Biosynthesis of Lobinaline[1, 2]

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The biosynthesis of lobinaline (**1**) in intact plants of *Lobelia cardinalis* L. was studied by tracer methods. The incorporation data are consistent with the view that the alkaloid is formed by dimerization of α-phenacylpiperidine (**3**), whose piperidine nucleus is derived from lysine via a nonsymmetrical intermediate, and whose sidechain originates, as an intact C_6–C_2 unit, from phenylalanine via cinnamic acid.

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Introduction

The cardinal flower, *Lobelia cardinalis* L., so called for its brilliant scarlet bloom, is one of the more spectacular North American wild flowers. Lobinaline (**1**), the major alkaloid of this plant species, was isolated by Manske (2) in 1938. Its structure was determined some 30 years later (3, 4, 5).

Visual dissection of its nucleus (dotted line) leads to two fragments, C_6–C_2–C_5N, whose skeleton corresponds to that of some simpler Lobelia alkaloids, 8-phenylnorlobelol (**4**) (6) and 8-phenyllobelol (**5**) (6), which occur in *Lobelia inflata*. Even though such monomeric structures have not hitherto been found in *L. cardinalis*, the hypothesis is biogenetically attractive, that the lobinaline skeleton originates by dimerization of a suitably functionalized C_6–C_2–C_5N monomer. Indeed, such a suggestion was made (7) long before the structure of lobinaline had been established.

The origin of the C_6–C_2–C_5N alkaloids of *Lobelia inflata* has not been investigated. However, the biosynthesis of sedamine (**5**), a base which differs from 8-phenyllobelol only in the configuration at C-2′, has been studied in *Sedum acre* (1, 8, 9). It was established that the piperidine nucleus of sedamine arises from lysine via a nonsymmetrical intermediate, and that the C_6–C_2 sidechain is derived as an intact unit from phenylalanine (Scheme 1).

The objectives of the present study of lobinaline biosynthesis were to identify primary precursors and to test whether the alkaloid does indeed originate by dimerization of a monomer related to sedamine, such as α-phenacylpiperidine (**3**).

Methods and Results

In 11 tracer experiments, specifically labelled samples of phenylalanine, cinnamic acid, benzoic acid, lysine, and α-phenacylpiperidine were administered by the wick method to intact plants of *Lobelia cardinalis* L. when these were in full bloom. In each experiment lobinaline (**1**) was extracted from the aerial parts of the plants and was purified to constant radioactivity as the *N*-acetyl derivative (**2**). The details of these experiments are recorded in Table 1.

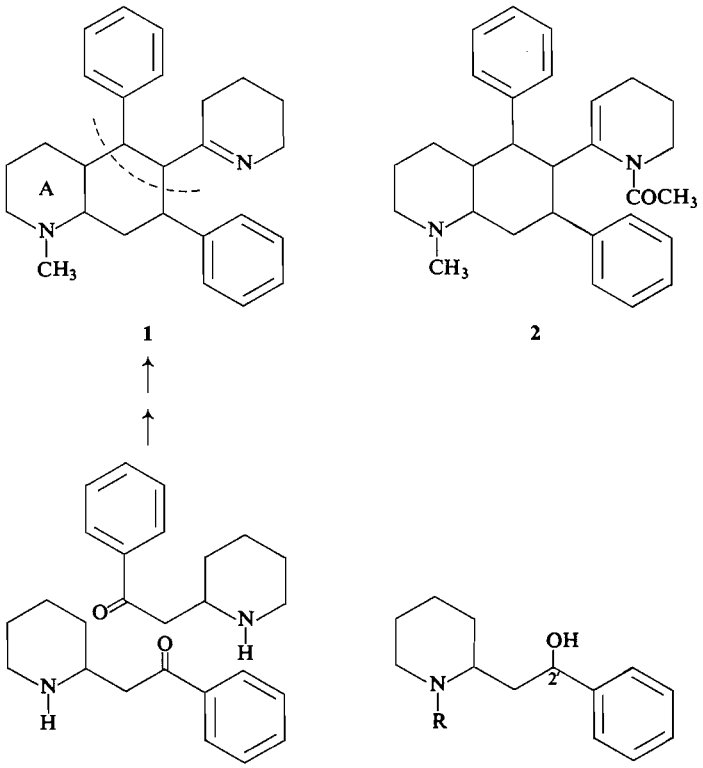
The labelled samples of acetyllobinaline (**2**) obtained from individual feeding experiments were degraded, by reactions described in the Experimental section, into the degradation products shown in Scheme 2. The specific activities of the degradation products are listed in Tables 2, 4, and 6 (indicated limits are standard deviation of the mean).

The samples of acetyllobinaline derived from 3-^{14}C-phenylalanine (expt. 2) and 3-^{14}C-cinnamic acid (expt. 5) yielded *p*-nitrobenzoic acid whose specific activity was half that of the intact alkaloid, and whose label was confined to the carboxyl carbon (*i.e.* C-5 and/or C-7 of lobinaline) (*p*-nitrobenzoic acid minus *p*-nitroaniline) (Table 2). The acetyllobinaline derived from the sample of 2,3-$^{14}C_2$-phenylalanine of known isotope distribution (see Experimental) (expt. 3) yielded *p*-nitrobenzoic acid whose specific activity, relative to that of the intact alkaloid, corresponded to the fraction of radioactivity at C-3 of the doubly labelled precursor (Table 3).

Activity from 2-^{14}C-phenylalanine (expt. 1) and from 2-^{14}C-cinnamic acid (expt. 4) was also

[1]This paper is dedicated to Professor Kurt Mothes, Director Emeritus of the Institute of Plant Biochemistry, German Academy of Sciences, Halle, West Germany on the occasion of his 70th birthday.

[2]A preliminary account of part of this work has been published (1).

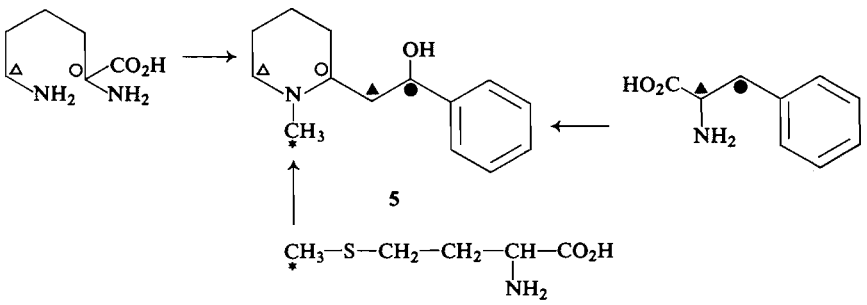


incorporated into lobinaline. These samples of the alkaloid were not degraded, since all attempts to develop reactions suitable for extracting the presumed labelled centers, C-6 and -8, were unsuccessful. Acetyllobinaline isolated after administration of carboxyl-^{14}C-benzoic acid (expt. 6) was essentially inactive.

