585. *The Biosynthesis of Ricinine.**

By HADASSAH TAMIR and DAVID GINSBURG.

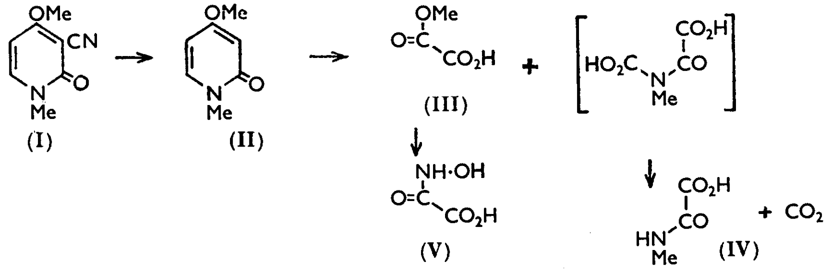
[2-^{14}C]Lysine hydrochloride, fed hydroponically to seedlings of *Ricinus communis* L., is incorporated in the ricinine produced by the plant. An ozonolytic procedure, which was developed in order to degrade 4-methoxy-1-methyl-2-pyridone formed from the labelled ricinine, showed that the latter is labelled solely in one position, α to the ring-nitrogen atom. Alternatively, α-amino[ε-^{14}C]adipic acid, similarly fed to the same plant, is also incorporated in the ricinine produced. In this case, however, both positions α to the ring-nitrogen atom were equally labelled in the alkaloid. These results are discussed.

FOR the first in a series of studies on the biosynthesis of alkaloids in plants, *Ricinus communis* L. which produces *ca.* 1% (dry weight) of a single alkaloid, ricinine [1] (I), was selected.

Seeds of this plant were permitted to sprout in the dark, the seedlings were transferred to a container in which the roots dipped into an aqueous solution of DL-[2-^{14}C]lysine hydrochloride and were grown for 14 days under controlled conditions. They were then harvested, dried, defatted (Soxhlet) with light petroleum, and then extracted (Soxhlet) with chloroform. After suitable working up, labelled ricinine was isolated.

Authentic ricinine was synthesised [2] and the unlabelled alkaloid was converted into 4-methoxy-1-methyl-2-pyridone (II) by heating it with 57% sulphuric acid at 140° for one hour.[3] Ozonolysis in ethyl acetate at −60° followed by oxidative decomposition with 30% hydrogen peroxide, then afforded three degradation products (scheme 1): methyl hydrogen oxalate (III), the *N*-methyloxamic acid (IV), and carbon dioxide. The last two substances are presumably formed from the unstable carbamic acid shown which is decarboxylated spontaneously. The carbon dioxide was isolated as barium carbonate (90% yield). Since the acid ester (III) could not be readily separated from the amidic acid (IV), the former was converted into hydroxyoxamic acid (V) under conditions which left the acid amide unaffected. The compounds (IV) and (V) were then isolated by paper chromatography in 85% and 65% yield, respectively.

Scheme 1.



The labelled ricinine produced by the plant was also converted into the pyridone (II) in yield (65%) similar to that from the unlabelled alkaloid.[3] The cyano-group was isolated in 97% yield as silver cyanide [4] and was shown to be unlabelled. Methoxyl and *N*-methyl

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* A preliminary note has been published (Abs. Internat. Congr. Biochem., Vienna, September, 1958, p. 142).

[1] Tuson, *J.*, 1864, **17**, 195.

[2] Schroeter, Seidler, Sulzbacher, and Kanitz, *Ber.*, 1932, **65**, 432.

[3] Späth and Tschelnitz, *Monatsh.*, 1921, **42**, 251.

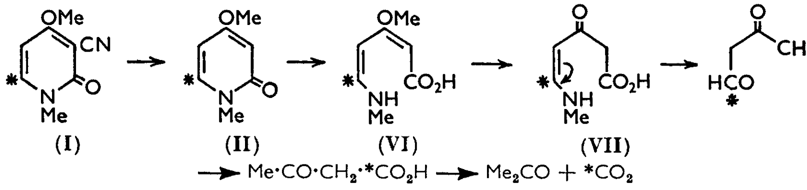
[4] Bottcher, *Ber.*, 1918, **51**, 673.

microdeterminations showed that no radioactivity resided at either the 1-methyl or the 4-methoxyl carbon atom. These methyl groups are known to be introduced into the ricinine molecule by methionine.[5]

Further, when the labelled pyridone (II) was ozonised, the hydroxylamine derivative (V) [produced from the acid ester (III)] and the acid amide (IV) were both unlabelled, and only the barium carbonate produced from the *non-carbonylic* α-carbon atom of the pyridone ring was labelled; its radioactivity fully accounted for that of the undegraded ring.

