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METABOLISM OF CHLOROPHYLL IN HIGHER PLANTS—I.

A THIN-LAYER CHROMATOGRAPHY FOR THE QUANTITATIVE DETERMINATION OF PHYTOL

SEKI SHIMIZU, HARUI FUKUSHIMA and EINOSUKE TAMAKI

The Central Research Institute, Japan Monopoly Corporation,
Yutaka-machi, Shinagawa, Tokyo, Japan

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Abstract—Thin-layer silicic acid chromatography has been shown to be a useful technique of the separation of phytol. The procedure can be applied to the quantitative separation and estimation of phytol of various plant materials.

INTRODUCTION

IN CONNECTION with our previous studies on the metabolism of chlorophyll,[1,2] it seemed to be of some importance to study the metabolism of phytol, especially at the times of chlorophyll synthesis and its degradation.

In 1907 Willstätter and Hocheder[3] discovered phytol in plant tissues and identified is as a component of chlorophyll. Recently, Fischer and his co-workers[4,5] described a paper chromatographic method for the quantitative determination of phytol in the plant tissue, and this has been utilized by Shlyk and Stanishevskaya.[6] We found, however, some difficulties in applying this method and our attention was drawn to the use of thin-layer silicic acid chromatography instead of a paper chromatography, for a simple and precise method for the quantitative determination of phytol.

RESULTS AND DISCUSSION

Determination of Phytol

As will be described in detail in the Experimental Section, phytol was separated from the alkaline hydrolysate of plant material by using thin-layer chromatography with silicic acid as adsorbent, and a mixture of benzene and ethyl acetate as solvent (Fig. 1). When the chromatogram was complete the appropriate area of silicic acid containing the phytol was scraped from the glass plate and eluted with acetone. The phytol in this eluate was determined by measuring the decolorization of potassium permanganate in the presence of magnesium sulphate.*

* A solution of potassium permanganate is decolorized by compounds having ethylenic linkage. This is known as Baeryer's test for unsaturation.

As the reaction proceeds the reaction mixture becomes alkaline.

$$3\text{>C=C<} + 2KMnO_4 + 4H_2O \rightarrow 3\text{>C(OH)—C(OH)<} + 2MnO_2 + 2KOH$$

It is undesirable that a reaction solution becomes alkaline, because no actual precipitate of manganese dioxide is observed and the purple color gradually changes to a reddish brown. In the presence of magnesium sulfate, however, the hydroxyl ion is precipitated in the form of insoluble magnesium hydroxide and the decolorization of the permanganate solution can be easily observed.[7]

[1] S. SHIMIZU and E. TAMAKI, *Botan. Mag., Tokyo* **75**, 462 (1962).
[2] S. SHIMIZU and E. TAMAKI, *Arch. Biochem. Biophys.* **102**, 152 (1963).
[3] R. WILLSTÄTTER and F. HOCHEDER, *Ann. Chem. Liebigs* **354**, 205 (1907).
[4] F. G. FISCHER and H. BOHN, *Ann. Chem. Liebigs* **611**, 224 (1958).
[5] F. G. FISCHER and W. RÜDIGER, *Ann. Chem. Liebigs* **627**, 35 (1959).
[6] A. A. SHLYK and E. M. STANISHEVSKAYA, *Biokhimiya* **27**, 984 (1962).
[7] R. L. SHINER and R. C. FUSON, *The Systematic Identification of Organic Compounds* (2nd Ed.), John Wiley & Sons, Inc., New York (1947).

The minimum limit of detection of this method was found to be 5 μg of phytol and the relationship between absorptivity and concentration was linear up to 60 μg.

The rate of decolorization of potassium permanganate rises with temperature but it was found most convenient to heat the reaction mixtures in a water-bath at 40° in order to minimize volume changes. Since potassium permanganate is decolorized itself during the assay period, it is necessary to run a blank in each assay. Although the reaction is not complete until about 6 hr, there is sufficient reaction (95%) after 2 hr for this to be used in the standard assay.