Radioactivity from 6-^{14}C-lysine (expts. 7 and 8) was recovered in the degradation fragments derived from the heterocyclic nuclei of lobinaline. Succinic acid, β-alanine, and a mixture of β-alanine and *N*-methyl-β-alanine, each showed a specific activity corresponding to approximately half that of the intact alkaloid (Table 4).

The same fragments, isolated from the acetyllobinaline from 2-^{14}C-lysine (expt. 9), were devoid of radioactivity, as was the formaldehyde (C-2) obtained from this sample of the alkaloid (Table 4).

The activity of the formaldehyde (C-2) obtained in an early experiment from 6-^{14}C-lysine-derived acetyllobinaline corresponded to one

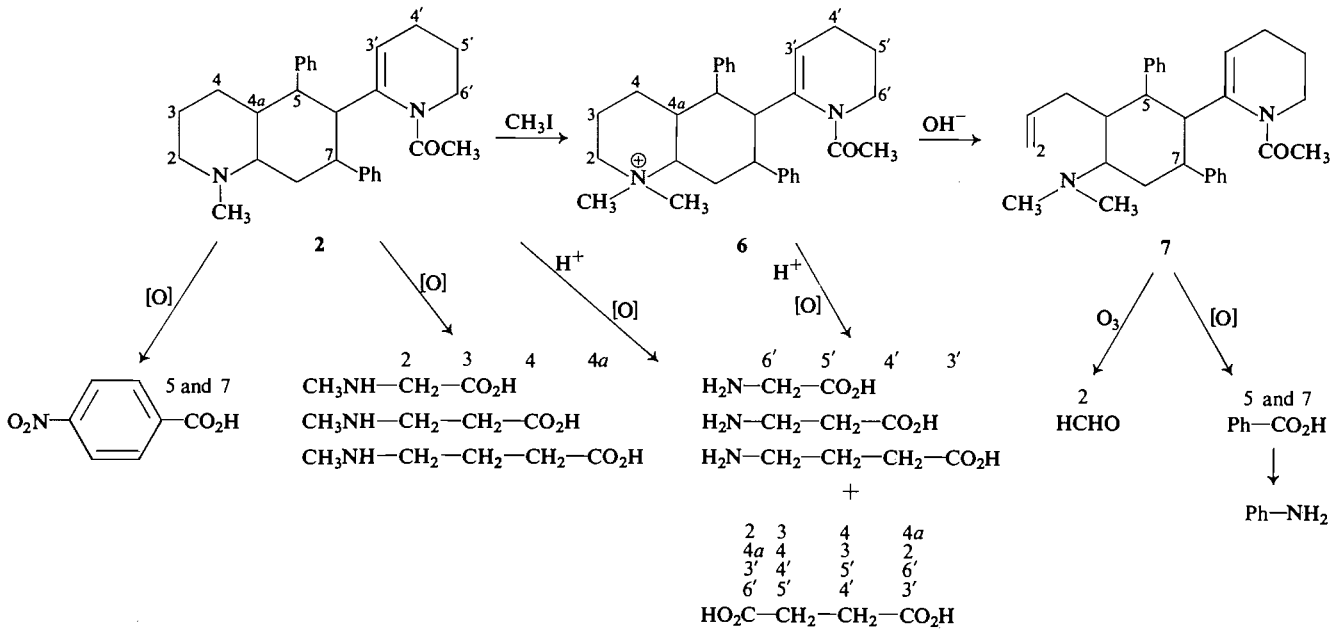


SCHEME 1. Incorporation of labelled precursors into sedamine (8).

TABLE 1. Incorporation of labelled compounds into lobinaline

Expt. no.	Compound administered	Nominal: Specific activity (mCi mmol^{-1})	Nominal: Total activity (mCi)	Date	Plants: No.	Plants: Dry weight (g)	Days	Acetyllobinaline: Yield (mg)	Acetyllobinaline: Specific activity (counts min^{-1} mmol^{-1}) $\times 10^{-4}$
1	2-^{14}C-DL-Phenylalanine*	1.1	0.1	September 1965	6	37	3	192	0.65 ± 0.02
2	3-^{14}C-DL-Phenylalanine†	10	0.1	September 1964	6	55	6	324	1.59 ± 0.05
3	2,3-$^{14}C_2$-DL-Phenylalanine‡			August 1966	15	156	3	956	5.21 ± 0.10
	from 2-^{14}C-DL-Phenylalanine§	1.8	0.1						
	from 3-^{14}C-DL-Phenylalanine‖	4.5	0.1						
4	2-^{14}C-Cinnamic acid¶	1.7	0.1	August 1965	8	23	3	60	2.02 ± 0.05
5	3-^{14}C-Cinnamic acid**	0.6	0.1	September 1965	12	46	3	270	3.95 ± 0.07
6	Carboxyl-^{14}C-Benzoic acid*	10	0.1	August 1965	8	26	4	91	0.06 ± 0.01
7	6-^{14}C-DL-Lysine††	2.3	0.05	September 1964	6	33	3	169	30.18 ± 0.70
8	6-^{14}C-DL-Lysine†	2.6	0.1	August 1966	6	68	3	260	29.47 ± 0.50
9	2-^{14}C-DL-Lysine‖	3.3	0.1	November 1966	8	87	3	480	13.58 ± 0.25
10	6-^{3}H,2-^{14}C-DL-Lysine‡			August 1968	10	90	3	215	19.74 ± 0.18 (^{14}C)
	from 6-^{3}H-DL-Lysine‖	6.9×10^3	0.8						
	from 2-^{14}C-DL-Lysine¶	1.3	0.1						
11	6-^{14}C-α-Phenacylpiperidine‡	1.0	0.075	September 1968	8	102	3	530	99.45 ± 1.03

*Volk Radiochemical Company.
†Commissariat à l'Énergie Atomique, France.
‡See Experimental.
§Nuclear Research Chemicals Inc.
‖New England Nuclear.
¶Tracerlab.
**Merck, Sharpe and Dohme.
††Calbiochem.



SCHEME 2. Degradation of acetyllobinaline.

TABLE 2. Incorporation of phenylpropanoid precursors into lobinaline

Precursor: Expt. no.:	3-^{14}C-DL-Phenylalanine 2		3-^{14}C-Cinnamic acid 5		2,3-$^{14}C_2$-DL-Phenylalanine 3	
Product	SA*	RSA†	SA	RSA	SA	RSA
Acetyllobinaline	1.59 ± 0.05	100 ± 3	1.64 ± 0.04‡	100 ± 2	5.21 ± 0.10	100 ± 2
p-Nitrobenzoic acid	0.76 ± 0.04	48 ± 3	0.78 ± 0.02	48 ± 2	1.43 ± 0.03	28 ± 1
p-Nitroaniline	0.01 ± 0.002	0.3 ± 0.1	0.01 ± 0.002	1 ± 0.3	—	

*Specific activity (counts min^{-1} $mmol^{-1}$) × 10^{-4}.
†Relative specific activity: percent (acetyllobinaline = 100).
‡Obtained from the original acetyllobinaline, specific activity (3.95 ± 0.07) × 10^4 counts min^{-1} $mmol^{-1}$, by dilution with inactive acetyllobinaline.