A complication, however, arose. The carbon dioxide formed by hydrolysis of the cyano-group [during the change (I) ⟶ (II)] was isolated as partially labelled barium carbonate. It appeared, at first, that this salt was labelled and thus, that the cyano-group of ricinine was partially labelled. This is clearly an artifact in view of the practically quantitative isolation of the cyano-group as unlabelled silver cyanide (see above). Indeed, 156% of the theoretical yield of barium carbonate was obtained from the transformation (I) ⟶ (II) so that it appears that unlabelled carbon dioxide resulting from hydrolysis of the cyano-group is diluted with radioactive carbon dioxide from the single labelled position in the ring. The latter labelled material is presumably formed from *ca.* 35% of the ricinine which is lost during the transformation (I) ⟶ (II) in a competitive reaction such as shown in the hypothetical reaction scheme 2.

Scheme 2.



It is here assumed that the " open " amino-acid (VI) formed during hydrolysis of ricinine under strongly acid conditions affords the β-oxo-acid (VII) which is both decarboxylated and hydrolysed as an enamine to afford formylacetone. This, by aerial oxidation, should give acetoacetic acid which on decarboxylation affords labelled carbon dioxide, diluting the unlabelled material from the cyano-group. Indeed, calculation of the radioactivity of this carbon dioxide, coupled with the specific activity and yield of the pyridone (II) and with the complete lack of radioactivity of the silver cyanide isolated in the alternative degradation procedure, justifies the conclusion that this carbon dioxide is, indeed, an artifact. The analogous degradations of ricinine formed from labelled α-aminoadipic acid further support this view (see below).

Since only one position in the ricinine, isolated after feeding with radioactive lysine, was labelled [scheme 2, (I) starred], the possibility presented itself that α-aminoadipic acid is an intermediate in the utilisation of lysine by the plant, through oxidative deamination of the ε-amino-group of the latter.[6] In a paper-chromatographic search this acid was indeed discovered.

In order to ascertain whether α-aminoadipic acid is formed from the lysine fed to the plant, this compound incorporating an ε-^{14}C atom was synthesised [6] and fed hydroponically to *Ricinus communis* L. This substance was incorporated by the plant more efficiently than lysine, and the labelled ricinine isolated was degraded as described above. In this case, only half of the radioactivity resided at the *non-carbonylic* α-carbon atom whilst the other half resided at the *carbonyl* carbon atom. The former conclusion was drawn in exact analogy to the result obtained for ricinine formed from lysine whilst the latter was drawn as follows: The hydroxy-amide (V) in this case was unlabelled, but the amide (IV) was labelled. The acid amide was submitted to the Schmidt reaction and 1·03 mol. of unlabelled

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[5] Dubeck and Kirkwood, *J. Biol. Chem.*, 1952, **199**, 307.

[6] Borsook, Deasy, Haagen-Smit, Keighley, and Lowy, *J. Biol. Chem.*, 1948, **176**, 1383, 1395.

carbon dioxide were isolated. The reaction mixture was then diluted with water and heated in order to hydrolyse the *N*-methylurea (VIII) which is the product of the Schmidt reaction. The carbon dioxide thus obtained (0·97 mol.), corresponding to the carbonyl-carbon atom of ricinine, was labelled.

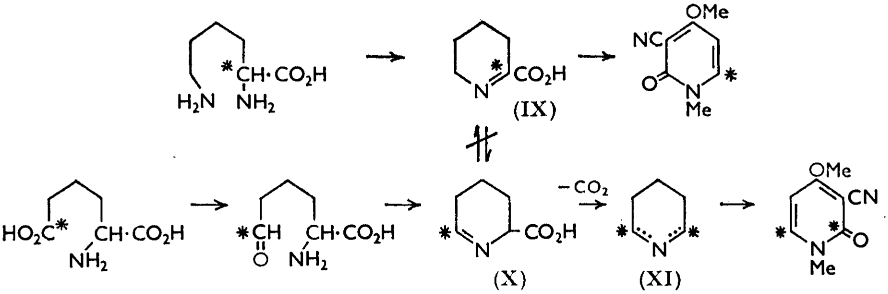
$$\underset{\text{(IV)}}{\mathrm{Me{\cdot}NH{\cdot}{*}CO{\cdot}CO_2H}} \longrightarrow \underset{\text{(VIII)}}{\mathrm{Me{\cdot}NH{\cdot}{*}CO{\cdot}NH_2}} + \mathrm{CO_2}$$

