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THE TERPENOIDS OF TISSUE CULTURES OF PAUL'S SCARLET ROSE

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Abstract—The carotenoids present in tissue cultures of Paul's Scarlet Rose stem are zeaxanthin, violaxanthin, auroxanthin and neoxanthin. β-Carotene, lutein and neoxanthin were the main pigments in stem and leaf. The concentration of total carotenoids in stem and leaf was four and thirty times greater, respectively, than the tissue culture.

The triterpenoids of the tissue culture and stem and leaf were the same: β-sitosterol was the main component, and traces of "γ-sitosterol" or stigmasterol, lanosterol, β-amyrin and squalene are also present.

INTRODUCTION

AS PART of a research programme designed to define plant differentiation more clearly in biochemical terms, the terpenoids of cultures of meristematic tissue from the stem of Paul's Scarlet Rose have been examined, and compared with those in the tissue of leaves from the parent plant. Functional chloroplasts contain a number of terpenoid compounds: sterols, carotenoids, the phytol residues in chlorophyll, vitamin K_1 and tocopherol, and the solanesol residue of plastoquinone. The aim of the present work was to see how many of these compounds are present in undifferentiated cells of tissue cultures which do not contain functional chloroplasts but which may contain plastid primordia. Preliminary reports of some of our findings have already appeared.[1,2]

RESULTS

Tissue Cultures

The carotenoid components. The unsaponifiable matter was dissolved in light petroleum (b.p. 60–80°) and a preliminary chromatographic separation was carried out on a column of zinc carbonate–celite (5:2) with light petroleum containing increasing amounts of diethylether as an eluant. Five fractions were obtained (Table 1). Fraction 1 was concentrated and examined spectrophotometrically; it showed no absorption between 240 and 400 mμ, which indicated that either C-40 polyenes, phytoene and phytofluene were completely absent from the cells, or only present in very minute amounts (less than 0·5 μg/g. dry wt.). Fraction 1 was then rechromatographed on Kieselgel G plates with light petroleum (b.p. 40–60°) as developing solvent. No fluorescent bands were observed under u.v. light, confirming the absence of phytoene and phytofluene. On staining the plates with I_2 vapour, a single brown spot was obtained. This had the same R_f as authentic squalene run alongside, and a mixture of another portion of Fraction 1 and authentic squalene did not separate. In preliminary experiments [2-^{14}C]mevalonic acid was strongly incorporated into this spot, which confirms the terpenoid nature of the compound.

[1] T. W. GOODWIN and B. L. WILLIAMS, *Biochem. J.* **85**, 12P (1962).
[2] D. R. THRELFALL and T. W. GOODWIN, *Biochim. Biophys. Acta* **18**, 532 (1963).

TABLE 1. SEPARATION OF UNSAPONIFIABLE MATTER FROM ROSE TISSUE CULTURES ON $ZnCO_3$–CELITE (5:2) COLUMNS

Fraction No.	Description	Proportion of ether in light petroleum for elution (% v/v)	Identified as
1	Colourless	0	Squalene
2	Faint yellow	5	Zeaxanthin
3	Light yellow	25	Violaxanthin
4	Greenish-yellow	40	Auroxanthin
5	Deep yellow	80	Neoxanthin

Fraction 2 was present only in small amounts and was purified on a small column of magnesium oxide; it was identified as zeaxanthin by the following criteria: (1) the same absorption maxima and curve shape as authentic zeaxanthin from *Polysiphonia fastigiata* in light petroleum (b.p. 60–80°) (451, 482 mμ), ethanol (452, 483 mμ) and chloroform (462, 494 mμ) (these also corresponded with published values for zeaxanthin):[3] (2) it could not be separated from authentic zeaxanthin in the following thin-layer systems, Kieselguhr G–benzene (R_f, 0·69); Kieselgel G–2% (v/v) methanol/benzene (R_f, 0·33); alumina–5% (v/v) methanol/benzene (R_f, 0·49) or on reversed phase paper chromatography (liquid paraffin as stationary phase with 70% (v/v) acetone/water) (R_f, 0·60).

