Phytochemisstry, Vol. 20, No. 8, pp. 1835–1839, 1981.
Printed in Great Britain.

0031-9422/81/081835-05 $02.00/0

5-HYDROXYLEVULINIC ACID, A NEW INTERMEDIATE IN THE BIOSYNTHESIS OF PROTOANEMONIN*

RUDOLF TSCHESCHE, WOLFGANG WIRTH and KARIN WELMAR

Institut für Organische Chemie und Biochemie der Universität Bonn, Gerhard-Domagk-Str. 1, D-5300 Bonn 1, West Germany

(*Revised received* 26 *January* 1981)

Key Word Index—*Helleborus foetidus*; Ranunculaceae; biosynthesis; protoanemonin; 5-hydroxylevulinic acid.

Abstract—Administration of 5-hydroxy [1-^{14}C]- and [4-^{14}C]levulinic acid to *Helleborus foetidus* led to the isolation of [1-^{14}C]- and [4-^{14}C]protoanemonin, respectively. There was also incorporation of radioactivity into the four glucosides ranunculin, isoranunculin, ranuncoside and ranunculoside. Acid hydrolysis of radioactive ranuncoside gave labelled 5-hydroxylevulinic acid (HKV). A study of the incorporation of various ^{14}C-labelled tracers into protoanemonin suggested that HKV is formed in higher plants by a new reduction of 2-ketoglutarate (2-KG) without free 4,5-dioxovalerate (DOVA) as an intermediate. A scheme for the biosynthesis of the antibiotic protoanemonin and its glucosidic precursors is proposed. It is shown that 5-(β-D-glucopyranosyloxy)levulinic acid could be the genuine precursor of all the compounds studied.

INTRODUCTION

5-Hydroxylevulinic acid (HKV)† is found as a secondary reaction product in the reaction catalysed by 2-KG: glyoxylate carboligase. The enzyme was detected in micro-organisms [2, 3] and in the mitochondrial fraction of rat liver [4] and beef heart [5] in the course of investigations on the metabolism of glyoxylic acid. The primary condensation product is 2-hydroxy-3-keto-adipate which is decarboxylated non-enzymatically to form HKV. Wang *et al.* [6] showed that radioactive ALA, 2-KG, glutamate and aspartate are synthesized from ^{14}C-labelled HKV when the latter is incubated with sonicated rat liver mitochondria. The results suggested that DOVA is an intermediate in the oxidation of HKV. It can be assumed that there is a cyclic mechanism of glyoxylate oxidation involving 2-KG, 2-hydroxy-3-ketoadipate and HKV as intermediates. A mechanism with 2-KG, 3-hydroxy-2-ketoadipate and 2-hydroxyglutarate was tentatively proposed by other authors [8, 9]. Yamasaki and Moriyama [3] reported that HKV is a competitive inhibitor of ALA dehydratase and might play an important role in the regulation of porphyrin synthesis.

HKV is found as its glucosidic derivative, ranunculoside (**6**) (Scheme 1), in *Helleborus foetidus* plants [1]. It is formed on acid hydrolysis of ranuncoside (**5**) [10, 11], which can be isolated from these plants.

We report here on *in vivo* incorporation experiments which demonstrate the biosynthetic priority of HKV during protoanemonin synthesis in *H. foetidus*. It is also shown that HKV is probably formed by direct reduction of the intact carbon skeleton of 2-KG.

RESULTS AND DISCUSSION

[1-^{14}C]HKV was prepared synthetically by the Grignard reaction of 1-benzyloxy-4-bromo-2-butanone ethylene acetal [12] with $^{14}CO_2$ and subsequent removal of the protecting groups [13]. Oxidation of the α-ketol grouping with cupric-II-acetate gave [1-^{14}C]DOVA [13]. [4-^{14}C]HKV was synthesized by deamination of commercial [4-^{14}C]ALA (Amersham) with nitrous acid according to Schlossberg *et al.* [5]. The other ^{14}C-labelled tracers were also purchased from The Radiochemical Centre, Amersham/Buchler. All the precursors tested were water-soluble and they were either administered to the plants by injection into the peduncles (Table 1, feeding time 8 days) or fed through the excised plant stems (Table 3, feeding time 84 hr).