$$\longrightarrow \mathrm{(Me{\cdot}NH_3)_2SO_4 + (NH_4)_2SO_4 + {*}CO_2}$$

The present type of study cannot clarify the exact nature and order of steps in the incorporation of lysine to afford ricinine. However, the unique incorporation of radioactive carbon into a single position within ricinine indicates that lysine is not broken up into small fragments, albeit the incorporation is only of about 1 part in 10,000.

The formation of αα′-doubly labelled ricinine from α-amino[ε-^{14}C]adipic acid renders untenable the hypothesis that the latter is formed from lysine by oxidative deamination of its ε-amino-group. This hypothesis would require the formation of carbonyl-labelled ricinine if α-aminoadipic acid were in fact an intermediate in lysine incorporation. However, the results obtained in this work fit into the known metabolic relationships between lysine and α-aminoadipic acid in *Neurospora* and in the rat.[7] They may be rationalised as in scheme 3.

Scheme 3.



This scheme presupposes that cyclisation of α-aminoadipic acid occurs at the aldehyde oxidation state or its equivalent and that no equilibrium exists within the plant between the acids (IX) and (X). Further, while the acid (IX) is oxidised at the unlabelled α-position and the product is transformed into ricinine through a series of as yet unelucidated steps, the acid (X) must be decarboxylated to a symmetrical intermediate (XI) which is then further transformed into ricinine.

Leete has shown that, although lysine is not a precursor of the pyridine ring of nicotine or of anabasine, it is the precursor of the piperidine ring of the latter.[8] This is a further indication that a more hydrogenated ring is formed from lysine by *Ricinus communis* L. and converted into the higher oxidation state of a substituted pyridone through a series of secondary transformations.

Even more relevant is Leete's clear-cut demonstration that a preformed pyridine derivative (nicotinic acid) is a precursor of ricinine in the plant.[9] [*carboxy*-^{14}C]Nicotinic acid afforded [*cyano*-^{14}C]ricinine.

In an attempt to find a connexion between them, labelled lysine and unlabelled nicotinic acid were fed simultaneously to sprouting seedlings of *Ricinus communis* L. This resulted in a two-fold increase in the yield of ricinine, compared with that obtained in absence of nicotinic acid. However, the specific activity was also higher by a factor of 2. This is

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[7] Cf. Work, in " Amino Acid Metabolism," Johns Hopkins Press, Baltimore, 1955, pp. 468–469, and references therein.

[8] Leete, *J. Amer. Chem. Soc.*, 1956, **78**, 3520.

[9] Leete and Leitz, *Chem. and Ind.*, 1957, 1572.

not a significant increase in specific activity but it appears that the clarification of the relationship, if any, between these two precursors requires further investigation.

EXPERIMENTAL

Growth Experiments.—Seeds of *Ricinus communis* L. were treated with a solution made up of 1% aqueous mercuric chloride (3 parts by wt.) and ethanol (96%; 1 part by wt.) for 1 min. They were then transferred to pre-boiled water at 85° for 2 min. All parts of the equipment, water, and sand were sterilised.

The sterilised seeds were spread on sand in enamel trays (40 by 60 cm.) allowing 10 cm. between seeds. The trays were covered with plate glass and placed in a sterile dark cupboard at 27°, and the seeds were watered every 48 hr. Sprouting occurred *ca.* 5 days after " planting." The most well developed seedlings were transferred 10 days after " planting " to cylindrical glass jars (20 cm. diam.; 10 cm. high). Excess of sand was removed from the roots by rapid washing with sterile water. In order to avoid contact between the upper part of the plant and the aqueous solution into which the roots were dipped, the latter were drawn through 15 holes (0·75 cm. diam.) in a Perspex plate supported on several pegs 2 cm. from the bottom of the jar. The seedlings were watered through an opening in the cover of the jar, and sterile air was circulated during the whole growth-period. The seedlings grow to a height of *ca.* 20 cm. during 3 weeks. During this time the cotyledons emptied almost completely.

