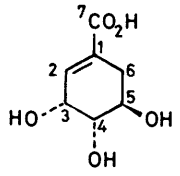
The Biosynthesis of Phenazines: Incorporation of [^{14}C]Shikimic Acid †

By **Richard B. Herbert, Frederick G. Holliman,* P. Nicholas Ibberson,** and **John B. Sheridan,** Department of Organic Chemistry, The University, Leeds LS2 9JT

Specific and self-consistent incorporations of [1-^{14}C]-, [6-^{14}C]-, and [1,6,7-$^{14}C_3$]-shikimic acid into iodinin in *Brevibacterium iodinum* closely define the orientation of the precursor molecule in the phenazine metabolite. [1,6,7-$^{14}C_3$]Shikimic acid gave phenazine-1-carboxylic acid with one fifth of the activity in the carboxy-group, which requires the involvement of two precursor molecules in biosynthesis or incorporation *via* a symmetrical intermediate derived from only one precursor molecule. Neither [^{3}H]anthranilic acid nor [^{14}C]dihydrohydroxyanthranilic acid was significantly incorporated into iodinin. Decarboxylation of [*ring*-^{14}C]pyrazinetetracarboxylic acid under various conditions has been studied; with copper chromite, but not with copper–bipyridyl–quinoline, radioactivity (up to 12%) appeared in the liberated carbon dioxide.

SHIKIMIC ACID (1), an intermediate in the biosynthesis of aromatic amino-acids, has been identified as an important precursor of microbial phenazines, *e.g.* phenazine-1-carboxylic acid (2) and pyocyanin (4).[1-5] Results of



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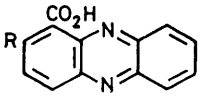
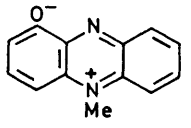


(2) R = H
(3) R = OH



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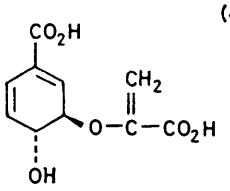

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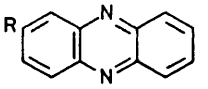


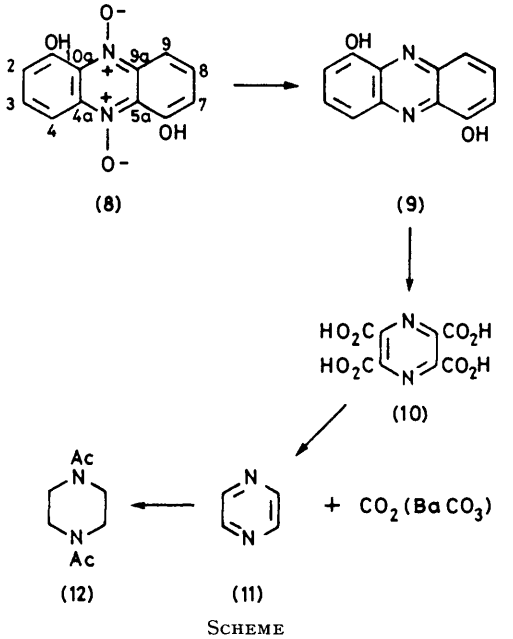
(6) R = H
(7) R = OH

studies with *Pseudomonas aeruginosa* mutants have led to the conclusion that the entire pyocyanin skeleton may be formed from shikimic acid [3] and that the branch-point for aromatic amino-acid synthesis and phenazine production occurs no later than chorismic acid (5).[6] Labelled chorismic acid has been found to be incorporated poorly into (2), but this may be attributed to lack of transport to the site of phenazine biosynthesis.[4]

Degradation of pyocyanin (4), phenazine-1-carboxylic acid (2),[4] and iodinin (8) [5] obtained after feeding (±)-[1,6-$^{14}C_2$]shikimic acid [as (1)] establishes that this compound is a specific precursor, but different results were obtained for the manner of this incorporation for iodinin (8) on the one hand, and phenazine-1-carboxylic acid (2) and pyocyanin (4) on the other. Apart from this lack of correspondence between the results, the use of this doubly labelled material does not allow unambiguous definition of the orientation of the shikimic acid units. We therefore set out to establish the mode of incorporation of (1) into the phenazine ring system using singly labelled shikimic acid samples.

