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EFFECT OF LIGHT ON THE INCORPORATION OF ISOPENTENYL PYROPHOSPHATE INTO PHYTOL IN THE LEAVES OF *PHASEOLUS VULGARIS*

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Abstract—Isopentenyl pyrophosphate has been shown to be incorporated into phytol in the dark by homogenates of etiolated leaves of *Phaseolus vulgaris*. There was an immediate stimulation of the incorporation after illumination of the leaves for a short period, but this stimulation diminished if the seedlings were returned to complete darkness.

INTRODUCTION

IT APPEARS probable that in the final stages of chlorophyll biosynthesis in higher plants the double bond between carbons 7 and 8 of the D ring of Mg vinyl phaeoporphyrin a_5 (protochlorophyllide) is reduced before the esterification of the propionic acid residue with phytol. That is, chlorophyll is finally formed from the reduced, but unesterified, chlorophyllide rather than from the esterified but unsaturated protochlorophyll. The evidence concerning this is however circumstantial and, at points, conflicting.

Granick[1] originally adduced evidence from solubility studies of the chlorophyll-like material of etiolated barley leaves for the existence of the phytyl ester of Mg vinyl phaeoporphyrin a_3 (protochlorophyll) i.e. the esterified but unreduced precursor. Wolf and Price[2] on the other hand concluded, largely on the basis of somewhat similar solubility studies of the pigments of etiolated bean seedlings (*Phaseolus vulgaris*), that, although some protochlorophyll was present, this material was not altered upon illumination *in vivo*, whilst the unesterified protochlorophyllide became reduced and subsequently esterified by phytol to give chlorophyll; the latter process did not require light. The *in vivo* spectroscopic observations of Shibata[3] were, to some extent consistent with this view. Further evidence based largely on the spectroscopic investigation of chlorophyll-like pigments separated before and after illumination, was produced by Virgin[4] and by Sironval *et al.*[5] who studied barley, for the view that both protochlorophyll and protochlorophyllide were present in etiolated leaves but that only the unesterified protochlorophyllide became reduced by light to chlorophyllide.

It is not even clear whether the biosynthesis of phytol itself occurs in the dark in flowering plants. Fischer and Rudiger[6] were unable to detect phytol after hydrolysis of the chlorophyll-like pigments of etiolated barley leaves and Hromatka *et al.*[7] were unable to detect phytol in

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[2] J. B. WOLF and L. PRICE, *Arch. Biochem. Biophys.* **72**, 293 (1957).
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[5] C. SIRONVAL, M. R. M. WOLWERTZ and A. MADSEN, *Biochim. Biophys. Acta* **94**, 344 (1965).
[6] F. G. FISCHER and W. RUDIGER, *Ann. Chem. Liebig.* **627**, 35 (1959).
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etiolated runner bean leaves. (*Ph. coccineus*). Godnev *et al.*[8] on the other hand, claimed that phytol could be detected in the chlorophyll-like pigments of barley, and Fischer and Rudiger[8] did report the presence of phytol in the protochlorophyll of etiolated *Cucurbita pepo.*

From this it seemed that some more direct evidence concerning the existence of phytol in etiolated leaves and any possible relation between chlorophyllide esterification and phytol synthesis might be obtained from an investigation of the incorporation of the isoprenoid monomer, isopentenyl pyrophosphate, into phytol by homogenates of etiolated leaves from *Ph. vulgaris* obtained before and after illumination.

RESULTS AND DISCUSSION

Etiolated seedlings of *Phaseolus vulgaris* grown in darkness for 7–10 days green considerably after exposure to light for 10–12 hr and it is therefore reasonable to assume that they will

TABLE 1. EFFECT OF LIGHT ON THE INCORPORATION OF [1-^{14}C] IPP INTO PHYTOL

Source of homogenate	Incorporation of IPP into phytol		Incorporation of IPP into "prenols"	
	(dpm)	(mμmoles IPP incorporated*)	(dpm)	(mμmoles IPP incorporated†)
Etiolated leaves	4,403	20·0	33,611	153
Illuminated leaves Period in darkness after illumination (hr)				
0	6,323	28·7	53,991	245
1	5,165	23·4	37,230	169
5	5,479	24·8	38,408	174
10	5,223	23·7	36,665	166

No incorporation of IPP into phytol or into the "prenols" fraction was observed for the incubations stopped at zero time.

