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TERPENE BIOSYNTHESIS FROM GERANYL AND NERYL PYROPHOSPHATES BY ENZYMES FROM ORANGE FLAVEDO

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Abstract—A cell free extract from orange flavedo forms phosphorylated derivatives of isopentenol, dimethylallyl alcohol and farnesol, as well as small amounts of hydrocarbons from 2-^{14}C mevalonic acid. Isopentenyl pyrophosphate 4-^{14}C is transformed into phosphorylated derivatives of dimethylallyl alcohol, nerol, geraniol, linalool and α-terpineol. The eventual formation of linaloyl pyrophosphate is discussed. Limonene is formed from 2-^{14}C neryl or geranyl pyrophosphate to the extent of 1–3%. α-Pinene is formed only from the *cis* isomer 2-^{14}C neryl pyrophosphate. There are differences between this system and a similar one from *P. radiata* seedlings. No interconversion of neryl and geranyl pyrophosphate was observed.

INTRODUCTION

THE BIOSYNTHESIS of cyclic monoterpenes involves the transformation of MVA (mevalonic acid) into 10 C pyrophosphorylated intermediates,[1] and subsequent elimination of the pyrophosphate moiety. The cyclization process leading to the formation of mono- or bicyclic-monoterpenes such as limonene or the pinenes apparently requires an adequate stereochemistry of the 10 C precursor. The *cis* isomer, NPP (neryl phyrophosphate) has been postulated as the more likely precursor of cyclic monoterpenes.[2] Experimental support for this hypothesis has been found both in chemical experiments,[3,4] in the formation of NPP from 2-^{14}C-MVA[5] and in the formation of α- and β-pinene from NPP by a cell free system obtained from seedlings of *Pinus radiata* D. Don.[6]

This system formed very small amounts of hydrocarbons from the *trans* isomer, GPP (geranyl pyrophosphate). This left the question open whether NPP had been formed by stereospecific synthesis or from GPP by a *trans–cis* isomerization. The idea of the former pathway seems more attractive since very few enzymic *trans–cis* isomerizations have been described,[7] whereas stereospecific condensations of 5 C units are the rule.[8] However recent work of Francis *et al.*[9] shows that rose petals form neryl and geranyl glucosides from stereospecifically labeled 4-^{3}H MVA with elimination of the *S* proton in both cases. This

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[1] W. D. LOOMIS, in *Terpenoids in Plants* (edited by J. E. PRIDHAM), p. 59, Academic Press, New York (1967)
[2] P. VALENZUELA, E. BEYTÍA, O. CORI and A. YUDELEVICH, *Arch. Biochem. Biophys.* **113,** 536 (1966).
[3] P. VALENZUELA and O. CORI, *Tetrahedron Letters* 3089 (1967).
[4] F. CRAMER and W. RITTERSDORF, *Tetrahedron* **23,** 3015 (1967).
[5] E. BEYTÍA, P. VALENZUELA and O. CORI, *Arch. Biochem. Biophys.* **129,** 346 (1969).
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[7] Y. J. TOPPER, in *The Enzymes* (2nd Edition, edited by P. D. BOYER, M. LARDY and K. MYRBÄCK), Vol. 5, p. 416, Academic Press, New York (1961).
[8] G. POPJAK and J. W. CORNFORTH, *Biochem. J.* **101,** 553 (1966).
[9] M. J. O. FRANCIS, D. V. BANTHORPE and G. N. J. LE PATOUREL, *Nature* **228,** 1006 (1970).

points to the formation of a *trans* compound and subsequent isomerization. Attaway[10] suggests that linaloylpyrophosphate, a tertiary isomer of NPP and GPP could be an intermediate in limonene biosynthesis in *Citrus*. He also showed the transformation of 3-^{14}C linalool into limonene and geraniol in whole *Citrus* fruits.[11]

It was thought worth exploring terpene formation in *Citrus* rind, which can be obtained in large quantities and contains appreciable amounts of several isoprenoids, limonene being one of the most abundant compounds in orange rind oil[12] and in orange leaves.[13] Some enzymes involved in the biosynthesis and utilization of MVA have been described in orange juice vesicles.[14–16]