Feeding with Labelled Amino-acids.—The solution was added through the aeration inlet. Feeding was begun 2 weeks after " planting ". Two batches of DL-[2-^{14}C]lysine hydrochloride (Tracerlab, Inc.) were used in this work: (*a*) 55·3 mg. (0·1 mc) in the preliminary experiments; (*b*) 285 mg. (1 mc).

Lysine hydrochloride (20—80 mg.) was dissolved in sterile water (12 ml.), and portions (1 ml.) of this solution were added to each of two jars containing seedlings, during 6 days.

Isolation of Ricinine.—The seedlings were harvested 20 days after " planting." The roots were separated and discarded as they contain a negligible quantity of ricinine.[10] The rest of the plant material was dried at 95° and ground to a powder corresponding in weight to 31% of the living plant. The powder (20 g.) was extracted (Soxhlet) with light petroleum for 24 hr. The lipid concentration in the plant varies (8—13%) and depends upon the degree of emptying of the cotyledons.

The powder was dried at 95°, re-ground, and extracted with chloroform for 70 hr., the powder being broken up occasionally. (After development of this procedure, Leete and Leitz reported an almost identical procedure.) The extract was dried (Na_2SO_4), and the solvent was removed. Crystallisation of the yellow amorphous residue from ethanol and sublimation at 120°/0·01 mm. afforded colourless crystals (170 mg., 0·85%), m. p. 201°.

A more effective isolation procedure was found when ethanol-extraction (40 hr.) followed the defatting by light petroleum. Removal of the solvent afforded a brownish-red resin which was dissolved in water; a small insoluble portion was removed by filtration through cotton. The aqueous solution was extracted (10 hr.) with chloroform in a liquid–liquid extractor. Working up as above gave ricinine in 1·1% yield.

Isolation by Weever's procedure,[10] in which organic acids are removed as lead salts before extraction of the alkaloid, afforded ricinine in 0·9% yield. Tuson's even more cumbersome method [1] did not increase the yield.

Radioactivity Measurements.—A solution of pure ricinine (10 mg.) in a minimal volume of ethanol was evaporated by means of an infrared lamp on the surface of an aluminium disc (4·5 sq. cm.), slowly revolving on a turn-table. The radioactivity was measured by means of a Geiger–Müller end-window tube connected to a decimal counter (Ekco Electronics Ltd., Scaler type N 529A). The sample was placed in a lead castle 1 cm. from the tube window. Two tubes were used throughout this work: (*a*) Tracerlab, Inc. (end window) type TGC2, mica window, 1·8 mg./sq. cm.; (*b*) Nuclear Chicago (end window) type D34, mica window, 1·4 mg./sq. cm.

Table 1 summarises the results obtained with ricinine produced after feeding with DL-[2-^{14}C]-lysine hydrochloride.

[10] Cf. Weevers, *Rec. Trav. botan. Neerland*, 1932, **30**, 336.

TABLE 1.

Lysine hydrochloride		Ricinine		
Wt. (mg.)	Activity (μc)	Wt. (mg.)	Activity (10^2 c.p.m./millimole)	Tube
10	18·2	5	2·3	TGC 2
42·2	76·3	11	4·0	TGC 2
81·7	286·6	21	6·1	D34
74·2	261	25	54	D34
60	210	25	25	D34

Ozonisation of 4-*Methoxy*-1-*methyl*-2-*pyridone*.—The pyridone (II) (42 mg.) was suspended in dry ethyl acetate (2·5 ml.), and dry ozone (0·91 vol. %; large excess) was bubbled through the suspension at −60° from a Welsbach ozonator (model T-23) during 6 hr. The ozonisation flask was attached to a trap containing carbonate-free sodium hydroxide to absorb any carbon dioxide formed. The mixture was then oxidatively decomposed by the addition of 30% hydrogen peroxide ("AnalaR"; 2 ml.) with stirring at room temperature during 12 hr. without disconnexion of the carbon dioxide absorption tube. The solvents were then removed in a vacuum-desiccator over phosphorus pentoxide.