TABLE 3. Incorporation of 2,3-$^{14}C_2$-phenylalanine into lobinaline (expt. 3); distribution of label in precursor and product

Precursor	RSA*	Product	RSA*
2,3-$^{14}C_2$-Phenylalanine	100 ± 2†	Acetyllobinaline (2α ± 2β)	100 ± 2‡
3-^{14}C-	56 ± 2†	*p*-Nitrobenzoic acid (β)	28 ± 1‡
$\frac{\text{2,3-}^{14}C_2\text{-Phenylalanine}}{\text{3-}^{14}\text{C-}}$ = 1.78 ± 0.08		$\frac{\text{Acetyllobinaline (2α ± 2β)}}{\text{2 × p-Nitrobenzoic acid (2 × β)}}$ = 1.82 ± 0.05	

*Relative specific activity, percent.
†See Experimental.
‡See Table 2.

quarter of the activity of the intact alkaloid (expt. 7). Modification of the degradation procedure in more recent work (expt. 8) led to formaldehyde containing 40% of the total activity. This discrepancy is discussed below.

Acetyllobinaline, obtained from the experiment with 6-^{3}H,2-^{14}C-lysine (expt. 10), showed a $^3H/^{14}C$ ratio identical with that of the doubly labelled precursor. The $^3H/^{14}C$ ratio of the pipecolic acid, isolated from the same plants, was approximately 20% higher (Table 5).

The distribution of radioactivity within the acetyllobinaline derived from 6-^{14}C-α-phenacylpiperidine (expt. 11) (Table 6) was similar to that of the alkaloid sample derived from 6-^{14}C-lysine (expt. 8).

Discussion

Lysine and phenylalanine, the primary metabolites which supply the skeleton of sedamine (**5**) also serve as precursors of lobinaline (**1**). The mode of incorporation of each of these substrates into sedamine (Scheme 1) was deduced unequivocally on the basis of a chemical degradation which permitted isolation of individual carbon atoms in such a way that the total activity of the intact alkaloid was accounted for in terms of activity at individual sites (8). Chemical degradation of lobinaline, on the other hand, was only partial. Due to the inertness of the alkaloid towards a wide variety of reagents (*cf.* ref. 3), sustained attempts to devise reactions capable of yielding degradation products representing C-6, -8, -8*a*, and -2′ failed to give useful results. Even though the interpretation of some of the incorporation data therefore rests on indirect argument, the conclusions are, for the most part, unambiguous.

The results of the experiments with phenylpropanoid precursors (expts. 1–5) are consistent with the view that an intact C_6–C_2 unit, derived from phenylalanine, is incorporated into each of the two "halves" of lobinaline, in analogy with the mode of entry of the phenylalanine-derived fragment into sedamine (Scheme 1).

Label from 3-^{14}C-phenylalanine (expt. 2) and from 3-^{14}C-cinnamic acid (expt. 5) is confined to the carboxyl group of the degradation product, *p*-nitrobenzoic acid, whose specific activity was half that of lobinaline in both experiments (Table 2).

Lobinaline contains two *C*-phenyl groups. *Provided that the yield of p-nitrobenzoic acid derived from each of these two moieties is equal*, isolation of a sample of this acid, with a specific activity half that of the original lobinaline, accounts for the total activity of the alkaloid and indicates that either one or both of the two

TABLE 4. Incorporation of lysine into lobinaline

Precursor:	6-^{14}C-DL-Lysine						2-^{14}C-DL-Lysine	
Expt. no.:	7				8		9	
Product	SA*	RSA†	SA	RSA	SA	RSA	SA	RSA
Acetyllobinaline	6.63 ± 0.11‡	100 ± 2	2.26 ± 0.03‡	100 ± 1	7.76 ± 0.20‡	100 ± 3	13.58 ± 0.25	100 ± 2
Formaldehyde (C-2) (as dimedone derivative)	[1.63 ± 0.04	25 ± 1]§	—		3.06 ± 0.05	39 ± 1§	0.002 ± 0.006	0
Succinic acid	—		1.02 ± 0.04	45 ± 2	4.00 ± 0.11	51 ± 2		
Mixed DNP-amino acids‖	—		1.13 ± 0.04	50 ± 2	4.07 ± 0.05	52 ± 2	0.05 ± 0.01	0
DNP-β-Alanine	—		—		4.27 ± 0.07	55 ± 2	—	
Benzoic acid	0.01 ± 0.003	0	—		—		—	

*Specific activity (counts min^{-1} $mmol^{-1}$) × 10^{-4}.
†Relative specific activity: percent (acetyllobinaline = 100).
‡Obtained from the original sample of acetyllobinaline (Table 1) by dilution with inactive carrier.
§See Discussion.
‖For the calculation of the molar specific activity the molecular weight of DNP-β-alanine was used.

TABLE 5. Incorporation of 6-^{3}H,2-^{14}C-DL-lysine (expt. 10)

	^{3}H/^{14}C Ratio
6-^{3}H,2-^{14}C-DL-Lysine	14.9 ± 0.5
Acetyllobinaline	14.4 ± 0.1
Pipecolic acid	16.8 ± 0.1

C_6–C_1 units are specifically derived from phenylalanine. The relative specific activity of the two units is not necessarily the same, but distribution of activity must be such that the relationship, relative specific activity at C-5 plus relative specific activity at C-7 equals 100, is obeyed. However, the two *C*-phenyl moieties of lobinaline lie in different chemical environments. There is therefore no *a priori* reason why *p*-nitrobenzoic acid should arise at the same rate from the two sites. Since oxidation of lobinaline is not necessarily complete, it is more likely that the two sites produce different amounts of degradation product, and that these yields differ somewhat from experiment to experiment. *If on oxidation each of the two sites yields a different quantity of p-nitrobenzoic acid*, the resultant product will have a specific activity corresponding to half that of the intact alkaloid either purely fortuitously,[3] or only if each of the two sites, C-5 and -7, contains one half of the total activity of the original lobinaline.

Even though we incline towards the last mentioned interpretation of the results, the other possibilities cannot be entirely ruled out on the basis of the present results.[4]

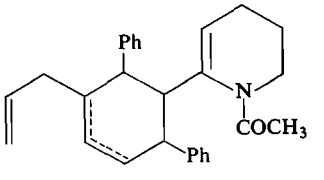
[3]Thus, *e.g.*, *p*-nitrobenzoic acid of the observed specific activity would be obtained from a sample of lobinaline containing 10% of its activity at C-5 and 60% at C-7, provided that four times as much *p*-nitrobenzoic acid is contributed to the final product by C-7 as is supplied by C-5. Under such circumstances the total activity of the intact alkaloid is not accounted for in the *p*-nitrobenzoic acid.