There are no data on the transformations of MVA in orange rind, where limonene is a very abundant component. The present communication establishes that water soluble enzymes from orange flavedo transform 2-^{14}C MVA and 4-^{14}C IpPP (Isopentenyl pyrophosphate) into terpene hydrocarbons and alcohols and form α-pinene from NPP. The pattern of limonene formation from 10 C precursors is different from the situation observed in *Pinus radiata* seedlings.[6]

RESULTS

A cell free extract obtained from orange flavedo (exocarpium) transformed 2-^{14}C MVA into hexane soluble compounds, as well as into water soluble compounds which became hexane soluble either after treatment with acid (allylic phosphates) or after treatment with an enzyme mixture which splits pyrophosphates into monophosphates (apyrase) and the latter into alcohols plus orthophosphate (phosphomonoesterase) (Table 1). These results are very similar to those reported for a cell free extract from *Pinus radiata* seedlings.[5]

Omission of KF from the reaction mixture increased the 'free lipid' fraction with a concomitant decrease of the 'prenyl phosphates'. In contrast with the results obtained in *Pinus*, the 'allylic phosphates' fraction was lower in the presence of fluoride (Table 1).

TABLE 1. ENZYMIC TRANSFORMATION OF MEVALONIC ACID 2-^{14}C INTO PRENOLS AND PHOSPHORYLATED COMPOUNDS

	Total Radioactivity (Δ counts/min per tube)		Radioactivity (Δ counts/min/mg protein)		Fraction of (RS) Mevalonic acid utilized (%)	
KF added, mM	0	30	0	30	0	30
'Free lipids'	26,594	20,636	20,400	15,800	5·9	4·6
'Allylic phosphates'	18,984	14,792	14,600	11,400	4·3	2·5
'Prenyl phosphates'	39,832	44,232	30,600	33,900	8 9	9 8

Temperature 37°; incubation time 2 hr. Total volume 1 ml. Composition of medium: 33 mM Tris-HCl buffer pH 7·4; 2·5 mM $MgCl_2$; 2·5 mM $MnSO_4$; 5 mM ATP; 20 mM 2-mercaptoethanol; 30 mM KF; 0·05 mM MVA 2-^{14}C (450,000 counts/min/tube); flavedo proteins 1·3 mg/ml. For analytical procedures and designation of fractions (see under Experimental).

[10] J. A. ATTAWAY, A. P. PIERINGER and L. J. BARABAS, *Phytochem.* **6**, 25 (1967).
[11] J. A. ATTAWAY and B. S. BUSLIG, *Biochem. Biophys. Acta* **164**, 609 (1968).
[12] E. GUNTHER, *The Essential Oils*, Vol. III, p. 125, Van Nostrand, New York (1949).
[13] J. A. ATTAWAY, A. P. PIERINGER and L. J. BARABAS, *Phytochem.* **5**, 141 (1966).
[14] V. H. POTTY, *J. Food Sci.* **34**, 231 (1969).
[15] V. H. POTTY and J. H. BRUEMMER, *Phytochem.* **9**, 99 (1970).
[16] V. H. POTTY and J. H. BRUEMMER, *Phytochem.* **9**, 1229 (1970).

Albedo (mesocarpium) extracts were completely inactive and those from whole orange rind were less active than flavedo extracts. Flavedo slices also formed hexane soluble compounds from 2-^{14}C MVA. There were marked differences in the total amount of MVA utilized by extracts from other varieties of oranges (Washington, Thompson, Valencia), and of lemons but the general pattern was the same as reported here.

The results were the same if the incubations were performed under nitrogen. Mg^{2+}, Mn^{2+} and SH groups were required for maximal activity. The rate of reaction was linear throughout 150 min. The rate was not strictly proportional to the amount of protein added, suggesting the presence of an inhibitor. In contrast to the *Pinus* extracts, our *Citrus* extracts were quite unstable. Their activity was completely lost after 18 hr at 4° or at −18°.

TLC of the 'free lipids' fraction showed that it was a mixture of hydrocarbons and of 5, 10 and 15 C prenols. GLC analysis of this fraction showed that the main components were isopentenol and dimethylallyl alcohol. 10 C alcohols were minor components, though radioactivity was found in the farnesol. The same pattern was obtained if the 'prenyl phosphates' fraction was analyzed by gas chromatography. We also observed the presence of radioactivity which had the retention volumes of 20 C prenols.