[1-^{14}C]HKV was incorporated into the four glucosides **3**, **4**, **5** and **6**, which were obtained by different extraction methods, as well as into the inhibitor protoanemonin (**1**). The latter was characterized as its crystalline dimer anemonin (**2**) and gave the highest specific activities when the plants were worked up by lyophilization. A part of the radioactive ranuncoside (**5**) was subjected to acid hydrolysis. The reaction products, Glc and HKV, were separated and characterized as β-D-pentaacetylglucose and HKV-*p*-bromophenacylester, respectively. About 90% of the label was recovered in the HKV (Table 2). The results suggest that in spite of its simple structure, HKV is probably not utilized for catabolic reactions in higher plants. Therefore, those steps which lead to the formation of HKV might have a regulatory function in protoanemonin biosynthesis.

Various ^{14}C-labelled compounds were tested for their effectiveness in protoanemonin biosynthesis. The plant material was worked up by water–steam distillation as described by Asahina [14]. Radioactivity was measured as silver valerate which was obtained upon reduction of **1** with hydrogen and Adam's catalyst [15]. After precipitation of the valerate with silver nitrate solution the silver salt was recrystallized to constant specific activity

*Part 7 in the series "On Glycosides with Lactone-forming Aglycones". For Part 6 see ref. [1].

†Abbreviations: ALA, 5-aminolevulinic acid; BzOKV, 5-benzyloxy-4-ketovaleric acid; DOVA, 4,5-dioxovaleric acid; Glc, glucose; HKV, 5-hydroxy-4-ketovaleric acid (5-hydroxylevulinic acid); 2-KG, 2-ketoglutaric acid.

and was then degraded when there was sufficient incorporation of label. C-1, C-2 and C-3 were obtained as barium carbonate by repeated Schmidt reaction and potassium permanganate oxidation. Kuhn–Roth oxidation of the silver valerate yielded C-4 and C-5 as acetic acid which was cleaved into carbon dioxide (as barium carbonate) and methylamine (as methylammonium picrate). Determination of ^{14}C in the barium carbonate samples was accomplished according to Frohofer [16]. After liberation of the carbon dioxide with concentrated sulphuric acid and complete trapping of the gas with methanolic ethanolamine solution, the radioactivity was measured by liquid scintillation counting. As can be seen from Table 3, the best precursor of protoanemonin of those tested was ^{14}C-labelled HKV. The radioactivity was mainly located at C-1 and C-4, respectively (Table 4).

Administration of [1-^{14}C]BzOKV (isolated during the synthesis of [1-^{14}C]HKV and fed to the plants as its water-soluble sodium salt [13]) gave a low but specific incorporation of label into C-1 of the inhibitor **1** which was probably attributable to decomposition or by enzymatic cleavage of the α-ketolbenzyl ether.

The involvement of 5-(β-D-glucopyranosyloxy)-levulinic acid (**7**) might explain the formation of the glucosides and the antibiotic **1** (Scheme 1). Investigations on α-methylen-γ-butyrolactone which was isolated from

Table 1. Incorporation of [1-^{14}C]HKV (4.2 mCi/mmol) into the glucosides **3**, **4**, **5**, **6** and into the inhibitor **1**.

Experiment No.	10^{-8} × total act of [1-^{14}C]HKV (dpm)	Metabolite	10^{-6} × sp. act. (dpm/mmol)	Incorporation (%)
1	0.487	ranunculin (**3**)	0.200	0.11
1	0.487	isoranunculin (**4**)	0.168	0.02
2	0.958	ranuncoside (**5**)	1.881	2.02
2	0.958	ranunculoside (**6**)	1.940	0.21
3	2.698	protoanemonin (**1**)*	7.592	0.66

*Isolated from the lyophilization distillate and measured as its crystalline dimer anemonin (**2**).

