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NON-UNIFORM LABELLING OF GERANIOL BIOSYNTHESIZED FROM $^{14}CO_2$ IN *PELARGONIUM GRAVEOLENS*

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Key Index Word—*Pelargonium graveolens*; Geraniaceae; geranium; monoterpenes; biosynthesis; geraniol; non-random labelling from $^{14}CO_2$; dimethylallyl pyrophosphate pool.

Abstract—Geranium (*Pelargonium graveolens*) cuttings were exposed to a 2 hr pulse of $^{14}CO_2$ and then allowed to metabolize the label in circulating air for an additional 22 hr. Geraniol isolated from cuttings 2, 4, 8, 12, 16, 20 and 24 hr after the start of the experiment revealed the label in this compound to suffer substantial turnover. Chemical degradation of the labelled geraniol to yield the C_3-isopropylidene fragment showed the distribution of label favored the isopentenyl pyrophosphate-derived half of the molecule. Between 2 and 12 hr of the time-course the distribution of label between the halves of the molecule showed the proportion of label associated with the isopentenyl pyrophosphate-derived half to increase to 78%. From 12 to 24 hr this preferential labelling declined and approached an equal distribution between the halves. Hypotheses presented to rationalize these observations include the existence of a dimethylallyl pyrophosphate pool and multiple compartments of isoprenoid biosynthesis.

INTRODUCTION

WITH THE exception of geraniol synthesis in rose petals[1] the incorporation of MVA-^{14}C* into monoterpenes is extremely poor.[2,3] Degradation of the monoterpene products from such biosynthetic experiments indicates direct incorporation of MVA-^{14}C through the isoprenoid pathway.[4–6] However, in some instances extensive randomization of the label has been observed.[2,3] Evidence for the compartmentalization of monoterpene synthesis from that of sesquiterpene synthesis has been obtained based upon the differential incorporation of labelled substrates into these products.[6,7] Croteau *et al.*[7] have found glucose and CO_2 to be the most effective and acetate and mevalonate the least effective as substrates for monoterpene synthesis in *Mentha piperita* under optimum conditions of monoterpene production.

In several cases in which monoterpenes and sesquiterpenes have been biosynthesized from MVA-2-^{14}C the distribution of label has been found to be present mainly in that part

* The following abbreviations are used throughout this paper: MVA—mevalonic acid; IPP—isopentenyl pyrophosphate; DMAPP—dimethylallyl pyrophosphate.

[1] FRANCIS, M. J. O. and O'CONNELL, M. (1969) *Phytochemistry* **8**, 1339.
[2] LOOMIS, W. D. (1967) in *Terpenoids in Plants* (PRIDHAM, J. B., ed.), p. 59, Academic Press, London.
[3] FRANCIS, M. J. O (1971) in *Aspects of Terpenoid Chemistry and Biochemistry* (GOODWIN, T. W., ed.), p. 29, Academic Press, London.
[4] BANTHORPE, D. V. and BAXENDALE, D. (1970) *J. Chem. Soc.* **C**, 2694.
[5] BANTHORPE, D. V., MANN, J. and TURNBULL, K. W. (1970) *J. Chem. Soc.* **C**, 2689.
[6] CROTEAU, R. and LOOMIS, W. D. (1972) *Phytochemistry* **11**, 1055.
[7] CROTEAU, R., BURBOTT, A. J. and LOOMIS, W. D. (1972) *Phytochemistry* **11**, 2459.

of the molecule derived from IPP.[5,8–10] For example, Banthorpe *et al.*,[5,8] found that from 90% to 99% of incorporated label in thujone, isothujone and sabinene, biosynthesized from MVA-2-^{14}C in cuttings of *Thuja, Tanacetum* and *Juniperus,* appeared in the IPP-derived moiety. More recently,[7] chemical degradation of pulegone biosynthesized from $^{14}CO_2$ in peppermint cuttings showed virtually no label to be in the isopropylidene group which represents 3/5 of the 'starter' DMAPP unit.