As described in *Pinus*, a crude preparation of phosphatidyl-ethanol-amine from beef brain[17] increased the total amount of 'free lipids'. The TLC pattern remained the same, although in some experiments an increase in hydrocarbons was observed. This point is being further explored.

When 4-^{14}C IpPP was used as substrate, the 'prenyl phosphates' fraction contained, in addition to isopentenol, derived from unreacted IpPP, dimethyl allyl alcohol, nerol, geraniol, linalool and α-terpineol (Table 2). The 'free lipids' fraction did not contain measurable amounts of radioactivity in hydrocarbons

TABLE 2. ENZYMIC FORMATION OF PRENOLS FROM 4-^{14}C ISOPENTENYL PYROPHOSPHATE

	Radioactivity (Δ counts/min)
Total radioactivity in "Prenyl phosphates"	107,000
Recovered as isopentenol	69,000
Recovered as Dimethylallyl alcohol	26,700
Recovered as linalool	4300
Recovered as α terpineol	2200
Recovered as nerol	1020
Recovered as geraniol	790
Total recovered	104,010

Temperature 30°; incubation time 2 hr. Total volume 1 ml. Composition of medium: 50 mM Tris-HCl buffer pH 7·4; 20 mM 2-mercaptoethanol; 30 mM KF; 30 mM $MgCl_2$; 10 mM $MnSO_4$; 0·015 mM IpPP 4-^{14}C (150,000 counts/min/tube); flavedo protein 1·5 mg/ml.

After extraction of the 'free lipids' with hexane, the deproteinized aqueous phase was treated with apyrase plus phosphomonoesterase, and the resulting prenols were extracted with hexane. The hexane extract was concentrated under gentle vacuum and 13,850 counts/min of the concentrated extract were injected into a gas chromatograph (see under Experimental). Isothermal temperature: 120°. Gas flow 40 ml helium/min. Injection port 150°; detector 250°.

[17] C. GEORGE-NASCIMENTO, E. BEYTÍA, A. R. AEDO and O. CORI, *Arch. Biochem. Biophys.* **132**, 470 (1969).

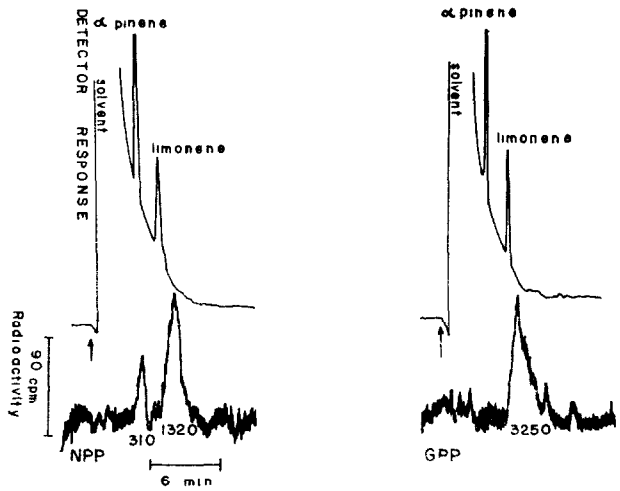

FIG. 1. RADIOACTIVITY IN HYDROCARBONS DERIVED FROM 2-^{14}C GERANYL PYROPHOSPHATE OR NERYL PYROPHOSPHATE.

Complete experiment described in Table 3. Details given under Experimental. Total radioactivity injected as indicated by the arrows: 4000 counts/min. The figures under the radioactivity peaks indicate the amount of counts per tube found in that particular fraction.

In contrast with this, Table 3 shows that limonene and α pinene were formed from 2-^{14}C NPP to the extent of 1% of the substrate added. Limonene was also formed from 2-^{14}C GPP, but no radioactivity was found with the α-pinene peak when this substrate was used (Fig. 1). About 3% of the counts added were found in limonene. The total amount of radioactivity released as hexane soluble compounds was much larger when GPP was the substrate, than when NPP was used (Table 3). The amount of limonene as percentage of this total radioactivity released was the same for both precursors. This is at variance with our results in *Pinus*.[6] However, in both organisms, no α-pinene formation could be detected from GPP.