The [1-^{14}C]- and [6-^{14}C]-shikimic acids [as (1)] used in our experiments were obtained from the appropriately labelled pyruvic acids using an excellent biochemical method *via* phosphoenolpyruvate.[7] However, lower yields than those reported were obtained for the conversion of phosphoenolpyruvate into shikimic acid [produced as the natural (−)-isomer].

The shikimic acids were administered to *Brevibacterium iodinium* cultures during the early stages of phenazine formation and the iodinin (8) produced isolated some 24—48 h later. Degradation of the iodinin was carried out (Scheme) with small but important modifications of the procedure used previously [4,5,8]



SCHEME

† Preliminary communications, R. B. Herbert, F. G. Holliman, and P. N. Ibberson, *Tetrahedron Letters*, 1974, 151; R. B. Herbert, F. G. Holliman, and J. B. Sheridan, *ibid.*, p. 4201: *ibid.*, 1976, 639.

TABLE 1

Incorporation of [^{14}C]shikimic acid into iodinin, and distribution of activity

Positions of ^{14}C label in shikimate precursors	Incorporation [a] (%)	Distribution of activity as % of phenazine-1,6-diol (9): Pyrazinetetracarboxylic acid (10) [b]	Pyrazine (11)	Carbon dioxide [c]
1	42	100	0	52, 67 [d]
		102	0	100, 101 [e]
6	31	102	113, [f] 100 [g]	1.2 [e]
1, 6, 7	34 [h]	99	55, [f] 47 [g]	49 [e]
			56, [f] 50 [g]	50 [e]

[a] Based on phenazine-1,6-diol. [b] Radioactivity determined on tetramethyl ester. [c] Radioactivity determined on barium carbonate. [d] Copper chromite decarboxylation. [e] Copper–bipyridyl–quinoline decarboxylation. [f] Radioactivity measured on pyrazine assayed by u.v. [g] Radioactivity determined on *NN*-diacetylpiperazine (12). [h] Allowing for loss of 1/3 of the activity.

(see Experimental section). The iodinin (8) obtained after feeding [6-^{14}C]shikimic acid [as (1)] at first gave an unexpected distribution of label (22% confined to C-1, C-4, C-6, and C-9 with 74% confined to C-4a, C-5a, C-9a, and C-10a), which appeared to be supported by the fact that the summed activity in the barium carbonate and *NN*-diacetylpiperazine (12) was close to that of the pyrazinetetracarboxylic acid (10) from which they were derived. However, results with [1-^{14}C]shikimic acid, which should have been complementary, were not. Up to 48% of the activity of (10) was unaccounted for in the *NN*-diacetylpiperazine (12) and barium carbonate (Table 1). The two results were however reconcilable if some of the carbon dioxide collected in the course of the copper chromite decarboxylation was to have its origins in the pyrazine ring of (10), label from the ring appearing in otherwise inactive carbon dioxide and inactive carbon dioxide from an unlabelled ring lowering the specific activity of radioactive carbon dioxide from the carboxy-groups of (10). This was supported when complementary results were obtained on the two samples of (10) using copper–bipyridyl–quinoline, rather than copper chromite, for the decarboxylation (Table 1). Proof was obtained by showing that synthetic [*ring*-^{14}C]pyrazinetetracarboxylic acid gave radioactive carbon dioxide on treatment with copper chromite; essentially inactive carbon dioxide was obtained using copper–bipyridyl–quinoline (Table 2). Other workers have used copper chromite for the decarboxylation of pyrazinetetracarboxylic acid and their results are seen to be numerically in error as a result of this observation. Our results (Tables 1 and 2) also show that estimation of activity by u.v. assay of the pyrazine, also used previously, may not be reliable.

Use of copper–bipyridyl–quinoline for decarboxylation of pyrazinetetracarboxylic acid (10) derived from (−)-[1-^{14}C]-, (−)-[6-^{14}C]-, and (−)-[1,6,7-^{14}C$_3$]-shikimic acid [as (1)] gave the results shown in Table 1 (careful drying of the *NN*-diacetylpiperazine was also necessary for satisfactory results). The data prove that for iodinin (8), shikimic acid (1) relates to the phenazine nucleus as in (13) (shikimic acid numbering). This corroborates other results [8] obtained for (8) and phenazine-1-carboxylic acid (2), and it follows that the metabolites derived from (2) [9] have a similar genesis, as probably do all microbial phenazines.