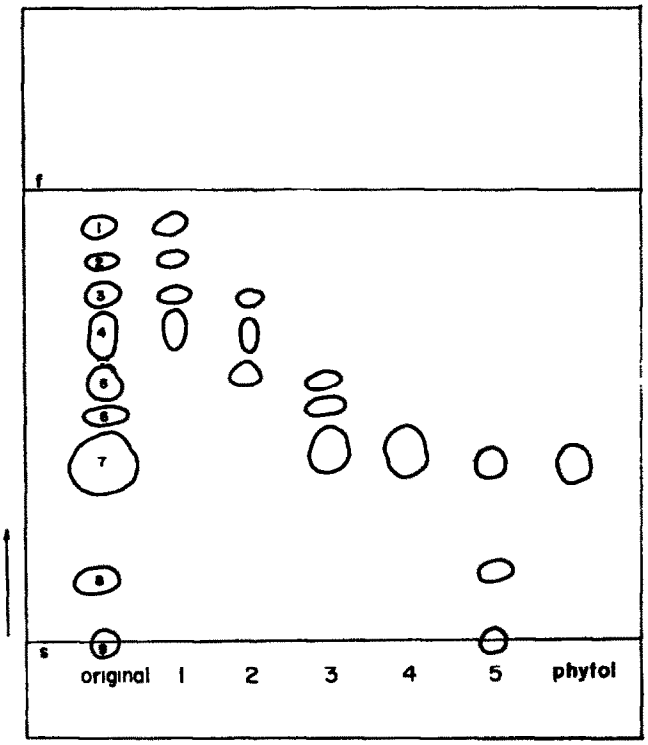

FIG. 1. THIN-LAYER CHROMATOGRAMS OF AN ALKALINE HYDROLYZED TOBACCO EXTRACT AND COMPONENTS OF COLUMN-CHROMATOGRAPHED FRACTIONS.

Thin-layer, silicic acid (Silica Gel D-5): Solvent, benzene: ethyl acetate; (19:1).
The fractions from the alumina column were eluted with petroleum ether containing 1, 0%; 2, 2%; 3, 4%; 4, 6%; and 5, 8% acetone (see Experimental).

Table 1 shows the recovery of the added phytol. For these experiments, aliquots of a phytol solution were added to a tobacco (*Nicotiana tabacum* "Bright Yellow") extract.

Change of the Phytol Content in Relation to Chlorophyll Formation in Tobacco Plants

The time course of chlorophyll and phytol synthesis in seedlings of *Nicotiana tabacum* "Bright Yellow" grown in both light and dark, is shown in Fig. 2. Before the light-induced stimulation of germination (6000 lx for 3 hr), seeds contained a detectable amount of phytol. After the stimulation was given, the content of phytol slightly increased at first and then more rapidly in the light.

In light-grown seedlings the molar ratio of phytol to chlorophyll was 121 on the 6th day (144 hr) but in the following two days the synthesis of chlorophyll was rapid and the ratio of

TABLE 1. RECOVERY OF ADDED PHYTOL FROM TOBACCO EXTRACT

Phytol		
Added (μg)	Found (μg)	Error (%)
89	84	5·6
89	82	7·8
89	94	5·6
207	193	7·3
207	223	7·7
207	189	8·7
326	311	9·5
326	308	8·6
326	306	9·2

phytol/chlorophyll finally (10 days) reached about 1. The phytol content of dark-grown seedlings decreased gradually during the whole period.