Fraction 3 was also purified on magnesia and was shown to be identical with violaxanthin (zeaxanthin 5,6,5′,6′-diepoxide) by the following criteria: (1) the same absorption maxima and curve shape as authentic violaxanthin from maize seedlings in light petroleum (b.p. 60–80°) (420, 442, 471 mμ), ethanol (420, 442, 471 mμ) and chloroform (424, 452, 482, mμ): (2) failure to separate it from authentic violaxanthin using the last three chromatographic systems mentioned above: R_f values were 0·67, 0·55, and 0·85 respectively; (3) addition of a drop of 0·1 N HCl to an ethanolic solution of the pigment resulted in a shift of 40 mμ in the absorption maxima to lower wavelengths; this also occurred with authentic violaxanthin and agrees with change reported by Krinsky and Goldsmith[4] for the conversion of a 5,6-diepoxide into an isomeric 5:8-diepoxide. The pigment so formed was indistinguishable from authentic auroxanthin (see Fraction 4) which is 5,8,5′,8′-diepoxyzeaxanthin.

Fraction 4 was present in larger amounts than Fraction 2; it was purified on magnesium oxide and shown to be identical with auroxanthin (zeaxanthin 5,8,5′,8′-diepoxide) by the following criteria: (1) the same absorption maxima and curve shape as authentic auroxanthin extracted from the petals of *Viola tricolor* in light petroleum (b.p. 60–80°) (382, 402, 427 mμ), ethanol (381, 402, 427 mμ) and chloroform (385, 413, 438 mμ): (2) failure to separate it from authentic auroxanthin in the following thin-layer systems: Kieselguhr G–5% (v/v) methanol/benzene (R_f, 0·57), alumina–5% (v/v) methanol/benzene (R_f, 0·33) and also on reversed phase paper (liquid paraffin as stationary phase using both 70% (v/v) acetone/water (R_f, 0·64) and 80% (v/v) acetone/water (R_f 0·68).

Fraction 5, the most strongly adsorbed compound, was purified on a column of powdered (icing) sugar. It was shown to be neoxanthin by the following criteria (1) the same absorption maxima and curve shape as authentic neoxanthin isolated from barley leaves in light petroleum (b.p. 60–80°) (417, 437, 469 mμ); ethanol (417, 441, 467 mμ) and chloroform (422,

[3] P. KARRER and E. JUCKER, *Carotenoids* (Trans. E. A. Braude), Elsevier, Amsterdam (1950).
[4] N. I. KRINSKY and T. H. GOLDSMITH, *Arch. Biochem. Biophys* **91**, 271 (1960).

445, 475 mμ); (2) failure to separate it from authentic neoxanthin in the following thin-layer systems: Kieselguhr G–5% ethyl acetate/hexane (R_f 0·64) and alumina–ethyl acetate (R_f 0·57); because of the high adsorptive affinity of neoxanthin no other system was effective; (3) addition of a drop of 0·1 N HCl to an ethanolic solution of the pigment resulted in a shift of 18 mμ in the absorption maxima to lower wavelengths; this also occurred with authentic neoxanthin and agrees with the change reported by Krinsky and Goldsmith[4] for the conversion of neoxanthin from a 5,6-epoxide into an isomeric 5,8-epoxide.

In early experiments violaxanthin (Fraction 3) was consistently absent from the tissue culture whilst in all later experiments it has replaced auroxanthin as the main pigment (cf. Table 2). Possible reasons for this are considered under "Discussion".

TABLE 2. SEPARATION OF UNSAPONIFIABLE MATTER FROM ROSE STEM AND ROSE LEAVES ON $ZnCO_3$:CELITE (5:2) COLUMNS

Fraction No.	Description	Proportion of ether in light petroleum for elution (% v/v)	Identified as
1	Yellow–orange	0	β-Carotene
2*	Yellow	5	Lutein
3	Yellow (trace)	15	Antheraxanthin?
4	Deep yellow	80	Neoxanthin

* In leaf extracts an additional yellow–orange band was observed running just before Fraction 2.