A similar preferential labelling pattern has been observed by Biollaz and Arigoni[11] in the sesquiterpenes coriamyrtin and tutin biosynthesized from MVA-2-^{14}C in *Coriaria japonica.* However, in a similar experiment performed with the same plant, Corbella *et al.*[12] found only tutin to be radioactive and no preferential labelling of that part of the molecule derive from the terminal IPP-unit. Croteau and Loomis[6] have suggested that the discrepancy between the two results may be a consequence of the developmental stage of the plants used, or of the different procedures used for incorporating the label. The latter workers[6] examined the distribution of label in the sesquiterpene caryophyllene biosynthesized from MVA-2-^{14}C by *Mentha piperita* and found that less than 12% of the incorporated label was associated with that portion of the molecule derived from DMAPP. The residual label is almost equally distributed between the two isoprenoid units derived from IPP but slightly favors the terminal moiety. They speculate that this may be indicative of the presence of a GPP pool.

We have examined the distribution of label in geraniol biosynthesized from $^{14}CO_2$ in *Pelargonium graveolens.* Measurement of the proportion of label in the isopropylidene C_3 fragment was made on the purified monoterpene obtained during a 24 hr period in which the label was allowed to metabolize.

RESULTS AND DISCUSSION

The biogenetic route from acetyl CoA to GPP has been thoroughly reviewed.[13] Although it was believed that plant monoterpenes were formed by the same pathway, the incorporation of such precursors as acetate and mevalonate into these products was so low[2,3] that, until recently, unequivocal evidence for the intermediacy of mevalonate in monoterpene synthesis was lacking. Francis *et al.*[14] found that rose petals incorporated *RS*-MVA-2-^{14}C to the extent of 11% into the monoterpenols and their glucosides. Incorporation into geraniol and nerol β-glucosides specifically was a remarkable 2·5–5·0% after a 1 hr infusion with substrate. Chemical degradation of these terpenes showed that mevalonate was incorporated via the isoprenoid pathway and that the label was equally distributed between the moieties derived from IPP and DMAPP.

In the present study we have examined the time-course of geraniol synthesis by leaves of *Pelargonium graveolens* and found changes occur in the contributions of label from DMAPP and IPP to geraniol which constitutes 30–60% of the oil from this species.[15] On the reasonable assumption that $^{14}CO_2$ would be incorporated via mevalonate we chose the gaseous substrate because of the vastly greater incorporation into the monoterpene fraction

[8] BANTHORPE, D. V. and TURNBULL, D. W. (1966) *Chem. Commun.* 177
[9] BANTHORPE, D. V., CHARLWOOD, B. V. and FRANCIS, M. J. O. (1972) *Chem. Rev.* **72,** 115.
[10] BANTHORPE, D. V. and CHARLWOOD, B. V. (1971) *Nature (New Biol.)* **231,** 285
[11] BIOLLAZ, M. and ARIGONI, D. (1969) *Chem. Commun.* 633.
[12] CORBELLA, A , GARIBALDI, P., JOMMI, G. and SCOLASTICO, C. (1969) *Chem. Commun.* 634.
[13] CLAYTON, R. B. (1965) *Quart. Rev. (Lond.)* **19,** 168.
[14] FRANCIS, M. J. O., BANTHORPE, D. V. and LEPATOUREL, G. N. J. (1970) *Nature* **228,** 1005.
[15] GUENTHER, E. (ed.) (1950) *The Essential Oils,* Vol. IV, p. 701, Van Nostrand, New York.

possible.[16] A short pulse of $^{14}CO_2$ for the fixation was to be preferred over a long term fixation in which all the metabolic pools would eventually approach identical specific radioactivities. The results of preliminary experiments to determine the incorporation of label from $^{14}CO_2$ into hexane-extractable material with time of fixation is shown in Table 1. From these data it was evident that a minimum of a 2 hr fixation was necessary for a reasonable level of radioactivity to be incorporated into geraniol for degradation studies.

TABLE 1. INCORPORATION OF $^{14}CO_2$ INTO HEXANE-EXTRACTABLE MATERIAL FROM *Pelargonium graveolens*

Time of $^{14}CO_2$* fixation (min)	Radioactivity in hexane extract (dpm)	Incorporation (%)
20	2710	0·0025
40	6650	0·0060
60	17620	0·0160
120	95880	0·0871

* 2 geranium cuttings per 125 ml Erlenmeyer flask were exposed to 50 μCi $^{14}CO_2$ for each experiment.

An experiment in which cuttings were exposed to a 1 hr pulse of $^{14}CO_2$ and then allowed to metabolize in a sealed chamber for a further 24 hr demonstrated a substantial regular increase in label into the hexane-extractable material with time from 20 000 dpm after 1 hr to 225 000 dpm after 25 hr. Under these conditions the refixation of $^{14}CO_2$ liberated by respiration must contribute substantially to the overall incorporation. Gas chromatography and effluent counting of the extract showed that most of the radioactivity is associated with geraniol.