[4]Mention should be made of an attempt, albeit unsuccessful, to show by means of mass-spectroscopy whether both or only one of the phenyl nuclei of lobinaline had acquired label. A major mass-spectrometric fragment (*m/e* 186) of lobinaline has been related to a segment of the molecule (5), which happens to correspond to one of the two "biogenetic halves" of lobinaline. Mass spectrometry of labelled lobinaline, obtained from a feeding experiment with [ring $^2H_5$]-phenylalanine, should demonstrate whether the molecular ion of lobinaline was accompanied by a signal M + 10 or M + 5, and whether the fragment, *m/e* 186, was accompanied by a corresponding fragment, *m/e* 191. A feeding experiment with [ring $^2H_5$]phenylalanine was carried out but the deuterium content of the lobinaline which was isolated was too low to yield the desired information (*cf.* footnote 7).

The remaining ambiguity might have been dispelled, had we succeeded in perfecting degradations of lobinaline, capable of isolating C-6 and/or C-8, the presumed centers of activity in lobinaline derived from 2-^{14}C-phenylalanine (expt. 1) and 2-^{14}C-cinnamic acid (expt. 4). Attempts to isolate α-picolinic acid, whose carboxyl group would have represented C-6, by aromatization of the piperideine nucleus followed by oxidation (3), failed to yield more than traces of the desired product. Equally futile were attempts to gain access to C-8, as the 2-carboxyl group of quinolinic acid, by demethylation of lobinaline (3) followed by dehydrogenation (3) and oxidation. Another possible approach to C-8, exhaustive methylation at N-1, which led, via **6** and **7** (*cf.* ref. 5), to a neutral methine, *m/e* 473, presumably **8** (two possible positions of the double bond shown by dotted lines), was also abandoned because of low yields.

Even though specific incorporation of label from 2-^{14}C-phenylalanine and 2-^{14}C-cinnamic acid into the predicted sites of lobinaline was therefore not demonstrated, the results of two further experiments are, nevertheless, consistent with the view that intact C_6–C_2 units derived from phenylalanine are incorporated into the alkaloid.

The labelling pattern established by partial degradation (Table 2), within the lobinaline obtained from plants to which intermolecularly labelled 2,3-$^{14}C_2$-phenylalanine of known distribution of radioactivity was administered (expt. 3), was found to correspond to that of the precursor (Table 3). Such a numerical relation-



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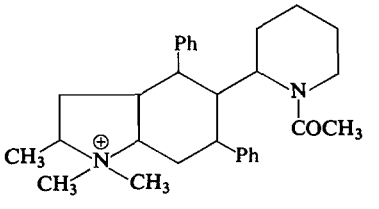

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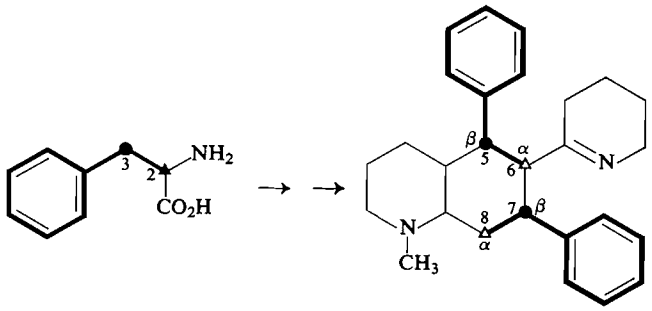
TABLE 6. Incorporation of 6-^{14}C-α-phenacylpiperidine into lobinaline (expt. 11)

Product	Specific activity (counts min^{-1} $mmol^{-1}$) $\times 10^{-4}$	Relative specific activity
Acetyllobinaline (diluted)	5.81 ± 0.06	100 ± 1
Formaldehyde (C-2) (as dimedone derivative)	2.30 ± 0.06	40 ± 1
Succinic acid	2.63 ± 0.08	45 ± 1
DNP-β-alanine	3.09 ± 0.06	53 ± 1



SCHEME 3. Incorporation into lobinaline of an intact C_6–C_2 unit derived from phenylalanine.

ship is as demanded by the hypothesis that intact C_6–C_2 units are incorporated and is most unlikely to be coincidental. The lack of incorporation of a C_6–C_1 precursor (expt. 6) is also consistent with this view.

Even though not entirely conclusive, the weight of evidence from expts. 1–6 favors the conclusion that, as in the case of sedamine, intact C_6–C_2 units supply the predicted moieties of lobinaline (Scheme 3).

The results of the experiments with labelled lysine (expts. 7–10) demonstrate that, in analogy with the biosynthesis of sedamine (8), a C_5N unit, derived from lysine by way of a nonsymmetrical intermediate, is incorporated into the piperidine nucleus of each of the two "halves" of lobinaline, and that each of the two "halves" of the molecule contains one half of the total activity (Table 4).

An early degradation result (July 1966) appeared to lead to a different conclusion. Formaldehyde, generated by ozonolysis of the Hofmann degradation product **7** (in the presence of unchanged lobinaline, see Experimental) obtained from lobinaline derived from 6-^{14}C-lysine, contained one quarter of the activity of the intact alkaloid (expt. 7, Table 4). This result demonstrated non-random entry of label from lysine into the alkaloid. Since it was presumed that the formaldehyde was derived solely from C-2 of lobinaline, the result appeared to indicate that four of the carbon atoms of the alkaloid were labelled. On this basis it might have been tempting to infer that the four labelled centers were C-2, -8*a*, -2′, and -6′, and that lysine had been incorporated into each of the two "halves" of lobinaline via a symmetrical intermediate. To provide further evidence in support of such an inference, confirmatory results had to be generated. At that time none of the crucial carbon atoms, other than C-2, were accessible. Evidence was sought in another way. If lysine had indeed entered by way of a symmetrical intermediate and activity from 6-^{14}C-lysine was distributed equally over four sites of lobinaline, it was an essential corollary that the same four sites would be labelled analogously when 2-^{14}C-lysine served as the precursor. Yet the formaldehyde derived from a sample of lobinaline isolated from the feeding experiment with 2-^{14}C-lysine (expt. 9) was completely inactive (Table 4). The earlier inference, that two symmetrical C_5N units had entered lobinaline, thus became untenable, since incorporation of the lysine-derived unit into ring A had clearly taken place in a non-symmetrical manner. The results might be plausibly interpreted to mean that the two "halves" of lobinaline were unequally labelled, and that they were therefore derived from related but unequal precursor units (10).

It became increasingly obvious that compelling evidence on the mode of incorporation of lysine into lobinaline would emerge only if the resistance of the molecule to degradation could be broken and degradation fragments could be obtained from each of the two hetero-rings, accounting for the total activity of the intact alkaloid. Success appeared assured when, on careful oxidation of lobinaline, a mixture of amino acids was obtained, from which β-alanine, contaminated with a little *N*-methyl-β-alanine, was isolated as the major component (Scheme 2). The specific activity of this mixture, obtained from the lobinaline derived from 6-^{14}C-lysine (expt. 7, Table 4), was half of that of the original alkaloid.