Table 2. Distribution of radioactivity (dpm) in ranuncoside (**5**) biosynthesized from [1-^{14}C]HKV

Ranuncoside*	D-Glucose	HKV	Pentaacetyl-glucose	HKV-*p*-bromo-phenacyl derivative
9.944×10^5	7.571×10^4 (11.1%)	6.082×10^5 (88.9%)	6.372×10^4 (10%)	5.656×10^5 (90%)

*From experiment No. 2 (Table 1).

Table 3. Incorporation of various precursors into protoanemonin (**1**) by *H. foetidus*

Experiment No.	Precursor	10^{-8} × total act. of precursor (dpm)	10^{-5} × total act. of hydrogenated steam-distillate* (dpm)	Amounts of silver valerate† (mg)	10^{-5} × sp. act. of silver valerate (dpm/mmol)	Incorporation (%)
4	[1-^{14}C]HKV	1.556	97.240	546.0	16.860‡	2.83
5	[4-^{14}C]HKV	0.728	5.664	754.3	1.132‡	0.56
6	[1-^{14}C]BzOKV	0.877	1.234	665.8	0.278‡	0.10
7	[2-^{14}C]glyoxylate	1.055	1.429	502.9	0.182	0.04
8	[1-^{14}C]glyoxylate	1.062	1.542	423.8	0.572‡	0.11
9	2-[5-^{14}C]KG	1.084	2.442	921.9	0.420‡	0.17
10	[1,5-^{14}C]citrate	1.001	1.294	598.6	0.109	0.03
11	[4-^{14}C]ALA	0.890	1.757	660.0	0.232	0.08
12	[1-^{14}C]DOVA	1.097	2.886	718.0	0.188	0.06

*See Experimental.
†Values are corrected corresponding to hydrogen uptake and titration results.
‡For degradation see Table 4.

Table 4. Distribution of label in protoanemonin (1) biosynthesized from various ^{14}C-labelled precursors

Carbon	10^{-3} × sp. radioactivity of barium carbonate samples (dpm/mmol)*				
	[1-^{14}C]HKV	[4-^{14}C]HKV	[1-^{14}C]BzOKV	[1-^{14}C]glyoxylate	2-[5-^{14}C]KG
1	931.097 (97.92)†	0.789 (0.80)	16.380 (93.26)	6.118 (20.39)	30.589 (86.60)
2	12.235 (1.29)	16.380 (16.60)	0.197 (1.12)	4.539 (15.13)	2.566 (7.26)
3	0.592 (0.06)	5.526 (5.60)	0.197 (1.12)	3.750 (12.50)	0.395 (1.12)
4	2.565 (0.27)	75.190 (76.20)	0.394 (2.25)	5.328 (17.76)	0.789 (2.23)
5‡	4.341 (0.46)	0.789 (0.80)	0.394 (2.25)	10.262 (34.22)	0.987 (2.79)

*Values shown are the average of three measurements.
†Values in parentheses represent distribution in percentages.
‡Counted as methylammonium picrate.

Erythronium americanum [17] and from tulips [18] showed that, contrary to our present findings, it is formed from an acylglucoside [19]. The HKV hydroxylglucoside (7) has possibly not yet been isolated from plants belonging to the Ranunculaceae because of its tendency to form lactones.

The biosynthetic pathway of HKV in higher plants has not been studied to our knowledge. The formation of the acid by a 2-KG:glyoxylate carboligase reaction as described in the Introduction was first examined. It was observed, however, that [1-^{14}C]glyoxylate was better utilized for protoanemonin synthesis than [2-^{14}C]glyoxylate although C-1 should be split off as carbon dioxide through the decarboxylation of the 2-hydroxy-3-ketoadipate. Incorporation of label from [2-^{14}C]glyoxylate was so poor that degradation of the metabolite was not performed. 2-[5-^{14}C]KG was incorporated into **1**, C-5 of the 2-KG becoming C-1 of the lactone. The results agree with those reported by Suga and co-workers [20] although they could not be supported by feeding [1,5-^{14}C]citrate. It was, therefore, assumed that HKV was synthesized from 2-KG by a two-step reduction with DOVA as an intermediate. Administration of synthetically prepared [1-^{14}C]DOVA and [4-^{14}C]ALA, which might be converted to DOVA by transamination reaction showed, however, that neither DOVA nor ALA were effective precursors of **1**. These results imply that HKV is biosynthesized by direct reduction of 2-KG (probably as 2-KG with activated C-1) with an enzyme-bound DOVA as an intermediate. The process could be analogous to the reduction of 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid during cholesterol biosynthesis [21]. The results also show that protoanemonin and porphyrin are biosynthesized by separate pathways.