After attempted paper-chromatographic separation of the products, the following procedure, for which we are indebted to Dr. J. Avidor of the Israeli Institute of Biological Research, Ness Ziona, effected their separation: A portion of the solid residue after ozonisation (up to 30 mg.) was dissolved in dry ethanol (1 ml.). To this solution were added ether (2 ml.) and a solution (1·5 ml.) made up of equal volumes of 5% methanolic hydroxylamine hydrochloride and 12·5% methanolic sodium hydroxide. The mixture was kept at room temperature for 1 hr. and was neutralised with a few drops of acetic acid. It was then quantitatively transferred to sheets (25 × 25 cm.) of Whatman No. 3MM paper. Butanol–acetic acid–water (4 : 1 : 5) was run for 20 hr., the paper was dried at room temperature, and strips were sprayed either with a solution of ferric chloride in butanol [brown spot corresponding to the hydroxylamide (V)] or with Ferron solution [red spot corresponding to the amide (IV) which does not give a hydroxy-amide under the conditions described above].

Products (IV) and (V) were eluted from the paper by means of 75% ethanol in 85% and 65% yield, respectively.

An authentic specimen of the amide (IV) was prepared by the published procedure.[11] Potassium methyl oxalate [12] (12 mg.) was dissolved in ethanol (2 ml.) and ether (4 ml.) was added, then hydroxylamine hydrochloride. Working up and chromatography as described above and elution with 75% ethanol gave the hydroxy-amide.

The synthetic amide (IV) and hydroxy-amide (V) were identical in infrared spectra with the corresponding ozonolysis products.

Isolation of Carbon Dioxide from Ozonolysis.—The carbon dioxide absorbed in the trap attached was converted into barium carbonate.[13] Table 2 summarises the results of the radioactivity measurements made in a typical experiment, starting with labelled lysine hydrochloride (210 μc).

TABLE 2.

Compound	Activity (c.p.m./millimole)
Ricinine (I)	$2{\cdot}95 \times 10^3$
4-Methoxy-1-methyl-2-pyridone (II)	$9{\cdot}8 \times 10^2$
Hydroxy-amide (V)	8
Acid amide (IV)	5
Barium carbonate ($\equiv C_{(6)}$)	$6{\cdot}08 \times 10^2$

Determination of Radioactivity in the Cyano-group.—(*a*) The published procedure was used.[3] Ricinine (20 mg.; $5{\cdot}4 \times 10^3$ c.p.m./millimole) was heated with 57·4% sulphuric acid (1 ml.) at 140° for 1 hr., the resulting carbon dioxide being swept out with a stream of helium and absorbed in carbonate-free sodium hydroxide and then converted into barium carbonate (38·6 mg., 156% yield; $8{\cdot}2 \times 10^2$ c.p.m./mmole). The mixture was neutralised (litmus) with sodium hydroxide

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[11] Weddige, *J. prakt. Chem.*, 1902, **12**, 435.

[12] Skrabal, *Monatsh.*, 1917, **38**, 35.

[13] Hutchens, Claycomb, Cathey, and van Bruygen, *Nucleonics*, 1950, **7**, 41.

and extracted with chloroform. Drying the extract (Na_2SO_4) and removal of the solvent afforded colourless crystals (II) (11 mg., 65%), m. p. 113° (lit.,[3] m. p. 112—113°). (*b*) Labelled ricinine was converted into ricininic acid,[4] and the latter (20 mg.; $9{\cdot}2 \times 10^3$ c.p.m./millimole), suspended in water (0·7 ml.), was oxidised [4] with chromic acid (0·2 ml. of a solution of 200 mg. of chromium trioxide in 1 g. of concentrated sulphuric acid), whilst helium was passed through the mixture. Silver cyanide was precipitated, centrifuged, and dried at 105° to constant weight (17 mg., 97%; 19 c.p.m./millimole).

Determination of Radioactivity in OMe *and N*-Me *Groups*.—The published procedure was used.[5] We are indebted to Mr. E. Meier of the Weizmann Institute for Science of carrying out these determinations.

Ricinine (10 mg.; $2{\cdot}95 \times 10^3$ c.p.m./millimole) afforded tetramethylammonium reineckate from the methyl iodide corresponding to the methoxyl group (33 mg., 95% yield; 3·4 c.p.m./millimole) and to that corresponding to the *N*-methyl group (32·4 mg., 94% yield; 3·2 c.p.m./millimole).