GLC of the 'free lipids' fraction from the same experiment showed that no geraniol was formed from NPP, nor nerol from GPP. If the experiments were run for shorter times to allow for some unreacted 10 C pyrophosphates to remain in the reaction mixture, and the sample was analyzed for 'prenyl phosphates' (i.e. radioactivity rendered hexane soluble by the effect of apyrase plus phosphomonoesterase) the results of GLC were substantially the same: No interconversion of the 10 C intermediates occurred in this flavedo extract.

DISCUSSION

The transformation of 2-^{14}C MVA by a cell free enzyme system from orange flavedo are very similar to those reported in *P. radiata* seedlings[5] and in juice vesicles from orange.[15,16] MVA is transformed into a mixture of phosphorylated prenols. A phosphatase which may be partially inhibited by fluoride[18] transforms these phosphorylated compounds into the corresponding unrearranged prenols, which were identified by gas chromatography.

[18] J. SCHORMÜLLER, N. PFROGNER and F. HOLZ, *Z. Lebensmitteluntersuch.Forschg.* **127**, 325 (1965).

The effect of fluoride on the phosphatase is less marked in *Citrus* than in *Pinus*. Another difference observed was a decrease of allylic phosphates produced by KF. This may be due to an inhibition of isopentenyl pyrophosphate isomerase. Direct measurement of the amount of dimethyl allyl pyrophosphate from IpPP 4-^{14}C in the presence and in the absence of KF confirms this assumption.

Loomis[1] stresses the fact that reported incorporations of label into monoterpenes are of the order of 0·1% or lower. Our cell free system incorporates from 1 to 3% of the radioactivity added as NPP or GPP into hydrocarbons. This and our previous communication in *Pinus*[6] seem to be the first reports of terpene hydrocarbons formed in a cell free system.

The fact that both GPP and NPP are precursors of limonene in *Citrus* suggests two main alternatives for the biosynthesis of this hydrocarbon: either isomerization of these two substrates and formation of limonene specifically from one of them or formation of the same hydrocarbon from two substrates through different pathways. One variant of this last alternative would be the confluence of these two pathways into one common intermediate different form GPP or NPP. Direct isomerization of GPP and NPP is excluded by the results in Table 3, where no geraniol was found to be formed from NPP nor vice versa.

We have not been able to observe a direct transformation of NPP or GPP into another phosphorylated 10 C intermediate. However, the possible existence of such an intermediate,[10,13,16,19] finds support in some of our data. The incorporation of radioactivity from IpPP into compounds which yield α-terpineol or linalool after enzymic hydrolysis points to phosphorylated α-terpineol or linalool as possible intermediates.

TABLE 3. ENZYMIC TRANSFORMATION OF NERYL PYROPHOSPHATE AND GERANYL PYROPHOSPHATE INTO HYDROCARBONS AND 10 C PRENOLS

	Radioactivity (Δ counts/min/tube)	
	From NPP	From GPP
Radioactivity in hexane extract	33,250	115,000
Radioactivity in Nerol	23,500	0
Radioactivity in geraniol	0	110,000
Radioactivity in limonene	1300	3200
Radioactivity in α pinene	300	0
Limonene as percentage of total radioactivity in hexane extract	3·9%	3·2%

Temperature 37°; time 120 min. Total volume 2·5 ml. Composition of medium: 33 mM Tris-HCl buffer pH 7·4; 2 mM $MgCl_2$; 2 mM $MnSO_4$; 2 mM 2-mercaptoethanol; 30 mM KF; 0·04 mM GPP or NPP (120,000 counts/min/tube); flavedo proteins 0·8 mg/ml.

Aliquots of the hexane extract were injected into gas chromatographic columns for hydrocarbon or prenol analysis, (see under Experimental). Results of the former analysis are detailed in Fig. 1.

Linaloyl pyrophosphate has been proposed by Attaway[10,13] as a precursor of limonene on the basis of seasonal fluctuations of linalool and limonene in orange leaves, as well as the incorporation of 3-^{14}C linalool into geraniol, α-terpineol and limonene, but not into α-pinene by whole tangerine fruits.[20]

While this manuscript was in preparation, Potty[16,19] reported the formation of a

[19] V. H. POTTY, M. G. MOSHONAS and J. H. BRUEMMER, *Arch. Biochem. Biophys.* **138**, 350 (1970).
[20] J. A. ATTAWAY and B. S. BUSLIG, *Phytochem.* **8**, 1671 (1969).

phosphorylated derivative of linalool in a cell free system from orange juice vesicles, and the cyclization of 3-^{14}C linalool to menthadiene-1-ol.

