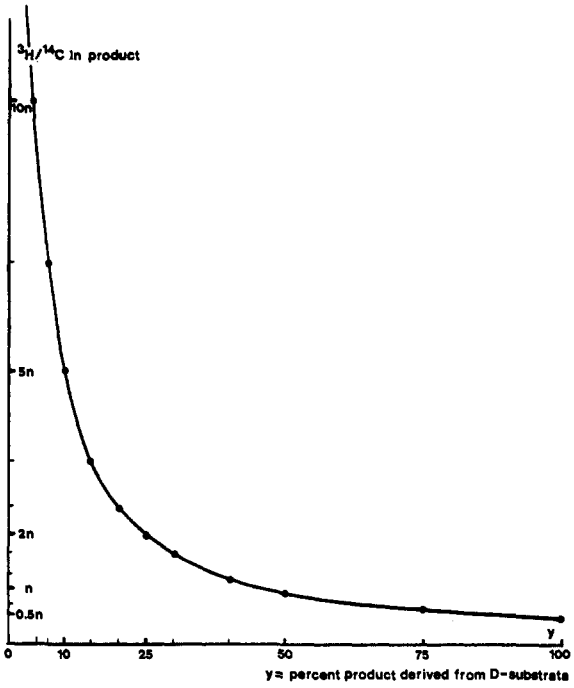
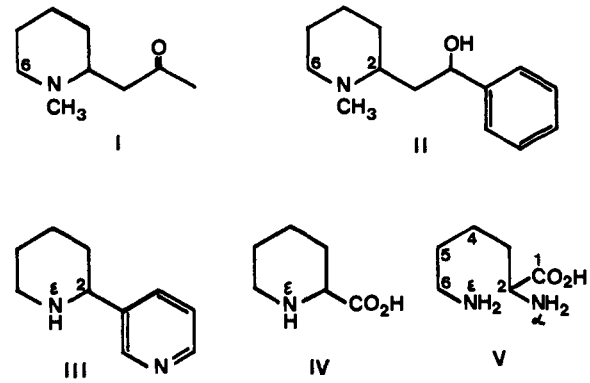


Figure 2. Variation of the $^3H/^{14}C$ ratio in a biosynthetic product as a function of the fraction of the product derived from the D enantiomer of a substrate, when DL-[^{3}H]/D-[^{14}C] labeled substance serves as the tracer ($^3H/^{14}C$ in tracer = n; % of product derived from D substrate = y; $^3H/^{14}C$ in product = $50n/y$).

Determination of the Enantiomeric Purity of a Labeled Substance. In connection with another investigation, it was necessary to determine the enantiomeric purity of a sample of [4,5-$^3H_2$]lysine monohydrochloride (nominal specific activity 18.8 Ci/mmol) which was claimed by the suppliers to contain 96% of the L isomer.[58] A small portion of this material was mixed with DL-[6-^{14}C]lysine to yield a doubly labeled sample ($^3H/^{14}C$ ratio = n = 8.3 ± 0.2) (*cf* Figure 1), which was subjected to enzymic decarboxylation in the presence of L-lysine decarboxylase [EC No. 4.1.1.18, L-lysine carboxylyase] from *Bacillus cadaveris*, an enzyme known to catalyse the decarboxylation of L-lysine, but not of D-lysine, to cadaverine.[59] If the sample of [4,5-$^3H_2$]-lysine consisted of the pure L species (*i.e.*, x = 100, *cf.* Figure 1), then the cadaverine resulting in the course of this reaction from the doubly labeled substrate can be predicted (Figure 1) to show a $^3H/^{14}C$ ratio of 16.6 ± 0.4 (*i.e.*, $nx/50$ = (8.3 ± 0.2)100/50). In fact, the cadaverine which was isolated showed the $^3H/^{14}C$ ratio 8.1 ± 0.1, *i.e.*, a ratio identical with that of the substrate, within experimental error. The percentage (x, *cf.* Figure 1) of the L species contained in the commercial sample of "L-[4,5-$^3H_2$]lysine" under investigation can then be calculated from the expression $nx/50$ = 8.1 ± 0.1. It follows (Figure 1) that the commercial sample of "L-[4,5-$^3H_2$]lysine" in fact contained 49 ± 1% L-lysine (*i.e.*, x = 50(8.1 ± 0.1)/(8.3 ± 0.2)) and 51 ± 1% of D-lysine. It is clear that, contrary to the claim of the supplier, the commercial sample was DL-[4,5-$^3H_2$]lysine and not L-[4,5-$^3H_2$]lysine.

The Chirality of Lysine Serving as a Precursor of Pipecolic Acid and of Other Naturally Occurring Piperidine Derivatives. Lysine has been shown, by means of tracer experiments, to serve as the precursor of many naturally occurring piperidine derivatives.[60] Among these are simple α-substituted piperidine bases such as the plant alkaloids *N*-methylpelletierine (I),[45,61] sedamine (II),[41,43,44] and anabasine (III),[46,62-64] whose hetero ring has been shown to be derived from a C_5N unit representing C-2 to -6 together with the ε-nitrogen of lysine. More complex polycyclic bases, *e.g.*, some belonging to the lupin,[60] lobelia,[61,65] and lycopodium alkaloids,[66,67] originate from more than one lysine derived C_5N unit.