The Triterpenoid Components

The fraction precipitated with digitonin. The sterols which were precipitated from the unsaponifiable matter with digitonin were regenerated and examined by gas–liquid chromatography with a 4 ft × 4 mm column of 5% Dow Corning Silicone QF1 on 80–100 gas Chrom. P solid support; temperature 219°, 1500 V, and gas flow 14/ft sec. Two components were observed with retention times of 4·71 and 3·98. The first corresponded with the value for β-sitosterol and the last to either "γ-sitosterol"* or stigmasterol (these cannot be separated in the system used). The same peaks were obtained when the tissue culture sterols were mixed with a mixture of β-sitosterol, "γ-sitosterol" and stigmasterol before chromatography.

The major component of the mixture was β-sitosterol. β-Sitosterol, "γ-sitosterol" and stigmasterol could not be separated on thin-layer or column chromatography. The same results were obtained from the sterol fraction obtained not by precipitation with digitonin but by chromatography of the total unsaponifiable matter on alumina (Brockmann Grade III), the fractions eluted with 8, 10 and 12% (v/v) ether in light petroleum contained the sterols.

The fraction not precipitated with digitonin. This fraction was found to contain lanosterol and β-amyrin. These compounds were suspected when a diffuse spot which stained brown with $SbCl_5$ was obtained on a Kieselguhr (25% benzene light petroleum) plate (R_f, 0·75) and on a Kieselgel (chloroform) plate R_f, 0·70). Lanosterol and β-amyrin run very close together on these systems with the R_f's indicated; when stained with $SbCl_3$ lanosterol becomes yellow

* It has very recently been shown that "γ-sitosterol" is probably a mixture of campesterol and β-sitosterol.[5]

[5] M. J. THOMPSON, W. E. ROBBINS and G. L. BAKER, *Steroids*, **2**, 505 (1963).

and β-amyrin pink; however, a mixture of the two stains brown. Furthermore, a Liebermann–Burchard reaction on this fraction showed a very gradual development of a yellow colour with a marked increase in absorption at 460 mμ with time. This is a characteristic of lanosterol. The same observations were made on the fractions which were eluted off an alumina (Brockmann Grade III) column with 4 and 6% (v/v) ether in light petroleum.

These fractions were examined by gas–liquid chromatography and two main peaks were observed with relative retention times of 5·21 and 4·78. The values corresponded very closely to those found for authentic β-amyrin and lanosterol, respectively. When the unknown fraction was mixed with authentic samples of these two substances and the mixture analysed only two peaks were obtained.

Paul's Scarlet Rose Stem and Leaf Tissue

The carotenoid components. The carotenoids present in the stem and leaves of Paul's Scarlet Rose were investigated in order to obtain an exact comparison with those in the cultures derived from the meristematic stem tissue of the same plant. Four coloured fractions were obtained from stem tissue unsaponifiable material chromatographed on a column of zinc carbonate–celite (Table 2). Fraction 1 was shown to be β-carotene by the following criteria: (1) same absorption maxima and spectral shape as authentic β-carotene (synthetic, crystalline) in light petroleum (b.p. 60–80°) (427, 452, 479 mμ), ethanol (427, 453, 480 mμ) and chloroform (441, 465, 496 mμ); (2) failure to separate it from authentic β-carotene on a thin-layer system of Kieselgel G–5% (v/v) ether in light petroleum (R_f 0·61) and on a column of magnesium oxide and alumina (Brockmann Grade III). Fraction 2 was purified on a magnesium oxide column and was shown to be lutein by the following criteria: (1) same absorption maxima and spectral shape as authentic lutein from grass in light petroleum (b.p. 60–80°) (423, 447, 475 mμ), ethanol (422, 447, 476 mμ) and chloroform (427, 455, 487 mμ); (2) failure to separate it from authentic lutein on the chromatographic systems described above for zeaxanthin. Fraction 3 occurred in small amounts and ran in between zeaxanthin and violaxanthin on the systems described above for these pigments. It was not identified but its spectrum and adsorptive power suggest that it was possibly antheraxanthin (zeaxanthin 5,6-epoxide). Fraction 4 was identified as neoxanthin by the criteria previously outlined for the corresponding pigment in the tissue cultures.