EXPERIMENTAL

Chromatography. TLC: Si gel HF_{254} (Schleicher & Schüll), visualization was achieved by spraying with conc H_2SO_4 (140°); CC: Munktell's cellulose powder, elution with system A, HOAc–pyridine–H_2O (72:20:23, upper phase), Sephadex G 10 (Pharmacia), elution with H_2O. Solvent systems: B, $CHCl_3$–MeOH–H_2O (13:7:2, lower phase); C, CH_2Cl_2–Me_2CO (100:1); D, $CHCl_3$–Me_2CO (4:1); H, C_6H_6–Me_2CO (4:1).

Radioactivity measurements. All labelled substances were purified and identified either by TLC or by recrystallization to constant sp. act. with authentic material. They were assayed for ^{14}C-activity by liquid scintillation spectrometry (Aqua-Luma (Baker) scintillation cocktail; quench corrections by external standard ratio method).

Labelled precursors. [4-^{14}C]ALA (58 mCi/mmol), [1,5-^{14}C]citric acid (20 mCi/mmol), [1-^{14}C]glyoxylate (10.9 mCi/mmol), [2-^{14}C]glyoxylate (2.5 mCi/mmol) and 2-[5-^{14}C]KG (20.2 mCi/mmol) were purchased from the Radiochemical Centre, Amersham/Buchler and had radiochemical purities of 99, 99, 97, 98 and 97%, respectively.

[1-^{14}C]BzOKV. $^{14}CO_2$ was liberated from 5 mCi barium [^{14}C]carbonate (20.9 mCi/mmol, Amersham) with 10 ml conc. H_2SO_4 and was reacted with the Grignard reagent prepared from 1.57 g (5.21 mmol) 1-benzyloxy-4-bromo-2-butanone ethylene acetal and 194.4 mg (8 mmol) Mg turnings in 17.5 ml dry THF to yield 0.87 mCi [1-^{14}C]BzOKV (4.5 mCi/mmol, by titration) via [1-^{14}C]BzOKV ethylene acetal (for more details see ref. [7, 13]. [1-^{14}C]BzOKV (71.77 μCi) was titrated with 0.05 N NaOH and used for expt No. 6.