The distribution of label in geraniol was determined from a separate experiment in which cuttings were exposed to $^{14}CO_2$ for 2 hr and then allowed to metabolize the fixed label for an additional 22 hr in circulating air. The results from this experiment are shown in Figs. 1 and 2. During the chase period geraniol may be synthesized from $^{12}CO_2$ and from the products of metabolism of cellular constituents some of which may have been labelled during the initial 2 hr $^{14}CO_2$ fixation.

From Fig. 1, it can be seen that substantial amounts of label are not incorporated into geraniol over the first 8 hr. Geranyl glucoside has been reported[17] to occur in *P. graveolens* and it is conceivable that the synthesis and/or transport of geraniol proceeds through the intermediacy of such a derivative so that labelled geraniol itself would not appear in the hexane extract. Not until 12 hr after the start of the experiment are high levels of ^{14}C-geraniol present. This high level is maintained through the 20th hr of the experiment after which a sharp decline occurs presumably as the ^{14}C-geraniol is metabolized and the pool diluted with geraniol synthesized from $^{12}CO_2$ or from degradation products of ^{12}C-containing cellular constituents. The metabolic turnover of geraniol in *P. graveolens* has previously been observed[16] as well as monoterpenes[18] and sesquiterpenes[6] in *Mentha piperita*.

[16] ALLISON, R. D. (1963) M.S. Thesis, Oregon State University, Corvallis.
[17] BOURQUELOT, M. E. and BRIDEL, M. (1913) *Compt. Rend.* **157**, 72.
[18] BURBOTT, A. J. and LOOMIS, W. D. (1969) *Plant Physiol.* **44**, 173.

Ozonization of the isolated geraniol yielded acetone, generated from the isopropylidene group, which was trapped as its 2,4-dinitrophenylhydrazone. If $^{14}CO_2$ had been incorporated into the geraniol molecule in a random fashion, we would expect 30% of the total radioactivity in geraniol to appear in this C_3 fragment. Furthermore, since this fragment represents 3/5 of the dimethylallyl moiety of the geraniol molecule, then the proportion of the total radioactivity in the gerainol molecule residing in this C_3 fragment permits an estimate to be made of the contributions of the dimethylallyl and, by difference, the isopentenyl moieties. From the results (Fig. 2) it can be seen that the distribution of label between the two C_5 units favors IPP for almost the entire experiment. Only at the 2 hr stage which represents the end of the fixation period does the label appear to be almost equally distributed and slightly favoring the DMAPP-derived half of the molecule.

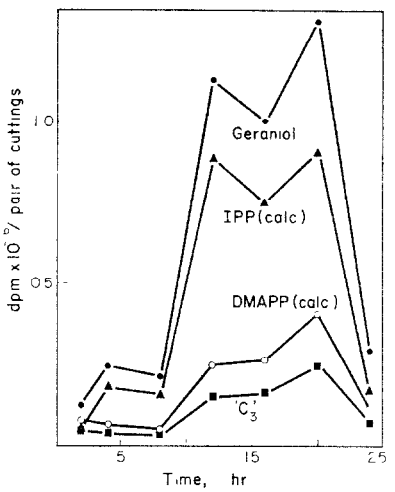

FIG. 1. RADIOACTIVITY IN GERANIOL AND ITS DISTRIBUTION BETWEEN THE MOIETIES DERIVED FROM IPP AND DMAPP.

14 geranium cuttings were exposed to 1·5 mCi of $^{14}CO_2$ for 2 hr in a 1000 ml bell jar. The cuttings were allowed to metabolize in circulating air up to an additional 22 hr. Pairs of cuttings were taken at 4 hr intervals from the start of the fixation. Geraniol isolated from the hexane-extractable material was degraded to give the isopropylidene group ('C_3') as described in the Experimental. The radioactivity in DMAPP is based upon the 'C_3' fragment representing 3/5 of the prenyl unit. The radioactivity in the IPP unit is then calculated by difference.