* Values quoted/25 mg dry wt. of leaf tissue.

† Tentative identification by TLC only (see Experimental).

The method of preparation of the homogenate and the composition of the incubation mixture are given in the text.

produce material active in chlorophyll synthesis. Homogenates of leaves derived both from completely etiolated plants and from plants exposed to light for 10 min and subsequently returned to darkness for varying periods were prepared and their capacity to incorporate [4-^{14}C] IPP into phytol was investigated. Although phytol, either free or combined, could not be detected in the etiolated leaves by the procedure of Shimzu and Fukushima[9] it is seen from Table 1, which gives details from a typical experiment from several carried out, that there is appreciable incorporation of [4-^{14}C]-isopentenyl pyrophosphate into phytol by homogenates derived from such leaves. When the leaves were illuminated there was an immediate increase in the capacity of the homogenates to incorporate IPP into phytol but this increased level of

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incorporation declined upon the subsequent return of the plants to absolute darkness. Similar effects were observed when the incorporation of IPP into the material which chromatographed in an identical manner to farnesol and geraniol on silica gel and silica gel-$AgNO_3$ thin layer plates, was measured. The results obtained on this material designated as prenols is shown in Table 1. These leaf homogenates were also found to be able to incorporate mevalonate into phytol in the presence of ATP.

These observations which were quite readily reproducible clearly show that light is not a prerequisite for phytol synthesis from IPP, and that if the homogenization has not destroyed any inhibiting mechanism; phytol could be available in etiolated leaves for the conversion of protochlorophyllide to protochlorophyll. The lag period in the conversion of chlorophyllide to chlorophyll observed spectroscopically does not therefore seem to arise from a time-dependent synthesis of phytol. It cannot however be deduced from the data presented whether the increased incorporation of IPP into phytol observed after illumination is due to a stimulation of the phytol synthesizing apparatus *per se*, to a diminution of the endogenous pool of precursors of phytol, or to a controlled replacement of endogenous phytol used for chlorophyll formation. Similarly, it is possible that the decreased incorporation of IPP into phytol found in homogenates derived from leaves returned to darkness after illumination arises either from an increase in the level of competing endogenous precursors or to a feed back inhibition arising from the phytol formation from these endogenous precursors.

EXPERIMENTAL

Preparation of [4-^{14}C] *Isopentenyl Pryophosphate (IPP)*

[4-^{14}C] isopentenyl pyrophosphate was prepared biosynthetically from 2 hr incubation at 30° of 200 μmoles [2-^{14}C] DL-mevalonate (MVA) (0·1 μc/μmole), 100 μmoles ATP, 100 μmoles $MgCl_2$, 250 μmoles phospho-enol pyruvate, 150 μmoles *N*-acetylcysteine 1 μmole KCl, 125 μg pyruvate kinase (specific activity 200 μmoles substrate converted/min/mg protein) with 300 mg reconstituted freeze-dried *Hevea* latex serum[10] made up to final volume of 20 ml with Tris-HCl buffer pH 7·0, 0·1 M. Protein was precipitated from the incubation mixture by boiling and then the IPP purified from the incubation products by chromatography on a 1 × 8 cm column of Dowex-1-formate (Bloch *et al.*[11]) followed by chromatography on a 1·5 × 25 cm of DEAE-cellulose according to the method described by Skilleter and Kekwick.[12]

Plant Material and Conditions of Incubation

The plant material used was *Phaseolus vulgaris* var. Canadian Wonder. After washing the seeds with commercial sodium hypochlorite (diluted 1:10) they were soaked for 24 hr in water and then planted in trays of vermiculite. The beans were grown in complete darkness with regular watering for 7–10 days after which the seedlings had reached a height of about 15 cm and the first formed leaves were exposed by the uncurling of the plumular hook.

When light treatment of the leaves was required the seedlings were illuminated for 10 min with artificial light comprising alternate rows of 40W Mazda Universal White fluorescent tubes and 15W pearl tungsten light bulbs in complete darkness again.

In all cases, after picking the leaves, the midribs were removed before the remainder of the leaf material was macerated for 1 min at 4° in phosphate buffer pH 7·0, 0·05 M containing *N*-acetylcysteine (10 mM) and $MgCl_2$ (5 mM) by means of a MSE top drive homogenizer for every 100 mg dry wt. of leaf material 6 ml of buffer was used in the homogenization procedure. The latter procedures were performed in the presence of a green safety light—a 15W tungsten light bulb with an Ilford Bright-Green filter No. 624.