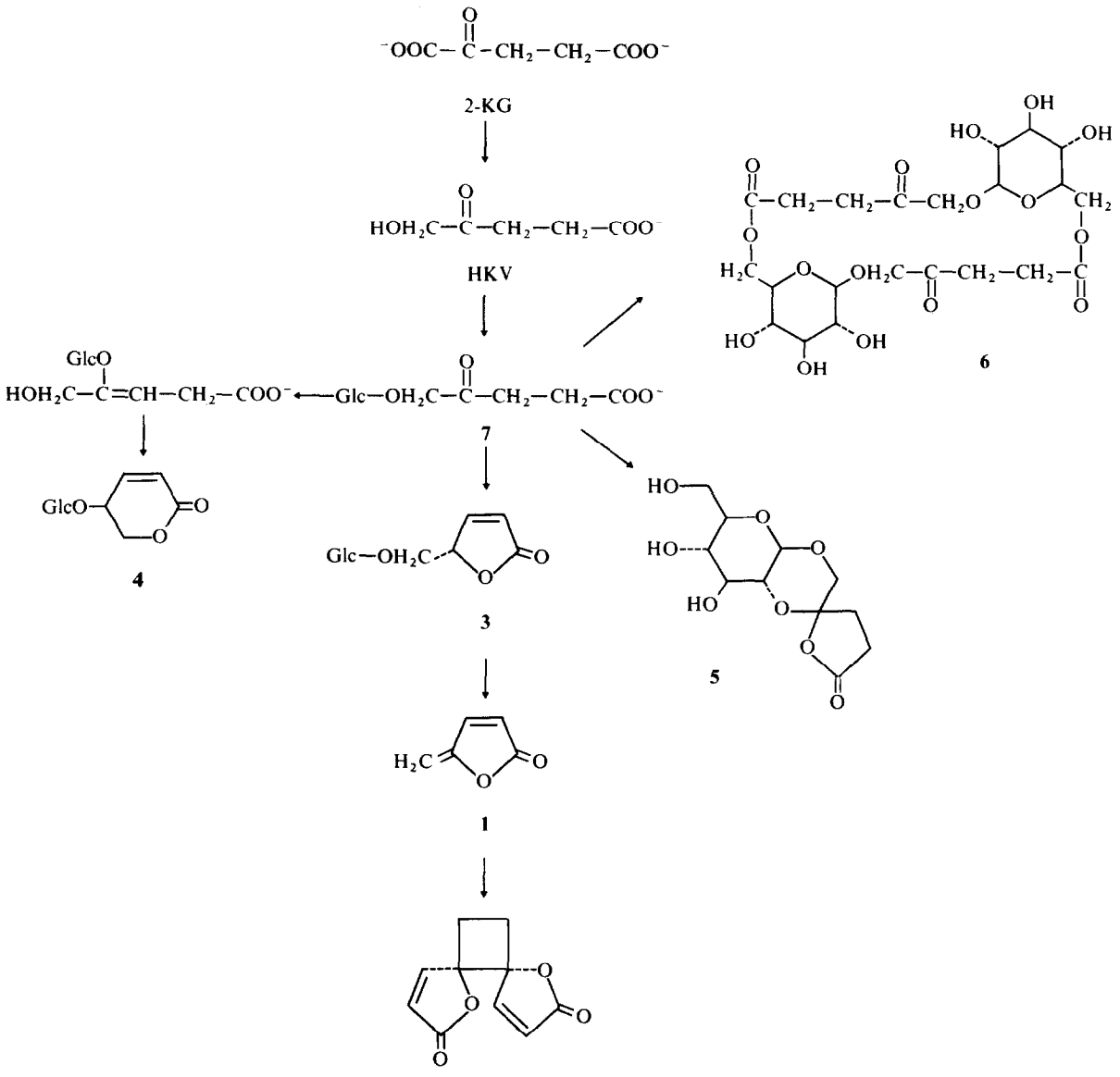
[1-^{14}C]HKV. Hydrogenolytic cleavage of 797.28 μCi [1-^{14}C]BzOKV with 40 mg Pd/charcoal in 4 ml EtOAc yielded, after purification by TLC (system B), 433 μCi [1-^{14}C]HKV (4.2 mCi/mmol, radiochemical purity 99% [13]. An aliquot was converted to [1-^{14}C]HKV *p*-bromophenacylester and characterized like the free acid by radio-TLC with authentic material.

[1-^{14}C]DOVA. 100 μCi [1-^{14}C]HKV was dissolved in 1 ml dist. H_2O and stirred with 140 mg cupric-II-acetate for 3 days under N_2 [13]. Purification by TLC (*n*-BuOH–H_2O–HOAc, 12:5:3) gave 58.72 μCi (3.9 mCi/mmol, 99% purity) [1-^{14}C]DOVA which was characterized as 4,5-dihhydroxyimino[1-^{14}C]valeric acid.

[4-^{14}C]HKV. Deamination of 250 μCi [4-^{14}C]ALA (6.5 mCi/mmol, Amersham) in 0.25 ml 1 N H_2SO_4 was performed with a soln of 25 mg $NaNO_2$ in 0.5 ml H_2O as described in ref. [5] and yielded, after repeated ion exchange chromatography on Dowex 1 X 1 columns (Roth), 38.59 μCi [4-^{14}C]HKV (5.3 mCi/mmol) [7, 13].