This result can be interpreted in a variety of ways, on the basis of arguments analogous to those adduced in the interpretation of the data obtained in the experiment with 3-^{14}C-phenylalanine, above. The most likely, though by no means the only, explanation of the activity pattern found in the amino acid mixture was that each of the components of the mixture, β-alanine and *N*-methyl-β-alanine, were equally labelled, presumably at the C-atoms derived, respectively, from C-6′ and -2 of the original alkaloid, and that the two "halves" of lobinaline contained equal activity. This inference, which was supported by the observation that succinic acid obtained in the same degradation also showed a specific activity corresponding to one half that of the alkaloid, was in direct contradiction to the earlier result, that formaldehyde (thought to be derived solely from C-2) showed only one quarter of the initial activity. This result thus became suspect. The mode of incorporation of 6-^{14}C-lysine into lobinaline was therefore reinvestigated (expt. 8).

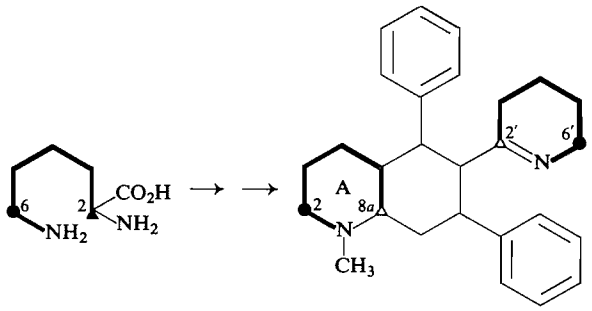
Succinic acid, and the mixture of the *N*-dinitrophenyl derivatives of β-alanine and *N*-methyl-β-alanine, obtained from the oxidation of this sample of lobinaline, again contained approximately one half of the activity of the intact alkaloid. So did a pure sample of *N*-dinitrophenyl-β-alanine, obtained by oxidation of the corresponding lobinaline methohydroxide (**6**) (Scheme 2). Since β-alanine represents C-6′, -5′, -4′, of the piperideine nucleus of lobinaline,[5] it follows that this fragment contains one half of the total activity of the alkaloid, presumably[6] at C-6′.

The other half of the activity of the intact alkaloid would be expected to reside at C-2. This carbon is contained in the degradation fragment, *N*-methyl-β-alanine. Since the specific activity of the mixture of β-alanine and *N*-methyl-β-alanine and that of β-alanine corresponded to approximately one half that of the intact alkaloid, *N*-methyl-β-alanine must also contain approximately one half the original activity. This activity is presumably contained at C-2, the carbon which is the source of the formaldehyde obtained on degradation. Yet, in the earlier experiment with 6-^{14}C-lysine (expt. 7), this formaldehyde had contained one quarter, rather than one half of the total activity. A modified procedure for the ozonolysis of the Hofmann product involving shorter exposure to ozonized oxygen yielded formaldehyde which contained approximately 40, and not 25%, of the activity of the original alkaloid. It must be concluded that in the course of the oxidation formaldehyde is generated not only from C-2, but also from one of the *N*-methyl groups of the methine base **7** or of the contaminating acetyllobinaline, presumably by way of the corresponding *N*-oxides. The inactive formaldehyde so produced then causes lowering of the specific activity of the formaldehyde derived from C-2. This contamination with inactive formaldehyde can be minimized but cannot be entirely prevented. Analogous findings in the course of the osmium tetroxide–periodate oxidation of a similarly ^{14}C-labelled Hofmann product have recently been reported (11).

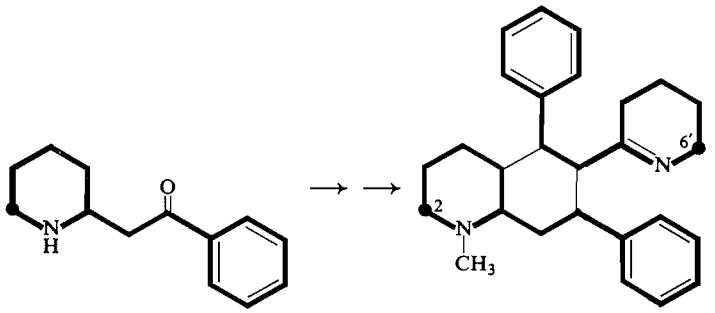
The evidence from expts. 7–9 indicates that activity from ^{14}C-lysine is equally divided between the two "halves" of lobinaline, and that the two heterocyclic rings are derived from lysine via a non-symmetrical intermediate (Scheme 4). Non-symmetrical incorporation of lysine is confirmed by the result of the experiment with 6-^{3}H,2-^{14}C-lysine (expt. 10). The ^{3}H/^{14}C ratio of this doubly labelled precursor was maintained in lobinaline (Table 5), indicating that loss of tritium relative to carbon had not occurred in the course of biosynthesis. Symmetrical incorporation of a lysine-derived precursor would have

[5]The possibility cannot be excluded, however, that β-alanine may originate also from ring A (C-2, -3, -4) of lobinaline, as a consequence of oxidative demethylation.

[6]Further degradation of the β-alanine was precluded by lack of material.



SCHEME 4. Incorporation into lobinaline of a C_5N unit derived from lysine.



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SCHEME 5. Incorporation into lobinaline of α-phenacylpiperidine.

placed C-6 of lysine at C-2′, as well as at C-6′ of lobinaline. Since C-2′ does not carry a hydrogen atom, such symmetrical incorporation would have demanded loss of tritium, relative to ^{14}C.

Incorporation of lysine, as well as of phenylalanine, into lobinaline, thus takes place in complete analogy with the entry of the two substrates into sedamine. Furthermore, it appears that the lysine-derived C_5N unit and the phenylalanine-derived C_6–C_2 unit enter both "halves" of lobinaline at the same rate, since in every experiment in which a degradation was performed, approximately half of the activity of the intact alkaloid was recovered in fragments representing each of the two "halves" of lobinaline.

The evidence is thus entirely consistent with the notion that lobinaline is, from the point of view of its biosynthesis, a modified dimer of a C_6–C_2–C_5N unit, related to sedamine.

A final experiment strengthened this view. 6-^{14}C-α-Phenacylpiperidine (3) (12) was administered to *L. cardinalis* (expt. 11). Partial degradation of the highly radioactive lobinaline which was isolated indicated, within the limitations of the degradation procedures discussed earlier, that both "halves" of the molecule were labelled at the predicted sites (C-2 and -6′) (Scheme 5), and that each of these sites carried one half of the activity of the precursor[7] (Table 6). The hypothesis that lobinaline originates from a monomer unit related to α-phenacylpiperidine demands exactly such a distribution of activity. The results here reported support this hypothesis.