Although we find, in agreement with Attaway's proposal, that a phosphorylated derivative of linalool is formed from a 5 C but not from a 10 C pyrophosphate, it would still be possible to visualize the transformation of GPP or NPP into linaloyl pyrophosphate similar to the intramolecular migration of pyrophosphate proposed by Popjak[21] for the formation of nerolidyl pyrophosphate. The distance between C_1 and C_3 of GPP or NPP is adequate to be spanned by an —O—P—O—P—O— bridge without strain. The *cis* or *trans* geometry at the double bond should not matter in this process, since the migration of the pyrophosphate group from C_1 to C_3 would occur perpendicularly to the plane of the two substituents at C_3. The 10 C intermediate could be visualized as bound to the enzymes involved in its biosynthesis. This may be an interpretation of the radioactivity from 2-^{14}C-MVA which we have found loosely bound to soluble proteins in *P. radiata*[5] and more recently in oranges.

The formation of GPP and NPP from 5 C intermediates is supported by Table 2. The discrepancy of these results with those of Potty[16,19] who does not find geraniol or nerol in his cell free preparations, may be due to differences between enzymic activity in flavedo and in juice vesicles. The former synthesizes substantial amounts of monoterpenes, and thus it is conceivable that the intermediates (NPP or GPP) may accumulate to higher steady state concentrations

The absence of interconversion of geraniol and nerol or their phosphorylated derivatives seems to favour the hypothesis of a stereospecific synthesis of the *cis* and *trans* 10 C-pyrophosphates over the possibility of an isomerization. Should NPP be an obligatory precursor of bicyclic monoterpenes, as shown in *Pinus* and now in orange, then it would be very difficult to reconcile the results presented here with the existence of an isomerization process, in which case GPP should be also a good substrate. The only possibility left open, would be to assume that the '10-C isomerase' would exist in whole tissue, but that it would either not be extracted or be destroyed by the process we have used to prepare the extract.

The results of Francis[9] in rose petals support the isomerization hypothesis for the formation of NPP from GPP. It is, however easy to conceive the existence of species differences between roses and oranges, and it is quite possible that isomerization and stereospecific synthesis are not mutually excluding processes. Experiments to analyze these alternatives in *Pinus* and *Citrus* are in progress.

EXPERIMENTAL

Preparation of Enzyme Extracts

Oranges of the 'Chilean' variety,* purchased from commercial sources, were cleaned with running tap H_2O and then with distilled H_2O. The flavedo (exocarpium) was grated off from the whole fruit with a hard polyethylene grater and the gratings were homogenized at 0° in an 'Omnimixer' (Ivan Sorvall, Norwalk, Connecticut) at top speed for 2–4 min with (2:3, v/w) buffer (100 nM Tris-HCl buffer pH 7·8; and 0·1 mM Na ethylenediaminotetraacetate). In some experiments the whole rind or the albedo (mesocarpium) was homogenized in a similar way.

* This variety was obtained about 80 yr ago through seed reproduction, and since then it has been reproduced through grafts selecting for juice production. It is a medium sized orange, of thin rind, and abundant seeds. It is the most common variety of juice oranges in this country. Information kindly furnished by the Instituto de Investigaciones Agropecuarias 'La Platina'.

[21] G. POPJAK, J. EDMOND, K. CLIFFORD and W. WILLIAMS, *J. Biol. Chem.* **244**, 1897 (1969).

The homogenate was centrifuged for 30 min at 32,000 *g* and 0°. The supernatant film and the precipitate were discarded, and the clear extract was used for all experiments. Centrifugation for 90 min at 104,000 *g* does not change the results reported. Proteins were determined by turbidimetry.[22] The extract contains from 1·5 to 4 mg of protein/ml.

All experiments were performed between May and September (late fall and early spring).

Enzymic Reactions

Incubations were performed in duplicate in glass stoppered conical tubes, under the conditions detailed in each table. Controls with boiled enzyme were always performed, and the results reported (Δ counts/min) are already corrected for these non-enzymic controls.