Plant material. Helleborus foetidus L. was collected from January to April in Brohltal, district Ahrweiler, W. Germany.

Isolation of ranuncoside (**5**), *ranunculoside* (**6**) *and protoanemonin* (**1**). [1-^{14}C]HKV was injected into the peduncles of flowering *H. foetidus*. After 8 days the blossoms (*ca* 120 g fr. wt) were harvested, ground under liquid N_2 in a mortar and lyophilized. The distillate was used for the isolation of **1**. It was satd with Na_2SO_4, extracted with Et_2O (×2) and after evapn was allowed to stand at room temp. for a few days. After recrystallization from CH_2Cl_2 22.5 mg anemonin (**2**) was obtained. Its radiochemical purity was confirmed by radio-TLC in system C (R_f 0.42) and system D (R_f 0.72) and cocrystallization with authentic material, mp 151°. **5** and **6** were isolated by extraction of the freeze-dried blossoms (*ca* 40 g) with 1 l. Me_2CO–H_2O (1:1). After careful evapn at low temp. the



Scheme 1. Proposed pathway for the biosynthesis of protoanemonin (**1**) and its glucosidic precursors ranunculin (**3**), isoranunculin (**4**), ranuncoside (**5**) and ranunculoside (**6**). Anemonin (**2**) is formed non-enzymatically on concentration of **1**.

extract was mixed with 15 g cellulose powder and was applied to a 400 g cellulose column. Elution with system A yielded first 302.8 mg **5** R_f (B) 0.66 and subsequently **6** R_f (B) 0.43. The latter (59.7 mg) was rechromatographed on a Sephadex G 10 column (× 2).

Acid hydrolysis of ^{14}C-*labelled* **5**. 155.6 mg **5** (9.944 × 10^5 dpm, expt No. 2) was heated with 10 ml 2 N HCl to 90° for 1 hr. The reaction mixture was mixed with 500 mg cellulose powder and chromatographed on an 8 g cellulose column. 58.2 g radioactive HKV, mp 100°, R_f (B) 0.75 and 65 mg non-crystalline D-Glc were eluted (Table 2). The derivatives were prepared in the usual ways.

Isolation of ranunculin (**3**) *and isoranunculin* (**4**) [22]. Eight days after administration of ^{14}C-labelled substrates the plants were ground with an Ultraturrax in a mixture consisting of 90 ml H_2O and 3 ml conc. HCl/100 g fr. wt. The extract was filtered through kieselgur, centrifuged and treated with 2.5 g charcoal for 20 min at room temp. It was again filtered through kieselgur and treated now for the absorption of the glucosides with 12 g charcoal. After standing for 1 hr the charcoal was thoroughly washed with H_2O and then eluted with EtOH–H_2O (1:1). Evapn yielded an oil which was rechromatographed on Sephadex G 10 columns (× 2). The mixture of **3** and **4** showed almost the same R_f value (0.65) as ranuncoside (**5**). **3** and **4** were separated as their tetraacetates [1] by chromatography on Si gel (× 3 development with system H) to give 75.0 mg **3**-acetate, R_f 0.46 and 18.3 mg **4**-acetate, R_f 0.42.

Isolation of protoanemonin (**1**) *according to Asahina* [14]. The precursors used in the expts outlined in Table 3 were fed through the excised plant stems of *H. foetidus* (170–350 g fr. wt/expt). Eighty-four hr later the plants were harvested and steam-distilled

for 4 hr to give 1 l. of a colourless distillate. It was then satd with Na_2SO_4, extracted with 200 ml Et_2O (×5) and dried over Na_2SO_4. On evapn **1** was obtained as a pale yellow oil with the known vesicant properties.