Incubations comprising 4 μmoles [4-^{14}C] IPP (0·1 μc/μmole), 5 μmoles $MgCl_2$, 20 μmoles *N*-acetylcysteine, 20–25 mg dry wt. of homogenized leaf tissue and phosphate buffer pH 7·0, 0·05 M to a final volume of 3 ml were carried out in the dark at 25° for 4 hr. In some cases the IPP was replaced by 8 μmoles [2-^{14}C] DLMVA (0·1 μc/μmole) and 15 μmoles ATP. The incubations were stopped by boiling at zero time.

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[11] K. BLOCH, S. CHAYKIN, A. H. PHILLIPS and A. DE WAARD, *J. biol. Chem.* **234, 2595 (1959).**
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Extraction of Phytol

A total lipid extraction was carried out on each of the boiled incubation mixtures by a method similar to that described by Galliard *et al.*[13] To each of the incubation mixtures was added 3·75 vol. of $CHCl_3$: methanol (1:2 v/v) and the single phase system allowed to stand with periodic shaking for 15–30 min. A biphasic system was then produced by the addition of 1·25 vol. of chloroform and 1·25 vol. of water. The lipid containing lower phase was then washed twice with 4·75 vol. of upper phase made from a similar mixture of solvents. The lower phase was blown to dryness under N_2 and redissolved in 6 ml methanol, then 0·2 ml 5M HCl added and the solution allowed to stand for 30 min in order to cleave the pyrophosphate groups from any allyl pyrophosphates present.[14] The solution was then adjusted to a final concentration of 6% KOH in methanol by the addition of solid KOH and the mixture saponified at 70° for 3–4 hr. The saponified mixture was extracted four times with 10 ml light petroleum (b.p. 40–60°) after the addition of 1 ml water, and these combined extracts further washed with an equal volume of water before finally being taken down to a small volume prior to TLC.

Purification of Phytol

The light petroleum extracts were applied as 3 cm lines to 300 μ 20 × 20 cm plates of silica Gel-G (System I) or silica Gel-G impregnated with silver nitrate (2·5 g/22·5 g silica Gel G, System II). All plates were activated at 110° for 1 hr immediately before use and developed in light petroleum (b.p. 60–80°)/diethyl ether 60:40. For radioactive assay, phytol and the "prenol" fraction from the petroleum extracts System II was used since this gave the best resolution of phytol from some of the possible radioactivity labelled contaminants of all the systems tried. In System I, R_fs were as follows: squalene 0·87 phytol 0·37 β-sitosterol/cholesterol 0·28, farnesol 0·31, geraniol 0·25. In System II, R_fs were, squalene 0·57, β-amyrin 0·43, phytol 0·34, β-sitosterol/cholesterol 0·23, farnesol 0·16, geraniol 0·13. The marker spots were visualized by spraying the plates with a 5% H_2SO_4 in ethanol followed by heating at 110° for 10–15 min.

Identification of Phytol

The material separated as phytol in System II above had identical chromatographic properties to authentic phytol in a number of TLC systems described by other workers[9,15,16] although these systems themselves were not as satisfactory as our System II for separating phytol from possible labelled contaminants. The material purified in System I was eluted from the silica gel with acetone and further identified by GLC on 15% ethylene glycol succinate (E.G.S.).[15,18] The GLC was carried out on a 110 cm column of 15% E.G.S on celite (80–120 mesh) in a Pye Argon Chromatograph using a temperature of 171° and an argon gas pressure of 15 lb/sq. in. The radioactive material eluted from the TLC plates was found to have identical GLC characteristics as authentic phytol. A major peak with a retention time of 22 min was observed in addition to a smaller peak having a retention time of 10 min. Since this smaller peak was also recorded for authentic phytol it may have been a breakdown product.[19]

Radioactive Counting

The regions of the TLC plates containing phytol and farnesol and geraniol were scraped into 20 ml glass vials and then 5 ml of scintillation fluid added. This fluid consisted of a 1:4 mixture of Triton X-100: phosphor toluene (3 g 2,5 diphenyloxazole (PPO) and 0·3 g p-bis (2,5-phenyloxazole)-benzene (POPOP) in 1 l. of toluene) after shaking the silica gel was allowed to settle and then the solution was counted in a Nuclear Chicago Scintillation Counter (Model 720).

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