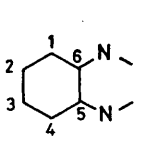
TABLE 2

Decarboxylation of [*ring*-^{14}C] pyrazinetetracarboxylic acid (9.98×10^3 disint. s^{-1} $mmol^{-1}$)

Reagent	Pyrazine (11) [a] (%)	*NN*-Diacetylpiperazine (12) (%)	Barium carbonate (%)
Copper–chromite	121	97	6.8
	111	96	12
Copper-bipyridyl–quinoline	121	95	1.4
	103	99	0.7

[a] Activity measured on pyrazine assayed by u.v.

The foregoing results do not allow distinction to be made between the use of one or two molecules of shikimic acid in phenazine biosynthesis. The incorporation [4] of



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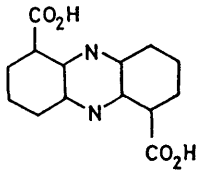

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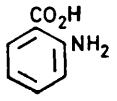

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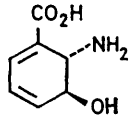

(16)

[G-^{14}C]shikimic acid into phenazine-1-carboxylic acid (2) in *Pseudomonas aureofaciens* cultures gave material in which the carboxy-group was labelled to the extent of 10.9%, a result which falls between the values of 14.3 and 7.9% required for one and two units of (1), respectively. In support, however, of the biosynthesis of (2), and also (3), involving two molecules of shikimic acid (1), we have found that (−)-[1,6,7-^{14}C$_3$]shikimic acid (equal labelling of each site) was incorporated in (2) and (3) in *P. aureofaciens* cultures with one fifth of the activity located in the carboxy-groups (Table 3). Our results suggest then that (1) is equally incorporated into both 'halves' of (2) and (3), but it must be noted that this result would inevitably be obtained if a symmetrical intermediate, *e.g.* (14), was involved in the pathway, even if only one shikimic acid unit was utilized for the biosynthesis of (2) and (3). (Further discussion relating to this problem is taken up in the following paper.)

In considering intermediates after shikimic acid in the biosynthesis of phenazines, anthranilic acid (15), itself

TABLE 3

Decarboxylation of phenazine-1-carboxylic acid and 2-hydroxyphenazine-1-carboxylic acid derived from [1,6,-7-$^{14}C_3$]shikimic acid (0.84 μCi) [a]

	Barium carbonate (%)	(6) (%)	(7) (%)
Phenazine-1-carboxylic acid (2)	21	79	
	22	79	
2-Hydroxyphenazine-1-carboxylic acid (3)	21		77
			76

[a] 7.5% Incorporation.

formed from shikimic acid *in vivo*, can appear as an attractive possibility. Although some results have been deduced in favour,[10] the weight of evidence is most strongly against its being involved in phenazine biosynthesis.[1,3,6] We have independently confirmed that it is a very poor precursor of iodinin (*ca.* 0.02%). An alternative possibility is (16) isolated from a *Streptomyces aureofaciens* mutant,[11] which, like anthranilic acid, has the nitrogen atom essential for phenazine ring formation. This also proved to be a poor precursor (0.3—0.7%) of iodinin (8) in support of other results.[3] This compound is structurally close to shikimic acid (1), however, and it may be that the results follow from transport difficulties, although preliminary experiments [12] with disrupted cell systems have not been encouraging, and use of lysozyme in an attempt to improve permeability was without observable effect.

EXPERIMENTAL

General Directions.—M.p.s were obtained on a hot-stage apparatus unless otherwise stated. Column chromatography was carried out using Kieselgel.[13] Micro-organisms used were *Pseudomonas aureofaciens* (Strain 9265 from the National Collection of Industrial Bacteria, Aberdeen) and *Brevibacterium iodinum* maintained on agar slants. Media and growth conditions were as described [14] for *P. aureofaciens*. The *B. iodinum* medium was yeast extract (0.3%), peptone (1.0%), Lablemco powder (1.0%), glucose (0.5%), and sodium chloride (0.5%); pH 7.2. Inoculation was performed into 500 ml conical flasks containing 100 ml of medium; after 24 h incubation the culture was transferred at 5—10% into 500 ml conical flasks containing 100 ml of medium; incubation proceeded at 27 °C in a rotary shaker. Radioactive compounds were administered over several hours during the early stages of phenazine formation and the phenazines were isolated after a further 24—48 h by extraction with chloroform (in the case of phenazine-1-carboxylic acid and 2-hydroxyphenazine-1-carboxylic acid after acidification).