When leaf tissues were examined for carotenoids all the pigments found in stem tissue were identified and in addition, small amounts of zeaxanthin (identified as described above) were present.

The terpenoids present. Gas–liquid chromatography of the sterol fraction revealed the same two peaks, corresponding to β-sitosterol and either "γ-sitosterol" or stigmasterol, as found in the tissue cultures. The relative amounts present were also approximately the same as in the tissue cultures. The non-sterol fraction was shown, by the same criteria as described previously, to contain β-amyrin and lanosterol.

Quantitative results. Table 3 records the amounts of unsaponifiable matter and sterol in tissue cultures and stem and leaf tissue; it will be seen that the overall picture is very similar in all cases, the percentage of unsaponifiable matter on a dry weight basis ranging from 0·15–0·19 of which some 21–25 per cent is sterol.

A comparison of the amount of carotenoids present shows a far greater difference (Table 4). The pigment concentration in the tissues is only one-thirtieth that in the leaf tissue. The lower level in the stem compared with the leaf is a reflection of the relative concentration of chloroplasts in the two tissues; quantitatively the pigments are the same

and are characteristic of chloroplasts. In the tissue cultures, however, three of the four pigments were not found in the chloroplasts of the whole plant.

TABLE 3. THE STEROL AND TOTAL UNSAPONIFIABLE MATTER IN ROSE TISSUE CULTURES AND IN ROSE STEM AND LEAVES

	No. of experiments	Total unsaponifiable matter	Sterol content	Sterol in unsaponifiable matter (%)
Tissue culture	4	18·9	4·5	23·8
Stem tissue	2	15·2	3·2	21·1
Leaf tissue	2	19·6	4·8	24·5

TABLE 4. QUANTITATIVE CAROTENOID DISTRIBUTION IN ROSE TISSUE CULTURES AND IN ROSE STEM AND LEAVES

	Concentration μg/g dry wt			
	Tissue culture*			
Pigment	(a)‡	(b)‡	Stem†	Leaf†
β-Carotene	0·0	0·0	15·2	96·3
Zeaxanthin	0·89	1·07	0·0	4·3
Antheraxanthin	0·0	0·0	0·8	3·6
Lutein	0·0	0·0	21·0	176·7
Violaxanthin	0·0	3·69	0·0	0·0
Auroxanthin	6·14	2·49	0·0	0·0
Neoxanthin	2·58	2·77	5·6	19·0
Total	9·61	10·02	42·6	299·9

* Mean of three experiments in each case.
† Mean of two experiments.
‡ (a) = values obtained during the first six months of culturing.
(b) = values obtained after the first six months of culturing.

Intracellular distribution. Differential centrifugation[6] was used to obtain fractions from homogenized tissue cultures: 600 *g* for 15 min, 20,000 *g* for 15 min, and 140,000 *g* for 90 min, and supernatant. No terpenoids could be detected in any fraction except the supernatant. In green tissues all the sterols and carotenoids are attached to particles prepared in the same way and only trace amounts appear in the supernatant fractions.[7]

DISCUSSION

Carotenoids

These investigations show that plant tissue cultures synthesize much less carotenoid than the green tissue of the plant from which they were derived and that they are qualitatively different. They are all oxidized derivatives of β-carotene and it would appear that plant tissues which actively respire rather than photosynthesize can carry out this type of oxidation. Epoxycarotenoids are characteristic of many fruit and flower petals[8] and recently it has been

[6] M. CALVIN, O. HOLM-HANSEN, N. G. PON, K. NISHIDA and V. MOSES, *Physiol. Plantarum*, **12**, 475 (1959).
[7] W. E. DAVIES, Ph.D. Thesis, University of Wales (1963).
[8] T. W. GOODWIN, *The Comparative Biochemistry of the Carotenoids*, Chapman & Hall, London (1952).

shown that the main carotenoid of the cotyledons of germinating french beans is auroxanthin whilst the main xanthophyll in the etiolated true leaves is lutein.[9] There is also other evidence which indicates that the incorporation of oxygen into carotenoids occurs at a late stage in the biosynthetic process.[10] The pattern of compounds found in the tissue cultures suggests the pathway of oxidation shown in Fig. 1. It is interesting that no unsymmetrical molecules (e.g. cryptoxanthin—3-hydroxy-β-carotene or antheraxanthin—5.6-epoxyzeaxanthin) were