Experimental

Isolation of Lobinaline

The dried aerial parts[8] of *Lobelia cardinalis* L. (40 g) were powdered in a blender (Osterizer Galaxie 10), the powder was moistened with aqueous ammonia (2 *M*, 25 ml), the mixture was allowed to stand for 1 h, and was continuously extracted with ether for 36 h. The ether extract was then extracted with hydrochloric acid (2 *M*, 5 × 5 ml). The acid extract was repeatedly washed with ether (10 ml portions), until the ether extract was no

An attempt to demonstrate, by mass-spectrometry, incorporation of ^{15}N from ^{15}N-α-phenacylpiperidine (prepared (*cf.* ref. 12) from ε-^{15}N-DL-lysine) failed to yield conclusive results. The ^{15}N content of the lobinaline which was obtained was too low for direct mass-spectrometric demonstration of heavy species.

[8]Preliminary experiments showed that the roots of the plant contained negligible amounts of the alkaloid.

longer colored. The acid solution was made strongly alkaline by addition of potassium hydroxide solution (10% w/v), and the basic components were extracted into hexane (5 × 30 ml). The combined hexane extract was dried (sodium sulfate) and the solvent evaporated, yielding crude lobinaline as a dark semicrystalline solid. Attempted purification of lobinaline by chromatography on alumina and crystallization as the hydrochloride (5) or the free base (3) was accompanied by significant losses of material.

Purification was achieved by conversion to acetyllobinaline. The crude product was refluxed with acetic anhydride for 5 min and solvent was evaporated *in vacuo*. The residue was dissolved in water, the aqueous solution was washed with ether and made alkaline by addition of aqueous potassium hydroxide (10% w/v). The mixture was extracted with ether (3 × 20 ml), the ether extracts were dried, solvent was evaporated, and the residue was recrystallized from hexane and sublimed at 190–200° and 3×10^{-3} mm. Acetyllobinaline (350 mg), m.p. 158–159°, was obtained (mass spectrum *m/e* 428). Reported m.p. 159–160° (3).

p-Nitrobenzoic Acid by the Oxidation of Acetyllobinaline

A mixture of acetyllobinaline (175 mg) and concentrated nitric acid (5 ml) was heated on the steam bath until nitric acid had evaporated. The residue was dissolved in sodium hydroxide solution (5% w/v, 10 ml), finely powdered potassium permanganate (1.2 g) was added, and the mixture heated overnight with continuous stirring. The mixture was cooled and acidified with dilute sulfuric acid, a stream of sulfur dioxide was passed through, and the solution was then extracted with ether. The ether extract was washed with water, dried, and evaporated. The residue was crystallized from aqueous methanol and sublimed at 120–125° and 5×10^{-3} mm, yielding a crystalline solid (40 mg), m.p. 239–240°, mass spectrum *m/e* 167, identical (mixture m.p., i.r. spectrum) with an authentic sample of *p*-nitrobenzoic acid.

p-Nitroaniline from p-Nitrobenzoic Acid (13)

p-Nitrobenzoic acid (50 mg) was dissolved in chloroform (5 ml), and a mixture of oleum (10%, 2.5 ml) and concentrated sulfuric acid (0.5 ml) was added, followed by sodium azide (5 mg). The mixture was refluxed 3 h with rapid stirring, cooled, and poured onto crushed ice (25 g). The chloroform layer was separated and rejected. The aqueous layer was made alkaline with sodium hydroxide (1 *M*) and extracted with ether (3 × 10 ml). The ether layer was washed with water, dried, and the solvent was evaporated. The residue was sublimed at 130–140° and 3×10^{-3} mm, yielding *p*-nitroaniline (25 mg) melting at 147–148°.

Hofmann Degradation of Acetyllobinaline

Acetyllobinaline (200 mg) was dissolved in dry acetone (4 ml), methyl iodide (2 ml) was added, and the mixture was allowed to stand 48 h at room temperature, and was then evaporated to dryness, yielding a foamy residue which did not crystallize. The residue was repeatedly triturated with ether, and the ether extracts containing unreacted acetyllobinaline were decanted. The residue was dissolved in hot aqueous methanol (50% v/v) and freshly precipitated silver oxide (300 mg) was added. The mixture was allowed to stand 4 h in the dark, solid was filtered off, the filtrate and washings were combined, solid potassium hydroxide (1 g) was added, and the solvent was evaporated.

Basic components were recovered from the residue by high vacuum sublimation at 190–200° and 5×10^{-3} mm. The sublimate was a mixture of unchanged acetyllobinaline (~70%) and the methine base (7) (~30%). In a pilot experiment the latter was separated by the general method described for the purification of methine bases derived from *trans*-decahydroquinoline systems (14). The mixture was refluxed 6 h with a solution containing acetic anhydride (2.5 ml) and glacial acetic acid (2.5 ml). The solvents were then evaporated, the residue was dissolved in water, and the solution made alkaline with potassium hydroxide (1 *M*), and was extracted with ether to remove unreacted acetyllobinaline. The aqueous layer was saturated with potassium iodide and extracted with chloroform, and the solvent evaporated. A quaternary salt, presumably 9 (*cf.* ref. 14) (n.m.r. spectrum: *C*-methyl, doublet, centered at 1.6 p.p.m.) remained. It was dissolved in hot water, freshly precipitated silver oxide was added, and the hot solution was left for 2 h in the dark. Solid was filtered off, the solution was evaporated, and the residue was heated *in vacuo*. The methine base 7 (*cf.* ref. 5) sublimed at 150–180° and 3×10^{-3} mm as a gummy mass which did not crystallize (mass spectrum, molecular ion *m/e* 442).

This separation was not used in the degradation of labelled samples. The mixed crude bases were dissolved in a mixture of ethyl acetate (3 ml) and acetic acid (2 ml). The solution was cooled in ice/salt mixture and ozonized oxygen was passed, in early experiments for 15 min, and in more recent runs for less than 5 min. The solution was then diluted with water (40 ml), zinc dust (50 mg) was added, and the mixture was concentrated until 25 ml distillate had been collected.

5,5-Dimethyl-1,3-cyclohexanedione (dimedone) (25 mg) was added to the distillate which was then allowed to stand at room temperature overnight. Formaldehyde dimethone (10 mg), which crystallized when the solution was concentrated, was recrystallized from aqueous methanol, and after sublimation at 120–140° and 3×10^{-3} mm melted at 186–187°.

The reaction mixture remaining after removal of formaldehyde by distillation was made alkaline by addition of aqueous sodium hydroxide (1 *M*), and was then extracted with ether. The ether extract was dried, solvent was evaporated, and water (20 ml) and powdered potassium permanganate (1 g) were added to the residue. The mixture was heated 24 h under reflux with constant stirring. The mixture was cooled and acidified (1 *M* sulfuric acid) and sulfur dioxide was passed until all manganese dioxide had dissolved. The solution was extracted with ether (3 × 10 ml), the extract was washed with water (1 ml), dried and evaporated, the residue was sublimed at 60° and 5×10^{-3} mm, and the sublimate was crystallized from aqueous methanol. Resublimation yielded benzoic acid (10 mg), m.p. 120–121°.

β-*Alanine from Acetyllobinaline*

(a) Chromic Acid Oxidation of Acetyllobinaline

Acetyllobinaline (200 mg) was suspended in aqueous sulfuric acid (5 ml, 30% v/v) and the mixture was refluxed for 1 h with continuous stirring. Chromic acid (1 g) in

water (1.5 ml) was then added and refluxing was continued for 36 h with stirring.