A cyclic amino acid, pipecolic acid (IV), retains the



intact carbon skeleton as well as the ε-nitrogen atom of lysine.[19,43,44]

Conversion of lysine into the piperidine nucleus has been most extensively studied in the case of the simple piperidine bases I–III. It has been demonstrated that activity from DL-[2-^{14}C]lysine is restricted to C-2 of the piperidine nucleus of *N*-methylpelletierine (I),[61] sedamine (II),[41] and anabasine (III)[46,62-64] and that label from DL-[6-^{14}C]lysine is confined to C-6 of *N*-methylpelletierine (I)[45] and sedamine (II).[41]

Further, it has been shown by tracer experiments with multiply labeled substrates that the nitrogen atom of the piperidine nucleus of anabasine (III) is supplied by the ε but not by the α-amino group of lysine.[63] That the nitrogen atom of I and II also originates from the ε-amino group of lysine can be inferred from the tracer experiments with DL-[^{14}C]- and with DL-[^{3}H]/DL-[^{14}C]-lysines as the substrate.[43-45]

Since the $^3H/^{14}C$ ratio of *N*-methylpelletierine (I) and sedamine (II), isolated from tracer experiments with DL-[2-^{3}H]/DL-[6^{14}C]-, with DL-[4,5-$^3H_2$]/DL-[6-^{14}C]-, and with DL-[6-^{3}H]/DL-[6-^{14}C]lysine, corresponds, in every case, to that of the doubly labeled substrate (Table III), it must be concluded that the intact chain $NCH_2CH_2CH_2$-CH_2CH, arising by loss of the carboxyl group and the α-amino group, either from L-lysine, or from D-lysine, but not from both, serves as the precursor of the piperidine nucleus of the bases.

All the necessary conditions have been met to settle

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the question of the chirality of the precursor by means of the tracer method outlined above.

The results of the experiments with L-[^{3}H]/DL-[^{14}C]-lysine (Table I) and the complementary results of those with DL-[^{3}H]/D-[^{14}C]lysine (Table II) clearly indicate that, within experimental error, only the L-enantiomeric component of the substrate supplies activity which is incorporated into the alkaloids.

The simplest interpretation of this result is that the alkaloids are derived entirely from L-lysine.

An alternative interpretation is possible, however. If it is assumed that only the L enantiomer of the substrate enters the plant system, whereas the D enantiomer does not penetrate, no information can be obtained on the stereochemistry of the precursor molecules which enter the product, since penetration of L-lysine into the system could be followed by epimerization to the D isomer, prior to incorporation into the product. This alternative interpretation of the results would be eliminated if it could be shown that D-lysine enters the system. Evidence that D-lysine does enter the plant will now be presented.

Pipecolic acid (IV), a cyclic amino acid which contains a lysine-derived piperidine nucleus[43,44,68–75] and is widely distributed in nature,[76] was isolated by carrier dilution from the plant material from each feeding experiment. The ^{3}H/^{14}C ratio of the samples of pipecolic acid isolated from *Nicotiana glauca* indicates (Tables I and II) that all activity is supplied by D-lysine. In the case of the pipecolic acid samples from *Sedum acre* and *S. sarmentosum* the ^{3}H/^{14}C ratios which were observed show that most of the activity (~90%) is supplied by D-lysine, but that the L-lysine component of the substrate does make a contribution.

Since in each of the three plant species simultaneous formation is demonstrated of a compound which derives its activity from D-lysine and one which derives its activity from L-lysine, it is clear that both enantiomers of the substrate enter the system.

It follows that the alkaloids are derived from L-lysine and that pipecolic acid is derived largely, if not entirely, from D-lysine.[92] It is not possible to decide on the basis of the present evidence whether the contribution of label from the L isomer of the substrate to the samples of pipecolic acid from the two *Sedum* species is due to direct conversion of a portion of the L-lysine into pipecolic acid, or due to the availability of a mechanism for the epimerization of L- into D-lysine within the plant. We tend to favor the second alternative.[94]

Evidence has accumulated recently that, contrary to earlier reports,[68–75,96,97] L-pipecolic acid is derived from D-lysine in the rat,[18,86,98] in several higher plants,[19,99] and in some microorganisms.[57,100] The conversion of D-lysine into pipecolic acid in an anaerobic reaction catalyzed by hog kidney D-amino acid oxidase [EC No. 1.4.3.3, D-amino acid: oxygen oxidoreductase (deaminating)] has also been demonstrated.[101]

Our results provide independent proof.

Acknowledgments. This work was supported by a grant from the National Research Council of Canada. E. L. thanks the Deutsche Forschungsgemeinschaft for an overseas fellowship.

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