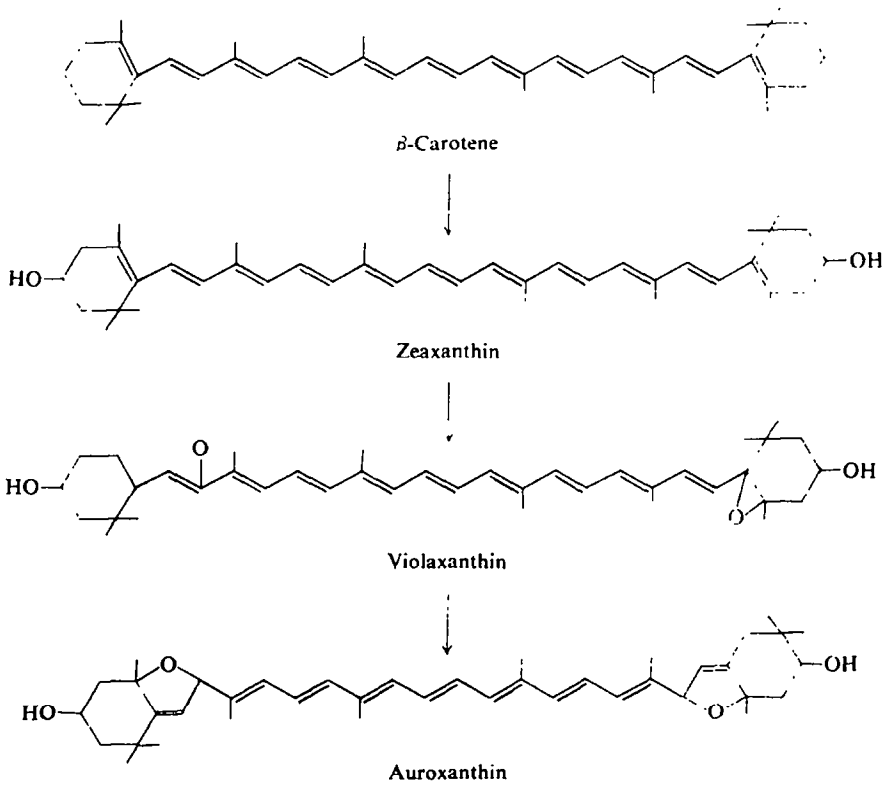

FIG. 1. SUGGESTED PATHWAY FOR THE OXIDATION OF β-CAROTENE.

observed in the tissue culture. The observations that cultures studied at the beginning of this investigation contained only auroxanthin and no violaxanthin whilst those examined during the later stages contained significant amounts of violaxanthin and a correspondingly less amount of auroxanthin suggest strongly that violaxanthin is a precursor of auroxanthin. The failure to find an intermediate between violaxanthin and auroxanthin in the later cultures is also significant and suggests that each step in the oxidation may be a two-stage process, with a transitory intermediate present only on an enzyme surface.

Triterpenoids

There are no significant differences either quantitative or qualitative between the triterpenoids of tissue cultures and the corresponding green plant tissues. It has been shown in

[9] T. W. GOODWIN and S. PHAGPOLNGARM, *Biochem. J.* **76**, 197 (1960).
[10] T. W. GOODWIN In *Modern Methods of Plant Analysis*, Vol. 3, p. 372. Springer, Heidelberg (1955).

: seedlings and in *Euglena gracilis*[7] that a considerable amount of sterols is associated the chloroplasts and all the remainder with other particles such as mitochondria. g the same analytical techniques we find that all the sterols appear in the supernatant on of the homogenized plant-tissue cultures. The significance of this cannot be assessed : present time and it may only be due to the comparative fragility of the tissue-culture ıelles; however, good pellets of particulate material were always obtained. Further es are required with different methods of homogenization.