Radioactivity was assayed by scintillation counting. Barium carbonate activity was measured in the same way: samples were satisfactorily counted settled on the floor of the counting vial with scintillant (NE250, Nuclear Enterprises, Edinburgh) above (particle size had no noticeable effect on counting efficiency). Counting efficiency was measured by use of prepared, standard barium carbonate samples with specific activity covering the range of activity in the barium carbonate samples obtained by degradation.

The quoted radioactivities of solids are those obtained after recrystallization to constant activity.

Radioactive Compounds and Feeding Experiments.—Our source of radiochemicals was the Radiochemical Centre, Amersham. The [1-^{14}C]-, [6-^{14}C]-, and [1,6,7-$^{14}C_3$]-shikimic acids were prepared by the published method [7] from, respectively, [2-^{14}C]pyruvic acid, [3-^{14}C]pyruvic acid, and [*U*-^{14}C]pyruvic acid, with activities 5—10 μCi mmol^{-1}. Approximately 1 μCi was used in feeding experiments. The amount of iodinin isolated in these experiments was *ca.* 60 mg; and of the phenazines from *P. aureofaciens*, *ca.* 50 mg.

[^{3}H]Anthranilic acid (200 μCi mg^{-1}) was prepared by heating the hydrochloride in tritiated water at 100 °C. A sample (290 μCi) was fed to *B. iodinum* cultures, 60 mg of iodinin being isolated.

[^{14}C]Dihydrohydroxyanthranilic acid was prepared as described,[11] except that the material was purified by an additional paper chromatogram (ethanol–isopentyl alcohol–1M-acetic acid, 2 : 1 : 1). This ensured, in particular, no contaminating shikimic acid. 1—3 μCi (specific activities 3—7 μCi mg^{-1}) were fed to *B. iodinum* cultures, 20—60 mg of iodinin being isolated. Lysozyme was found to decrease iodinin production at greater than 2 mg per 100 ml. It was fed with dihydrohydroxyanthranilic acid at this concentration.

Degradation of Iodinin.—The iodinin isolated from the *B. iodinum* cultures was crystallized from chloroform; m.p. 239° (capillary; decomp.; lit.,[15] 236°), *m/e* 244 (M^+), 228 ($M^+ - 16$), 212 ($M^+ - 32$), 211, 210, and 199 (Found: C, 59.1; H, 3.3; N, 11.6. Calc. for $C_{12}H_8N_2O_4$: C, 59.1; H, 3.3; N, 11.5%). It was degraded as follows.

(a) *Phenazine-1,6-diol.* Iodinin (64.8 mg) was dissolved in aqueous sodium hydroxide (1M, 10 ml), and zinc dust (40 mg) added. The mixture was refluxed for 1 h. The solution was cooled, made just acid with 2M-sulphuric acid, and extracted with chloroform. The extracts were dried (Na_2SO_4) and evaporated. The residue was recrystallized from benzene to give yellow needles of phenazine-1,6-diol (47 mg, 82%), m.p. 281.5—282.5° (sealed capillary; decomp.; lit.,[15] 273—274°), $\nu_{max.}$ ($CHCl_3$) 3 392 cm^{-1}; $\lambda_{max.}$ 273, 371, and 443 nm; *m/e* 212 (M^+), 184 ($M^+ - 28$), and 155.

(b) *Pyrazinetetracarboxylic acid.* Ruthenium trichloride hydrate (20 mg) was dissolved in water (5 ml) and sodium hypochlorite (14% available chlorine, 15 ml) added with stirring. A suspension of phenazine-1,6-diol (70 mg) in carbon tetrachloride was added to the now yellow aqueous solution and the mixture was stirred for 3.5 h. (The oxidant for this reaction,[16] ruthenium tetraoxide, is yellow. Complete consumption of hypochlorite results in a permanent black precipitate of spent oxidant which may be recharged by the addition of further hypochlorite). 1-Methylbutyl alcohol was added to give a black precipitate, which was removed by centrifugation. The organic phase was removed and extracted with water. The combined aqueous solutions, after filtration, were passed through an ion-exchange column (Dowex 50-W-X8, 20—50 mesh; 2.4 cm × 43 cm; H^+ form; three passages generally sufficed with regeneration in between) to remove sodium chloride, and evaporated to dryness. Recrystallization from hydrochloric acid (10M; 5 ml) gave pyrazinetetracarboxylic acid as off-white needles (39 mg, 46%), m.p. 196—198° (lit.,[17] 199°), $\nu_{max.}$ (KCl) 1 730 cm^{-1}; $\lambda_{max.}$ 292 nm. Radioactive samples of the acid were assayed for activity *via* the tetramethyl ester. This was prepared from the acid by refluxing a dry methanolic solution in the presence of conc.