The enzymic reaction was stopped by heating for 3 min at 100°. The denatured proteins were removed by centrifugation and aliquots of the supernatant were used for further analysis, to differentiate between different radioactive products, as already described.[5]

Direct treatment of the deproteinized supernatant with 2 ml of hexane extracts hydrocarbons and prenols. We have called this fraction 'free lipids.' Acid treatment of the supernatant releases radioactivity from allylic phosphates as rearranged alcohols, which become thus extractable with hexane. ('Allylic phosphates'). Treatment with potato apyrase plus alkaline phosphomonoesterase from *E. coli* (Prenyl phosphates) releases isopentenol and unrearranged allylic alcohols from the corresponding pyrophosphorylated compounds.[5] The two last fractions contain in addition the 'free lipids' fraction; the results reported are already corrected by substraction of this value.[5]

Aliquots of the hexane extract were counted in a liquid scintillation spectrometer[5] and other aliquots were used, after the addition of 300 μg of each of the appropriate carriers, for thin layer or gas–liquid chromatography.

Heat inactivation of the enzyme leads to a substantial loss of hydrocarbons. Thus, when GPP or NPP were used as substrates (Table 3), the reaction was stopped by cooling the tubes to 0°, the prenols and hydrocarbons were extracted with 2 ml of hexane and analyzed by GLC.

Thin Layer Chromatography

Silica gel G plates were developed in ethyl acetate–benzene–hexane 12:25:63 (v/v). Spots were located with I_2 vapours. The silica gel was scraped from the plates in 7 mm portions into scintillation vials and counted. This solvent system does not differentiate 5 C from 10 C prenols, nor limonene from the pinenes, but the separation of hydrocarbons from alcohols is very satisfactory.[6]

Gas–Liquid Chromatography

The instrument used was a Varian Aerograph 1800 equipped with a thermal conductivity detector programmer. The effluent from the gas chromatograph was carried into a heated proportional counter (Nuclear Chicago, Biospan 4998) through a heated stainless steel line. Mass and radioactivity peaks were recorded by a two channel instrument (Varian 20). The lag between carrier and radioactivity peak was about 18 sec. The area of the radioactivity peak was measured by cutting out and weighing the paper, and by comparison with peak areas obtained with known amounts of radioactivity. In the experiments with IpPP, radioactivity was measured by collecting the effluent in scintillation vials and subsequent counting.[5]

Hydrocarbons were separated on a 300 × 0·62 cm column of 2% β,β′ oxidipropionitrile on Chromosorb W 60/80 mesh. Temperature 50°, helium flow 30 ml/min. The injection port was kept at 135°. No cyclization of nerol or geraniol by dehydration in the instrument occurs under these conditions.[6] Prenyl alcohols were separated on a similar 2% ethylene glycoladipate column either at 110° (Table 2) or with programmed temperature between 60 and 190° with a flow of He of 30 ml/min.

Radioactive Substrates

(RS) MVA 2-^{14}C (5–7 μc/μM) was purchased from New England Nuclear Corporation, with chromatographically controlled radiochemical purity. IpPP 4-^{14}C was prepared from MVA 2-^{14}C by means of reconstituted serum from Hevea brasiliensis.[23]

NPP and GPP 2-^{14}C were synthesized chemically from 6-methyl heptenone and 2-^{14}C methyl bromoacetate (New England Nuclear Corp.) as previously outlined.[6] The radiochemical purity of the prenol moiety was determined by extraction with hexane after hydrolysis of the pyrophosphate by means of apyrase plus phosphomonoesterase and subsequent gas chromatography of the hexane extract. Nerol thus obtained from NPP was always completely free of geraniol. However, some batches, of GPP contained measurable amounts of radioactivity as NPP. It is not quite clear to us, whether this contamination, which is always one sided, is due to faults in the separation of the geranyl ester during the chemical synthesis, or to some chemical rearrangement in later steps. Non enzymic controls of the substrates were always performed to exclude any effects of this contamination.

[22] E. R. Stadtman, G. D. Novelli and F. Lipmann, *J. Biol. Chem.* **191**, 365 (1951).
[23] C. J. Chesterton and R. G. O. Kekwick, *Arch. Biochem. Biophys.* **125**, 16 (1968).

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