Neither Fischer and Rüdiger[5] nor Hromatka *et al.*[8] observed the presence of any form of phytol in etiolated shoots and they concluded that light is indispensable for phytol synthesis in plants. The present results shown in Fig. 2 seem to support their result. However, Shlyk

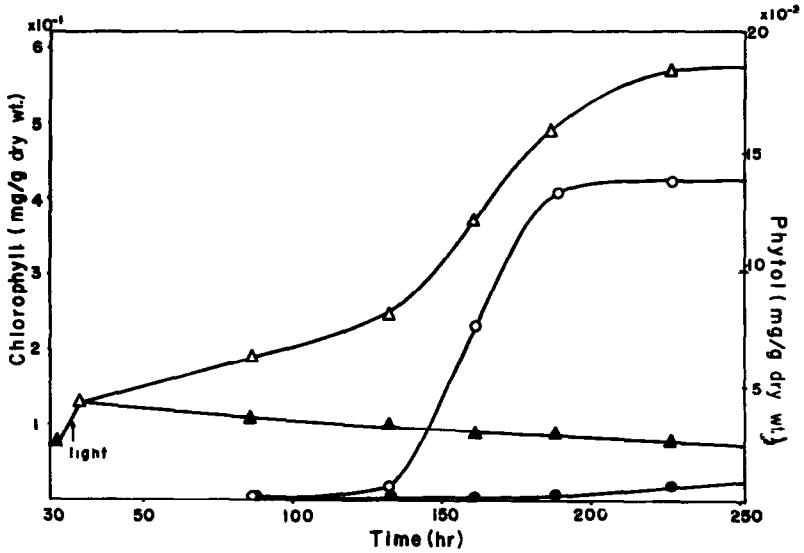

FIG. 2. TIME COURSES OF CHLOROPHYLL AND PHYTOL SYNTHESIS IN TOBACCO SEEDLINGS GROWN IN DARK AND LIGHT CONDITIONS.

○—○: Chlorophyll, in the light,
●—●: Chlorophyll, in the dark,
△—△: Phytol, in the light,
▲—▲: Phytol, in the dark.

and Stanishevskaya[6] reported that the formation of phytol is possible even in the dark although the process takes place more slowly than in the light. Since tobacco plants need the light stimulation for germination, however, no conclusive discussion on whether or not the dark formation of phytol is possible can be given at present.

[8] O. HROMATKA, W. BRÖLL and L. STENTZEL, *Monatsh.* **89**, 126 (1958).

EXPERIMENTAL

Reagents

Phytol was purchased from Nutritional Biochemical Corp., U.S.A., and further purified by distillation under reduced pressure (b.p. 202°, 10 mm Hg). For the preparation of standards it was dissolved in peroxide-free acetone.

Potassium permanganate 4·16 mM in water,

Magnesium sulfate 10 mM,

"Silica Gel D-5" and the apparatus for its application were purchased from CAMAG, Muttenz, Switzerland.

Aluminium oxide: Merck, for chromatographic absorption analysis.

Quantitative Estimation of Phytol

Thin-layer (300 μ) plates (20 × 20 cm) of silicic acid were prepared with "Silica Gel D-5" (contains 5% calcium sulfate). The plates were dried for 1 hr in an oven at 120° and stored in a desiccator. Demole[9,10] and Reitsema[11] both used *n*-hexane–ethyl acetate (85:15) as a solvent, but we have found that benzene: ethyl acetate (19:1) have a better resolution. After development, the plates were dried and sprayed with 0·25% aq. solution of $KMnO_4$: unsaturated compounds yield yellow spots on a pink-red background which on standing at room temperature turn to dark brown on a pale brown background.

For preparing the standard curve 0·1 ml of a known concentration of phytol was applied to a plate prepared as described above, at 1-cm intervals along the start line leaving room at each end for markers. The plate was developed until the solvent had travelled ~ 15 cm, dried and the center of the plate was covered with a band of paper for protection while spraying the marker spots with 0·25% potassium permanganate. The corresponding areas of the unsprayed part of the plate were then scraped off with a spatula and eluted with 5 ml of acetone. The filtrate was made up to 10 ml with acetone in a volumetric flask. Eight ml of the acetone solution, 1 ml of potassium permanganate and 1 ml of magnesium sulfate were added to a test tube. The tubes were then heated for 2 hr in a water-bath at 40°, cooled and re-made up to 10 ml. The absorptivity was then read using a Hitachi's photoelectric photometer with a No. 53 filter. A blank determination using 8 ml of acetone in place of the phytol solution was carried simultaneously. The chromatoplates were run in triplicate for each analysis and average density value was used.