Excess chromic acid was destroyed by addition of a few drops of methanol and the mixture was continuously extracted with ether for 24 h. The ether extract was worked-up for succinic acid, the residue was diluted with water (100 ml), and worked-up for amino acids.

The ether layer was extracted with water (5 × 1 ml), the aqueous extract was washed with ether (2 ml) and was then evaporated to dryness. The residue was sublimed at 110–130° and 5×10^{-3} mm, yielding succinic acid (5 mg), m.p. 181–182°, identical with an authentic sample.

The aqueous solution containing the residue remaining after extraction of the chromic acid oxidation mixture with ether was heated on the steam bath and neutralized (p*H* 6–7) by addition of hot barium hydroxide solution. After 1 h the mixture was filtered, the precipitate was washed with hot water (3 × 10 ml), and the combined filtrate and washings were concentrated, treated with charcoal and filtered. Sodium carbonate (25 mg) and methanolic 2,4-dinitrofluorobenzene (10% v/v, 1 ml) was added, and the mixture was allowed to stand at room temperature with occasional stirring. The solution whose p*H* was still above p*H* 8 was extracted with ether, and the extract discarded. The solution was then acidified with hydrochloric acid (2 *M*) and was extracted with ether. The ether extract was dried, solvent was evaporated under reduced pressure, and the residue was heated at 80° and 3×10^{-3} mm to remove 2,4-dinitrophenol by sublimation. The deep yellow residue was dissolved in chloroform (0.5 ml) and the solution was applied to two plates (20 × 20 cm) coated with silica gel (2 mm thickness). Development with benzene/pyridine/acetic acid 80:20:2 (15) led to the appearance of three well separated major bands, whose R_f value corresponded to those of authentic samples of the *N*-dinitrophenyl derivatives of glycine (R_f 0.28), β-alanine (R_f 0.72), and γ-aminobutyric acid (R_f 0.82). A minor band of higher R_f value was not identified.

The band corresponding to *N*-dinitrophenyl-β-alanine was scraped off the plate and eluted thoroughly with methanol. The methanol was evaporated, the residue was dissolved in hydrochloric acid (1 ml, 1 *M*) and the solution extracted with ether (3 × 5 ml). The ether extract was washed with water (1 ml) and dried (sodium sulfate), the solvent evaporated, and the residue sublimed at 150–160° and 3×10^{-3} mm.

The sublimate did not crystallize. Its mass spectrum showed, in addition to the expected molecular ion of *N*-dinitrophenyl-β-alanine, *m/e* 255, a signal at *m/e* 269, presumably due to *N*-dinitrophenyl-*N*-methyl-β-alanine. The sublimate was esterified by treatment with diazomethane in ether. The n.m.r. spectrum of the product in deuterochloroform showed a singlet (3.03 p.p.m.) characteristic for *N*-methyl. The mixture of the *N*-dinitrophenyl derivatives of β-alanine and *N*-methyl-β-alanine, or of their methyl esters, resisted all attempts at separation by thin layer, column, or gas–liquid chromatography.

(b) Chromic Acid Oxidation of Acetyllobinaline Methohydroxide

Methyl iodide (2 ml) was added to a solution of acetyllobinaline (300 mg) in acetone (2 ml), the mixture was allowed to stand 48 h at room temperature and was then evaporated to dryness. The residue was triturated with ether to remove unreacted acetyllobinaline. The residue was dissolved in hot aqueous methanol (5 ml, 50% v/v), freshly precipitated silver oxide (400 mg) was added, and the mixture was kept in the dark for 4 h. Solid was filtered off and washed with hot water (2 × 5 ml), and filtrate and washings were combined and evaporated, yielding a residue which was dissolved in aqueous sulfuric acid (5 ml, 30% v/v) and refluxed 1 h with continuous stirring. Chromic acid (1 g) in water (1.5 ml) was then added and refluxing was continued for 36 h with stirring.

Further work-up of the reaction mixture was as described in the case of the direct chromic acid oxidation of acetyllobinaline, above. Succinic acid was isolated from the ether soluble fraction of the oxidation mixture. Amino acids, present in the aqueous layer, were converted into their *N*-dinitrophenyl derivatives, which were separated by thin layer chromatography, as described above. Three major bands were again observed.

The band corresponding to *N*-dinitrophenyl-β-alanine was scraped off the plate and eluted thoroughly with methanol. The methanol was evaporated, the residue was dissolved in dilute hydrochloric acid, and the solution extracted with ether. The ether extract was washed with a little water, the solution was dried (sodium sulfate), the solvent was evaporated and the residue sublimed at 150–160° and 3×10^{-3} mm, yielding *N*-dinitrophenyl-β-alanine (10 mg), m.p. 142–144° (reported m.p. 145–146° (16)), which was further purified by crystallization from a mixture of methanol and ether, followed by resublimation (mass spectrum, molecular ion *m/e* 255).

N-2,4-Dinitrophenyl Derivatives

Authentic samples of the *N*-dinitrophenyl derivatives of ω-amino acids, which were required for comparison with the samples derived from acetyllobinaline, were obtained as follows.

N-Dinitrophenylglycine, N-Dinitrophenyl-β-alanine, and N-Dinitrophenyl-γ-aminobutyric Acid

These samples were commercial products (Sigma Chemical Co.).

N-Dinitrophenyl-N-methyl-β-alanine

3-Methylaminopropionitrile (420 mg, Aldrich Chemical Co.) was dissolved in methanol (5 ml), and sodium hydroxide (1 *M*, 5.2 ml) was added. The mixture was refluxed 6 h, the solvents were then evaporated and the residue triturated with ether to remove unchanged nitrile or amide. The residue was then dissolved in water (5 ml) and the solution was brought to p*H* 10 by addition of a few drops of 1 *M* hydrochloric acid. Methanolic 2,4-dinitrofluorobenzene (10% v/v, 5 ml) was added and the mixture was allowed to stand 2 h at room temperature with occasional shaking. Water was added, the solution was extracted with ether, and the ether extract rejected. The aqueous solution was acidified and extracted with ether, the ether extract was washed with water, dried over anhydrous sodium sulfate, and concentrated. *N-Dinitrophenyl-N-methyl-β-alanine* crystallized spontaneously, and was further purified by sublimation at 5×10^{-3} mm and 130–140°, m.p. 142–143°. Mass spectrum, molecular ion *m/e* 269.

Anal. Calcd. for $C_{10}H_{11}N_3O_6$: C, 44.61; H, 4.12; N, 15.61. Found: C, 44.45; H, 4.05; N, 15.37.