Degradation of labelled protoanemonin (**1**). The degradation procedure used was the same as that used by Suga *et al.* [20], except that all radioactivity measurements were carried out by liquid scintillation counting. Crude **1** was reduced to valeric acid according to Kipping [15] soon after its isolation (see above). It was dissolved in 2 ml EtOH, added to 30 mg hydrogenated PtO_2 in 2 ml EtOH and stirred under H_2 for 6 hr at room temp. H_2 uptake was equivalent to 0.11 ± 0.01% based on fr. wt. Pt was removed, washed with EtOH and the filtrate assayed for radioactivity (total act. of hydrogenated steam distillate, Table 3). After titration with 1 N NaOH (phenolphthalein) and evapn of the solvents, the residual sodium valerate was dissolved in 10 ml H_2O–EtOH (1:2) and pptd as silver valerate by the dropwise addition of equimolar $AgNO_3$ in H_2O–EtOH (7:3) [23]. The dark ppt. was filtered off and washed with H_2O, a little Me_2CO and Et_2O. Recrystallization was achieved with boiling H_2O–Me_2CO mixtures to which small amounts of pyridine were added and was continued until the sp. act. of the silver salt remained constant (Table 3). Its IR spectrum was identical with that of synthetic material. The amounts of silver valerate were corrected corresponding to hydrogen uptake and titration results. C-1, C-2 and C-3 of labelled **1** were obtained as $BaCO_3$ by the stepwise degradation of the silver valerate involving successive application of the Schmidt decarboxylation and $KMnO_4$ oxidation. In a typical experiment one part of the silver salt (*ca* 40 mg) was mixed with an 1.4 molar excess of powdered NaN_3. *Ca* 700 mg polyphosphoric acid was added in an evacuated apparatus and the mixture was heated to 80° for 1 hr. CO_2 was trapped with $Ba(OH)_2$–$BaCl_2$ soln, pptd $BaCO_3$ was washed with hot, double distilled H_2O (×5), MeOH (×5) and Et_2O (×5) and was dried at 60°/0.01 torr for 6 hr, yield 70–80%. 4 ml 30% aq. NaOH was carefully added to the resulting butylamine polyphosphate soln and the mixture was distilled into 5 ml 0.2 N H_2SO_4. Oxidation of the amine was performed by the addition of 5 ml 5% aq. $KMnO_4$ and 0.5 N NaOH (> pH 8) and by refluxing for 0.5 hr. After acidification with 0.5 N H_2SO_4 the resulting butyric acid was recovered by steam distillation and if necessary purified by column chromatography on moist (0.9 ml 0.5 N H_2SO_4–15 g Si gel) celite [24]. Final identification was by TLC of its *p*-bromophenacyl ester derivative (Si gel, C_6H_6). Yields ranged from 70 to 90% based upon silver valerate. Dried sodium butyrate was subjected Schmidt decarboxylation as described above to yield C-2 of **1** as $BaCO_3$ and after further use of the reaction series C-3 of **1**.

Another part of the silver valerate was used for the determination of label at C-4 and C-5 of **1**. *Ca* 30 mg was oxidized according to Kuhn–Roth by refluxing with 20 ml 5 N CrO_3 and 5 ml conc H_2SO_4 for 1 hr. The HOAc produced was steam-distilled and identified by the position of its *p*-bromophenacyl ester derivative on the chromatogram. Schmidt procedure yielded CO_2 (C-4 of **1**) and methylamine which was trapped by an 1.4 molar excess of ethanolic picric acid soln. The picrate was crystallized to constant sp. act. (C-5 of **1**, Table 4).

Radioactivity of all $BaCO_3$ samples was measured as previously described by Frohofer [16]. The weight of 1–5 mg $BaCO_3$ was exactly determined. CO_2 was then liberated by the addition of conc. H_2SO_4 in a special apparatus and was trapped by 1.5 ml of $H_2NC_2H_4OH$–MeOH (1:14). The apparatus was swept with 3.5 ml MeOH and the trapping soln was combined with dioxane scintillation cocktail (10 ml of a soln of 10 g PPO, 0.5 g POPOP and 50 g naphthalene in 830 ml dioxane and 170 ml ethylene glycol monomethyl ether) for radioactivity measurement (Table 4).

Acknowledgement—This work was supported by the Deutsche Forschungsgemeinschaft.

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