Isolation of Amino-dicarboxylic Acids from Seedlings of Ricinus communis *L.*—Three-weeks-old etiolated seedlings (200 g.) were dried, powdered, extracted with ethanol, and worked up in the usual way.[14] Paper chromatography of the concentrated solution of amino-dicarboxylic acids [15] identified aspartic, glutamic, and α-aminoadipic acid. Two spots were not identified.

Isotopic Dilution Experiment with Nicotinic Acid.—Seedlings of *Ricinus communis* L. were grown as described above. In a control experiment feeding was carried out with DL-[2-^{14}C]-lysine hydrochloride (0·503%; 3 ml.; 100 μc) whilst in the parallel experiment the two-week-old seedlings were fed during 3 days with DL-[2-^{14}C]lysine hydrochloride (same amount) and nicotinic acid (1·91%; 6 ml.). After 3 additional days the seedlings were harvested and the alkaloid isolated in the usual way from each batch. The control gave ricinine (11·5 mg., $1{\cdot}84 \times 10^3$ c.p.m./millimole) whilst the nicotinic acid batch gave ricinine (17 mg.; $4{\cdot}5 \times 10^3$ c.p.m./millimole).

Labelled Ricinine from α-Amino[ε-^{14} *C*]*adipic Acid.*—The amino-acid (240 mg.; $1{\cdot}2 \times 10^6$ c.p.m./millimole) was prepared by the published procedure [6] in similar yield. It was fed to seedlings of *Ricinus communis* L. as described for the lysine-feeding experiments. Ricinine (15 mg.; $2{\cdot}6 \times 10^4$ c.p.m./millimole) was isolated in the usual way.

Determination of Radioactivity of the Cyano-group.—(*a*) Ricinine (11 mg.; $2{\cdot}6 \times 10^4$ c.p.m./millimole) was diluted with unlabelled ricinine (15 mg.) and hydrolysis of the cyano-group was effected by 57·4% sulphuric acid at 140° for 1 hr. Barium carbonate was isolated (77·5 mg.; 160%; $5{\cdot}3 \times 10^3$ c.p.m./millimole).

(*b*) Labelled ricinine (4 mg.; $2{\cdot}6 \times 10^4$ c.p.m./millimole) diluted with unlabelled ricinine (2 mg.) gave silver cyanide (3 mg.; 50 c.p.m./millimole).

Ozonisation of the Pyridone (II) *resulting from α-Amino*[ε-^{14}C]*adipic Acid.*—The pyridone was obtained in the usual way. Ozonisation of it (11·6 mg.; $2{\cdot}1 \times 10^4$ c.p.m./millimole) as described above afforded unlabelled hydroxy-amide (V) [corresponding to half-ester (III)], labelled carbon dioxide isolated as barium carbonate (9·3 mg.; $1{\cdot}09 \times 10^4$ c.p.m./millimole) and labelled amide (IV) (8 mg.; $0{\cdot}95 \times 10^4$ c.p.m./mmole).

Degradation of Labelled Amide (IV).—In a Schmidt reaction, a mixture of labelled amide (8 mg.; $0{\cdot}95 \times 10^4$ c.p.m./mmole) with unlabelled amide (IV) (4 mg.), sodium azide (23·5 mg.), and dry benzene (0·4 ml.) was treated with concentrated sulphuric acid (0·5 ml.) in two portions at room temperature. The flask was connected to a trap containing carbonate-free sodium hydroxide while helium was passed through the reaction mixture. The mixture was kept at 80° for 4 hr. Barium carbonate was prepared from the material in the trap in the usual way (24·2 mg., 103%; $1{\cdot}1 \times 10^2$ c.p.m./mmole).

To the cooled mixture attached to the trap was added 2N-sulphuric acid (0·4 ml.), helium was passed through the mixture, and the temperature was kept at 110° for 6 hr. Barium carbonate was isolated from the material in the trap (23 mg., 97%; $0{\cdot}9 \times 10^4$ c.p.m./mmole).

DEPARTMENT OF CHEMISTRY, ISRAEL INSTITUTE OF TECHNOLOGY,
HAIFA, ISRAEL.
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[14] Pucher, Abrahams, and Vickery, *J. Biol. Chem.*, 1948, **172**, 579.

[15] Bergstrom and Paabo, *Acta Chem. Scand.*, 1949, **3**, 202.