sulphuric acid for 12 h; m.p. 183—184.5° (from methanol) (lit.,[18] 182, 184°) (62% yield); $\nu_{max.}$ (KCl) 1 746 and 1 729 cm^{-1}; $\lambda_{max.}$ (MeOH) 277 (log ε 3.60); *m/e* 312 (M^+), 281 ($M^+ - 31$), and 250 (Found: C, 46.4; H, 4.0; N, 9.0. Calc. for $C_{12}H_{12}N_2O_8$: C, 46.2; H, 3.8; N, 9.0%).

(c) *Decarboxylation of pyrazinetetracarboxylic acid.* Assembly and dismantling of the apparatus used were carried out in a CO_2-free atmosphere, as were the preparation and introduction of materials. Pyrazinetetracarboxylic acid (17 mg) and copper chromite [19] (CO_2-free by heating at 290 °C under high vacuum; *ca.* 20 mg) were intimately ground and introduced into a flask connected in sequence to two traps (10 ml filter tubes) of aqueous (CO_2-free) mercury(II) chloride (1.0 g in 10 ml water; first trap cooled in ice) and then two of aqueous barium hydroxide (0.4 g in 10 ml water). The train was completed by attaching a soda-lime guard-tube. The mixture was heated at 290 °C for 2 h while a very slow stream of dry CO_2-free nitrogen was passed through the apparatus.

The precipitated barium carbonate (17—27 mg) was collected, washed with CO_2-free water, and dried over phosphorus pentaoxide. The first tubes contained a precipitate of the mercury(II) chloride adduct of pyrazine [20] [also some mercury(II) chloride]. The suspension was diluted with water (*ca.* 10 ml), shaken, and centrifuged. The supernatant liquid was separated from the precipitate, which was re-suspended in water, and basified with aqueous potassium hydroxide (2M), used first to recover any solid material clinging to the first trap. The suspension was extracted with ether (8 × 5 ml) and the combined extracts were dried ($MgSO_4$). Acetic anhydride (10 ml) was added and the solution reduced in volume (to 10—15 ml) by distillation at atmospheric pressure. Hydrogenation of this solution with one drop of acetic acid added and over platinum oxide (5 mg) was carried out at room temperature and atmospheric pressure for 16 h. Filtration and then evaporation gave a residue which was sublimed at 100 °C and 0.2 mmHg to give *NN*-diacetylpiperazine (1.1—2.1 mg), m.p. 140—142.5° (lit.,[21] 138.5°). This material is hygroscopic. It was kept dry (silica gel) and was handled with exclusion of moisture; $\nu_{max.}$ (Nujol) 1 639 cm^{-1}; *m/e* 170 (M^+), 155 ($M^+ - 15$), 127 ($M^+ - 43$), and 112 (Found: C, 56.4; H, 8.2; N, 16.6. Calc. for $C_8H_{14}N_2O_2$: C, 56.5; H, 8.2; N, 1.56%).

The procedure adopted when using copper plus quinoline instead of copper chromite was as described above except that the flask contained pyrazinetetracarboxylic acid (17.5 mg), copper powder (25 mg), 2,2′-bipyridyl (5 mg), and CO_2-free quinoline (distilled from BaO) (1.5 ml). The reaction temperature was 215 °C. This gave *NN*-diacetylpiperazine (1.97 mg) and barium carbonate (34.3 mg).

Degradation of Phenazine-1-carboxylic Acid and 2-Hydroxyphenazine-1-carboxylic Acid.—The chloroform extract from the *P. aureofaciens* cultures was subjected to repeated chromatography on Kieselgel to give phenazine-1-carboxylic acid (with chloroform as eluant) and 2-hydroxyphenazine-1-carboxylic acid (with chloroform containing 5% methanol and 2% acetic acid), recrystallized, respectively, from ethanol containing 5% dimethylformamide and benzene containing 15% ethanol. They were decarboxylated as follows.