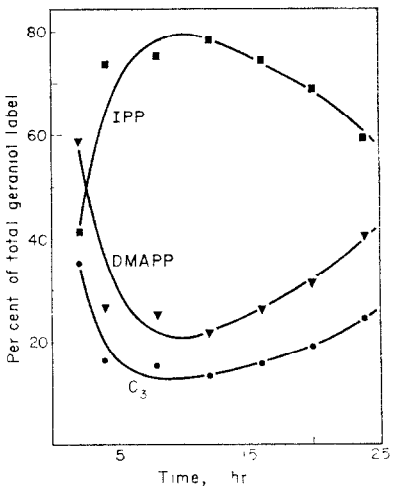

FIG. 2. PERCENTAGE DISTRIBUTION IN THE 'C_3' FRAGMENT, DMAPP AND IPP FROM LABELLED GERANIOL.

Geraniol was labelled by exposure to $^{14}CO_2$ for 2 hr of geranium cuttings which were then allowed to metabolize for an additional 22 hr in circulating air. Descriptions of the isolation and degradation of geraniol are given in the Experimental. The 'C_3' fragment represents the isopropylidene moiety of geraniol The radioactivity in DMAPP is based upon the 'C_3' fragment representing 3/5 of the prenyl unit. The radioactivity in the IPP unit is then calculated by difference.

A distribution of label favoring the DMAPP moiety may be rationalize by assuming that two compartmented isoprenoid pathways generate the halves of the geraniol molecule which is then synthesized at an intra-cellular surface. This model was earlier proposed by Francis to explain the non-equivalent labelling from MVA-2-^{14}C of the halves of certain monoterpenes.[3] The phenomenon of compartmentation as a regulatory device in isoprenoid biosynthesis is well known.[19] In the present case, under the CO_2-rich conditions

[19] ROGERS, L. J., SHAH, S. P. J. and GOODWIN, T. W. (1968) *Photosynthetica* **2**, 184.

(approx. 2× times the normal atmospheric concentration) used during the fixation period, the compartment generating DMAPP must be more accessible to the substrate than that generating the IPP. An alternate explanation is that some DMAPP may not originate via mevalonate but be synthesized from CO_2 by a different route when excess of the gas is available. However, there is no supporting evidence for the synthesis of monoterpenes by a non-mevalonoid route.

The preferential labelling of the IPP-derived half of the molecule observed throughout the remainder of the experiment may result from the condensation of IPP, labelled from $^{14}CO_2$ with an endogenous pool of DMAPP.[20] Clearly, this represents a site of synthesis distinct from the one generating early-labelled geraniol. Loomis and Croteau[21] have likewise suggested that our data may be interpreted by invoking the presence of two geraniol pools; the early one in which both halves of the geraniol molecule are almost equally labelled, represents 'leakage' of GPP originally destined for the synthesis of higher terpenoids; the samples obtained later in the time-course represent the C_{10} essential oil pool. In support of multiple sites of terpenoid synthesis, the same authors have already demonstrated the presence of separate sites for monoterpene, sesquiterpene and squalene synthesis in *Mentha piperita*.[6] Interestingly, although the labelling patterns of pulegone and caryophyllene from MVA-2-^{14}C supported the presence of a DMAPP pool, the label in squalene was equally distributed between the six prenyl units indicating a separate site for higher terpenoid formation.[22] Indirect evidence for the presence of two compartmented prenyl transferases in germinating pea seeds has also been reported.[23] The contribution to labelled geraniol by leakage from other sites of terpenoid synthesis is supported by the fact that the amount of leakage need be very small to account for the low level of label in early-labelled geraniol. Furthermore, consideration of the wide range of classes of terpenoids and partial terpenoids present in plants would suggest that there are potentially many different sites of higher terpenoid synthesis, several of which might be a trace source of GPP and thereby geraniol. Arguing against the leakage rationalization is the finding by Holloway and Popják[24] that the ratio of enzymic activities catalyzing the condensation of IPP with DMAPP, and IPP with GPP remained constant during the purification of *trans*-diprenyltransferase from pig liver. Consequently, they have concluded that both reactions are catalyzed by one and the same protein. Thus, under these circumstances GPP would not be expected to accumulate. Also, Green and Baisted[23] found no label accumulate in GPP from incubation of MVA-2-^{14}C with pea seed homogenates under conditions of steady state synthesis of squalene. Such *in vitro* conditions would favor leakage more so than the *in vivo* condition of the present study. However, Pollard *et al.*[25] in a similar *in vitro* study with pea seeds under slightly different conditions have reported the appearance of small amounts of radioactive geraniol generated by acid hydrolysis of water-soluble radioactive metabolites of MVA-2-^{14}C.