Application of the Quantitative Method of Phytol to Plant Material

About 30 g of fresh leaves were blanched by immersion in hot water and then rapidly cooled. They were ground with a small amount of sand and magnesium carbonate in a motor-driven mortar and extracted exhaustively with methanol. Part of the methanol extract was used for the determination of chlorophyll content.[12] The other part of the methanol extract (~ nine-tenths) was used for the quantitative determination of phytol. To the extract was added solid KOH to make a 2·5% solution, and the mixture heated for 30 min at 65–70° to give all the phytol in the free form.

The mixture was concentrated 8-fold under reduced pressure and repeatedly extracted with peroxide-free ether until the extract was colorless. The ether extracts were combined, washed

[9] E. DEMOLE and E. LEDERER, *Bull. Soc. chim. France* **1958**, 1128 (1958).
[10] E. DEMOLE, *J. Chromatog.* **6**, 2 (1961).
[11] R. H. REITSEMA, *Analyt. Chem.* **26**, 960 (1954).
[12] D. I. ARNON, *Plant Physiol.* **24**, 1 (1949).

with water until neutral and dried over Na_2SO_4. After removal of the ether the residue was dissolved in a small amount of petroleum ether (b.p. 30–50°) and the solution transferred to an aluminium oxide column (200 × 15 mm), previously saturated with petroleum ether. The compounds were eluted with mixtures of petroleum ether containing increasing proportions of acetone from 0 to 8% (see Fig. 1). Fractions of 10 ml each were collected, and after evaporation of the solvent, the components of each fraction were examined by thin-layer chromato-

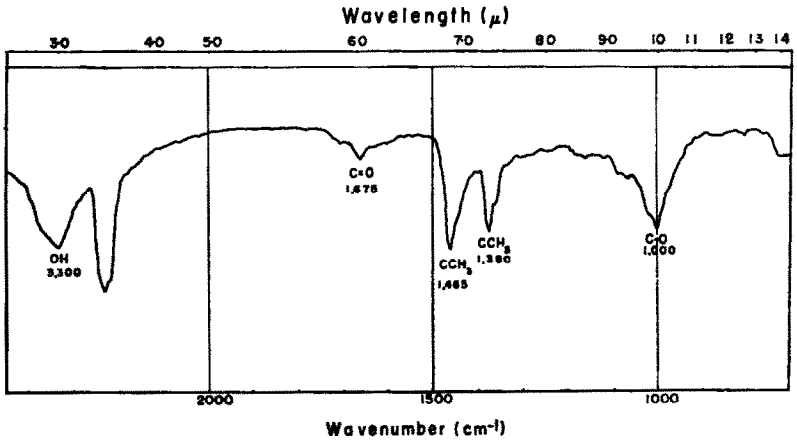

FIG. 3. INFRARED SPECTRUM OF THE COMPOUND CORRESPONDING TO SPOT 7 (Fig. 1).

graphy (Fig. 1). Fractions which had contained Spot 7 were combined and rechromatographed on a fresh plate, and the quantity of phytol was determined by the method mentioned above.

Identification of Phytol

After chromatography, the fractions containing Spot 7 were combined and applied to a preparative chromatoplate (1000 μ thickness). The substance corresponding to Spot 7 was eluted with re-distilled and peroxide-free acetone, filtered and the solvent evaporated under reduced pressure, yielding pale yellow oil. The i.r. spectrum of this compound (Fig. 3), which shows bands for a primary alcohol (3300 cm^{-1}), with C=C bonds (1675 cm^{-1}) and C—CH_3 groups (1380 and 1465 cm^{-1}), was identical with that of authentic phytol[9].