A two-necked flask containing phenazine-1-carboxylic acid (20 mg), copper powder (5 mg), and quinoline (0.5 ml; distilled from barium oxide) was coupled in series, in a CO_2-free atmosphere, to two tubes (10-ml filter tubes) each containing 5 ml of freshly filtered aqueous barium hydroxide (38 g l^{-1}) and finally a soda-lime guard-tube. The apparatus was flushed with CO_2-free nitrogen (10 min). The flask was heated at 216—220 °C for 1 h in a slow stream of CO_2-free nitrogen. The precipitated barium carbonate in the first tube was collected in a CO_2-free atmosphere, washed, and dried (P_2O_5). The residue in the reaction flask was taken into chloroform. The solution was filtered and evaporated, finally under high vacuum at 65 °C (bath temp.). The residue of crude phenazine was purified by sublimation and recrystallization (aqueous ethanol); yields 12 mg of phenazine and 10 mg of barium carbonate.

2-Hydroxyphenazine-1-carboxylic acid (9.6 mg) was decarboxylated by heating at 214—218 °C for 1.5 h. The evolved carbon dioxide was trapped as above. The residual 2-hydroxyphenazine was purified by sublimation and recrystallization (benzene containing 15% ethanol); yields 4.8 mg of 2-hydroxyphenazine and 4.2 mg of barium carbonate.

Preparation of [ring-^{14}C]*Pyrazinetetracarboxylic Acid.*—The method used followed a route to tetramethylpyrazine *via* butane-2,3-dione mono-oxime.[22]

[3-^{14}C]*Pentan-3-one and its oxime.* Barium propionate (5 g) and sodium [1-^{14}C]propionate (50 μCi) were dissolved in water (15 ml) and the solution was evaporated, finally at 100 °C under high vacuum. The mixture (3.5 g) was pyrolysed in a long flame-dried Pyrex tube at 550—600 °C and 0.07 mmHg, the product being collected in a trap cooled by liquid nitrogen. The crude pentan-3-one was taken up into ether (5 ml), and the solution was dried, (Na_2SO_4), filtered, and cooled in ice. Pentyl nitrite (1.3 ml) and conc. hydrochloric acid (0.04 g) were added, and the mixture was allowed to warm to room temperature. After 16 h it was extracted with aqueous sodium hydroxide (2M; 7 ml). The alkaline solution was extracted with ether, cooled in ice, and acidified with sulphuric acid (2M). Extraction with ether, drying, and evaporation gave [3-^{14}C]-pentane-82,3-dione 2-mono-oxime (0.643 g, 45%).

(b) 2,5-*Diethyl*-3,6-*dimethyl*[2,5-$^{14}C_2$]*pyrazine.* Crude oxime (0.643 g) from the previous step was added over 2 h to a stirred solution of tin(II) chloride (2.27 g) in conc. hydrochloric acid (3.5 ml) at 5—10 °C. Stirring was then continued for a further 30 min. The mixture was diluted with water (5 ml) and aqueous sodium hydroxide (9 ml, 33%) was added dropwise at 40 °C. After 10 min a solution of mercury(II) chloride (1.5 g) in water (3.5 ml) was added and the product was collected by steam distillation. The distillate was extracted with ether, and the extract was dried (Na_2SO_4) and evaporated at 35 °C (bath temp.) to give 2,5-*diethyl*-3,6-*dimethyl*[2,5-$^{14}C_2$]*pyrazine* (203 mg, 44%); $\lambda_{max.}$ (EtOH) 281.5 (log ε 3.60) and 297 nm (3.30); *m/e* 164 (M^+, 58%) and 149 ($M^+ - Me$, 100%); δ($CDCl_3$) 1.29 (6 H, t, *J* 7 Hz), 2.54 (6 H, s), and 2.83 (4 H, q, *J* 7 Hz) (*cf.* ref. 23).

(c) [ring-^{14}C]*Pyrazinetetracarboxylic acid.* A mixture of 2,5-diethyl-3,6-dimethyl[2,5-$^{14}C_2$]pyrazine (0.171 g), potassium permanganate (2.1 g), and aqueous potassium hydroxide (0.2%; 30 ml) was refluxed for 3 h. Ethanol was added, and the brown precipitate was removed by filtration. The precipitate was washed with hot water and the filtrate and washings were freed of inorganic cations by ion-exchange chromatography (Dowex 50-W-X8; H^+ form; 200 ml wet resin). The portion of the eluate containing pyrazinetetracarboxylic acid ($\lambda_{max.}$ 292 nm) was evaporated

to dryness. The residue was recrystallized from hydrochloric acid (10M) to give [ring-^{14}C]*pyrazinetetracarboxylic acid* (0.135 g, 50%), m.p. 180—183°. Further identification was carried out *via* the tetramethyl ester, m.p. and mixed m.p. 181—184°. Decarboxylation was carried out essentially as described above.

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