Between 2 and 12 hr of the time-course (Fig. 2) the distribution of label between the halves of the molecule showed the proportion of label associated with the IPP-derived half to increase to 78%. From 12 to 24 hr this preferential labelling declined and approached an

[20] Banthorpe, D. V., Charlwood, B. V. and Francis, M. J. O. (1972) *Chem. Rev.* **72,** 115.
[21] Loomis, W. D. and Croteau, R. (1973) in *Recent Advances in Phytochemistry* (Runeckles, V. C., ed.), Vol. 6, Academic Press, New York.
[22] Croteau, R. and Loomis, W. D. (1973) *Phytochemistry* **12,** (7), in press.
[23] Green, T. R. and Baisted, D. J. (1971) *Biochem. J.* **125,** 1145.
[24] Holloway, P. and Popják, G. (1967) *Biochem. J.* **104,** 57.
[25] Pollard, C. J., Bonner, J., Haagen-Smit, A. J. and Nimmo, C. C. (1966) *Plant Physiol.* **41,** 66.

equal distribution between the halves. At 24 hr when this approach to an equal distribution is greatest, 80% of the label in geraniol has turned over (Fig. 1). Thus, there must be a reversal in the preferential labelling pattern of early-labelled and late-labelled geraniol synthesized at the essential oil site. A plausible explanation of the observations made in this experiment would be that the site at which the essential oil is synthesized initially has a large, endogenous unlabelled DMAPP pool. During 24 hr this pool becomes labelled. The rate at which label appears in this pool is slow, however, because of its size. Geraniol is then synthesized at this site initially by condensation of labelled IPP with this endogenous DMAPP pool. Later however, condensation occurs with unlabelled IPP and an increasingly labelled DMAPP. The geraniol pool itself is sufficiently large that these changes can be observed over a 24 hr period.

The discrepancy between these results and those of Francis *et al.*[14] who found an equal distribution of label between DMAPP and IPP in geraniol biosynthesized from MVA-2-^{14}C in rose petals no doubt lies in the physiological uniqueness of the type of oil gland found in rose petals. As Loomis and Croteau[21] have pointed out the ready incorporation of MVA-^{14}C into rose petal monoterpenes indicates that their synthesis is not subject to the strict compartmentalization of monoterpene synthesis observed in other tissues. Furthermore, there can be no participation of an endogenous DMAPP pool in geraniol synthesis in order that there be an equal distribution of label between both halves of the geraniol molecule.[7]

EXPERIMENTAL

Materials. Pelargonium graveolens L'Her plants were grown in the greenhouse with a photoperiod of at least 15 hr and a day–night temp. cycle of 24° and 18°. The plants were grown in Perlite (Supreme Perlite Company, Portland, Oregon) and were irrigated 3× week with Hoagland and Arnon[26] nutrient solution No 2. Geraniol was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin, U.S.A. It was found to be >99% pure by GLC $^{14}CO_2$ was obtained as $Ba^{14}CO_3$ from New England Nuclear Corporation, Boston, Massachusetts, U.S.A. It had specific activity 62·2 μCi/μmol and was converted to $Na_2{}^{14}CO_3$ for use in the labelling experiments. In the latter form the stock solutions were prepared at a concentration of 2·5 μCi/ml.

Methods. Incorporation of $^{14}CO_2$ into P. graveolens. Cuttings with *ca* 2 cm stems and having no more than 3 developing leaves and the growing tip were used. Just before use they were severed from larger shoots under H_2O and two cuttings were kept in each small vial with sufficient water to cover the cut stem. All experiments were started in the morning. The $^{14}CO_2$ fixation was carried out in sealed bell jars (325 or 1000 ml capacity) or conical flasks (125 ml) filled with entry and exit tubes, dependent upon the number of cuttings used. The vials with the cuttings were arranged around a central vial containing $Na_2{}^{14}CO_3$ solution. The vessel was first flushed with CO_2-free air. A slight vacuum was then applied and the $^{14}CO_2$ released by injecting excess 10% $HClO_4$ acid into the central vial. Fixation was carried out at 24° under 10000 lx supplied by either Sylvania VHO Gro-Lux lamps supplemented with incandescent bulbs or an equal mixture of VHO Gro-Lux and VHO Gro-Lux wide spectrum lamps At the end of the fixation period, excess $^{14}CO_2$ was flushed into 4 N KOH and the radioactivity measured by scintillation counting.