N-Dinitrophenyl-N-methyl-γ-aminobutyric Acid

A mixture of *N*-methyl-2-pyrrolidone (500 mg, Aldrich Chemical Co.) and concentrated hydrochloric acid (2 ml) was refluxed 4 h. The solution was evaporated to dryness and the residue was repeatedly extracted with ether to remove unchanged starting material, and was then recrystallized from methanol/ether (17). The *N*-methyl-γ-aminobutyric acid hydrochloride so obtained was converted, by the standard procedure outlined above, into *N-dinitrophenyl-N-methyl-γ-aminobutyric acid*, m.p. 104–105°, after recrystallization from aqueous methanol. Mass spectrum, molecular ion *m/e* 283.

Anal. Calcd. for $C_{11}H_{13}N_3O_6$: C, 46.64; H, 4.63; N, 14.84. Found: C, 46.87; H, 4.57; N, 14.74.

Methyl Esters of N-Dinitrophenyl-ω-amino Acids (cf. ref. 18)

The *N*-dinitrophenyl-ω-amino acid (20 mg) was dissolved in methanol (5 ml), a solution of diazomethane in ether was added, and the mixture was allowed to stand at room temperature overnight. Solvents were evaporated under reduced pressure and the dark red oily residue was dissolved in chloroform (0.5 ml) and applied to a column (1 × 10 cm) packed with neutral alumina, activity I. The product was eluted with benzene, the eluate was concentrated to dryness, and the residue crystallized from methanol and sublimed at $\sim 10^{-3}$ mm.

The following esters were prepared:

Methyl 3-[2,4-dinitrophenylamino]propionate (methyl ester of *N*-dinitrophenyl-β-alanine) m.p. 97°; mass spectrum, molecular ion *m/e* 269.

Anal. Calcd. for $C_{10}H_{11}N_3O_6$: C, 44.61; H, 4.12; N, 15.61. Found: C, 44.49; H, 3.98; N, 15.46.

Methyl 4-[2,4-dinitrophenylamino]butyrate (methyl ester of *N*-dinitrophenyl-γ-aminobutyric acid) m.p. 52°; mass spectrum, molecular ion *m/e* 283.

Anal. Calcd. for $C_{11}H_{13}N_3O_6$: C, 46.64; H, 4.63; N, 14.84. Found: C, 46.43; H, 4.60; N, 15.07.

Methyl 3-[N-2,4-dinitrophenyl-N-methylamino]propionate (methyl ester of *N*-dinitrophenyl-*N*-methyl-β-alanine) m.p. 103°; mass spectrum, molecular ion *m/e* 283, n.m.r. spectrum, *N*-methyl, singlet 3.03 p.p.m.

Anal. Calcd. for $C_{11}H_{13}N_3O_6$: C, 46.64; H, 4.63; N, 14.84. Found: C, 46.77; H, 4.71; N, 15.04.

Methyl 4-[N-2,4-dinitrophenyl-N-methylamino]butyrate (methyl ester of *N*-dinitrophenyl-*N*-methyl-γ-aminobutyric acid) oily, mass spectrum, molecular ion *m/e* 297.

Administration of Labelled Compounds to Lobelia cardinalis

Plants of *Lobelia cardinalis* L. were obtained from Sheridan's Nursery, Port Credit, Ontario. Plants in full bloom were used in all feeding experiments. The labelled compound was dissolved in distilled water (10 ml), and equal portions of the solution were administered to individual plants by aspiration into the stem through a cotton wick. In most experiments the plants were kept in contact with tracer for 3 days. The labelled compounds which were administered to the plants in separate experiments are listed in Table 1.

The sample of doubly labelled phenylalanine (expt. 3) was prepared by mixing [2-^{14}C]-DL-phenylalanine (Nuclear Research Chemicals Inc., 9.3 mg, total activity $(4.75 \pm 0.17) \times 10^7$ c.p.m., specific activity $(0.84 \pm 0.03) \times 10^9$ c.p.m. mmol^{-1}) and [3-^{14}C]-DL-phenylalanine (New England Nuclear, 3.7 mg, total activity $(6.07 \pm 0.22) \times 10^7$ c.p.m., specific activity $(2.71 \pm 0.10) \times 10^9$ c.p.m. mmol^{-1}). This gave intermolecularly labelled [2,3-$^{14}C_2$]-DL-phenylalanine (13.0 mg, total activity $(10.82 \pm 0.28) \times 10^7$ c.p.m., specific activity $(13.73 \pm 0.34) \times 10^8$ c.p.m. mmol^{-1}), with a specific activity at C-2 of $(6.03 \pm 0.22) \times 10^8$ c.p.m. mmol^{-1} ($43.9 \pm 1.9\%$) and a specific activity at C-3 of $(7.70 \pm 0.27) \times 10^8$ c.p.m. mmol^{-1} ($56.1 \pm 2.4\%$).

The sample of doubly labelled lysine (expt. 10) was prepared by mixing [2-^{14}C]-DL-lysine (Tracerlab, 14.5 mg, nominal total activity 0.1 mCi) and [6-^{3}H]-DL-lysine (New England Nuclear, 0.021 mg, nominal total activity 0.8 mCi. The ^{3}H/^{14}C ratio of the sample (Table 5) was determined by liquid scintillation counting.

The sample of [6-^{14}C]-α-phenacylpiperidine (expt. 11) was prepared from [6-^{14}C]-DL-lysine (Commissariat à l'Énergie Atomique, France) by the method described in an earlier paper (12).

Isolation of Labelled Lobinaline and Pipecolic acid from L. cardinalis

Labelled lobinaline was isolated from the dried stems and leaves of the plants used in each of the 11 tracer experiments. Chemical and radiochemical yields are summarized in Table 1.

Radioactive pipecolic acid was isolated by carrier dilution from plants of *L. cardinalis* to which 6-^{3}H,2-^{14}C-DL-lysine had been administered. The dried plant material was first extracted with ether to isolate lobinaline (see above), and was then further extracted with methanol (12 h). Solvent was evaporated, and unlabelled pipecolic acid (250 mg) was added to the residue which was then extracted with water (5 × 5 ml). Pipecolic acid was then re-isolated by a method described in an earlier publication (19).

Determination of Radioactivity

Radioactivity was assayed, on samples of finite thickness on aluminum planchettes, with a gas-flow system (Model 4342, Nuclear Chicago). The usual corrections for background and self-absorption were applied. The counting efficiency for ^{14}C was approximately 30%. For plating, a 1% solution of collodion in dimethylformamide was used as the solvent. In the cases of benzoic and *p*-nitrobenzoic acid, a drop of a 5% solution of sodium hydroxide in 50% aqueous methanol was added to prevent loss by evaporation.

The ^{3}H/^{14}C ratios were measured by liquid scintillation counting (Mark I liquid scintillation computer, Model 6860, Nuclear Chicago). Activity due to ^{3}H and ^{14}C was determined simultaneously, by external standardization counting with ^{133}Ba. Samples were dissolved in methanol or methanol–water and the solution was dispersed in a solution of Liquifluor (Nuclear Chicago) diluted 25 times with toluene. Duplicate samples of each compound were counted under comparable conditions of quenching. For highly quenched samples the confidence limits of the quench correction curves were ±5%.

Confidence limits shown in the Tables are standard deviation of the mean.

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