Isolation and purification of geraniol-^{14}C. The cuttings were ground in a mortar with anhydr. Na_2SO_4, sand and hexane. Carrier geraniol (20 mg) was added to minimize evaporative losses of geraniol-^{14}C during extraction. The extraction was repeated until the final extract was colorless The combined extracts were decolorized with activated charcoal and, after centrifugation, the vol. carefully reduced under a slow stream of N_2 at room temp. Geraniol was isolated from the resulting oil by preparative TLC on 1/4 mm layers of adsorbent activated at 100° for 25 min. The first TLC was in dim light on $AgNO_3$-impregnated silica gel G (prepared in dim light by slurrying the adsorbent with 4% aq. $AgNO_3$, 1:2, w/v) with EtOAc–hexane (1:2, v/v), and the second on silica gel G in EtOAc–hexane (12:88, v/v). Standard geraniol was run as a marker and located with Rhodamine 6G under UV light (254 nm). The radioactive zone scraped from the glass and the geraniol was eluted with Et_2O. More carrier geraniol (20 mg) was added during the elution. The final Et_2O eluate was evaporated to a vol. of 10 ml in a stream of N_2 at room temp. Examination by GL-radiochromatography showed the samples to be radiochemically pure.

GL-radiochromatography of geraniol-^{14}C. Samples of the purified geraniol-^{14}C were chromatographed

[26] HOAGLAND, D. R. and ARNON, D. I. (1950) *Calif Agr. Exp. Sta. Circ.* **347**, 31.

on a Beckman GC4 instrument equipped with a FID, a 7:1 stream splitter, and a gas ionization detector (Nuclear Chicago, model 4998). The column was aluminum tubing (8·4 m × 3 mm) packed with 100–120 mesh Chromosorb G coated with 1 5% sucrose acetate butyrate and 1% phenyldiethanolamine succinate operated isothermally at 140° with He at 12 ml/min. Specific activity measurements of the low activity geraniol-^{14}C samples were made by counting aliquots on a scintillation counter, identical aliquots being analyzed quantitatively by GLC.

Radioactivity measurements. Non-aqueous samples were counted on a Packard, model 574, in scintillation vials containing 10 ml of a mixture of 2,5-diphenyloxazole (4 g) and 1, 4-bis-(5-phenyloxazol-2-yl)benzene (30 mg) in toluene (1 l.). For counting of radioactivity of aqueous samples, each vial contained 10 ml of Bray's scintillation fluor. Quenching corrections were made by 'spiking' vials with a known amount of toluene-^{14}C. Planchet counting of the 2,4-dinitrophenylhydrazone of acetone-^{14}C was carried out on a NMC Nuclear Measurements instrument, model PC 3A. 100 μl of $CHCl_3$ containing 1 mg of the derivative on a 3 cm dia. planchet was used for each measurement. Counting of all samples was to the 99% confidence limit, but errors in weighing and volume measurement gave specific activities accurate to *ca.* ±10%.

Ozonolysis of geraniol-^{14}C. A standard solution of geraniol in MeOH was added to each radioactive sample to give a known amount of geraniol (~ 100 mg) of known specific activity in 5 ml MeOH. The sample was ozonized with 2% O_3–O_2 mixture at 17 ml/min generated by a Welsbach ozonator, model T-23. The reaction was carried out in a dry ice-acetone bath and was complete in about 12 min (as measured by release of I_2 from acidic KI). The ozonide was decomposed by addition of HOAc (1 ml) at −10°. The mixture was then brought to room temp. during which time 1 ml of 50% H_2O_2 was added. The vessel was shaken at room temp. for 1 hr and an additional 2 ml of 25% H_2O_2 was added while allowing the mixture to stand at room temp. for a further hr. The mixture was heated at 100° under reflux for 1 hr and H_2O (2 ml) then added. Finally, the pH was brought to 5 with 2 N NaOH and the liberated acetone distilled directly into 2,4-dinitrophenyl hydrazine solution. The distillation temp. was kept below 65° (bp of MeOH). The crude product was re-crystallized repeatedly from hot EtOH. Specific activity measurements were made after each crystallization.

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