EXPERIMENTAL

e cultures

he cultures used were derived from the cambial tissue of the stem of Paul's Scarlet Rose were kindly provided by Prof. P. W. Brian who also supplied details of the following um which was used throughout the investigation. One litre of medium contains: (A) oelements: $Ca(NO_3)_2$, $4H_2O$ (1·42 g); KNO_3 (0·81 g); $MgSO_4$, $7H_2O$ (0·49 g); KH_2PO_4 g); NaCl (0·12 g); $CaCl_2$, $6H_2O$ (0·30 g); (B) microelements: $MnSO_4$, $4H_2O$ (66·5 mg); $)_4$, $7H_2O$ (26·8 mg); H_3BO_3 (15 mg); KI (7·5 mg), Fe-EDTA complex (sequestic acid) ; (C) growth factors: glycine (3 mg); nicotinic acid (0·5 mg); thiamine hydrochloride ng); pyridoxine (0·1 mg), 2,4-D (6 mg); coconut milk preparation (100 ml); and (D) ›hydrate: sucrose (25 g). The pH is adjusted to 5·5 and 5 g agar (Oxoid Ionagar No. 2) dded if a solid medium is required. The medium (150 ml) was dispensed in 250-ml al flasks and sterilized at 15 lb/in^2 for 15 min. The coconut milk was prepared by autoclaving at 15 lb/in^2 for 10 min, filtering off the proteinaceous precipitate and toclaving. It was kept sterile at 2° until required.

he flasks were inoculated with 10 ml of inoculum from a 14-day culture and incubated ɔm temperature in a shaker for 14 days. The cells were then harvested by centrifugation 00 revs/min in a bench centrifuge (Martin Christ). Yields were of the order of 200 g veight per litre of medium.

ɪction Procedures

he cells were freeze-dried and placed in a bucket of an M.S.E. homogenizer, and covered acetone. The cells were homogenized, and the homogenate filtered through a sintered funnel (Quickfit) attached to a suction flask. The process was repeated with fresh nt until the homogenate was white and the solvent colourless (usually five times).

he coloured extract was transferred to a separating funnel, and an equal volume of ether ıly distilled over iron to remove peroxides) was added, followed by an excess of distilled r until two layers were formed. All the pigment was then transferred to the ethereal ıase. The aqueous acetone phase was rejected, and the ether fraction then shaken with al small volumes of distilled water in order to remove any remaining acetone. The ether ct was dried by standing over anhydrous sodium sulphate overnight in the cold-room, hen filtered through a sintered glass funnel attached to a suction flask. Care was taken ısh the sodium sulphate several times with ether to remove any pigment adhering to it. ether was removed by distillation under reduced pressure, and the extract was then nified by the standard procedure.[10]

mn Chromatography

he carotenoids and squalene were separated on a $ZnCO_3$:Celite column ($ZnCO_3$, H. for chromatography; Celite, Hyflo-super Cel, L. Light & Co.) with light petroleum

containing increasing amounts of diethylether as developing solvent. Purification was carried out on a column of MgO ("MFC"-heavy, Hopkins and Williams) (less strongly adsorbed xanthophylls) and icing sugar (more strongly adsorbed xanthophylls). Sterols were purified on columns of alumina (Brockmann Grade III).

Thin-layer Chromatography

The standard procedures were used[11] and the details of adsorbent and solvent are detailed in the Results. Reversed phase partition chromatography[12] was also used for separating the highly polar xanthophylls. Liquid paraffin was the stationary phase and 70% (v/v) aqueous ethanol the solvent. The same system on paper was also used on some occasions, but not routinely. Sterols and β-amyrin were detected on thin plates by spraying with antimony pentachloride (saturated solution in chloroform) and heating the plates in an oven at 100° for 5 min. On reversed-phase paper systems they were detected by spraying with 5% (w/v) phosphomolybdic acid in ethanol[13] and heating at 100° until the background began to turn green; the sterols showed up as dark-blue spots. Squalene was identified after staining with I_2 vapour.[14]

The Liebermann–Burchard Reaction

This reaction was carried out by dissolving the sample in glacial acetic acid and to a portion adding two parts of the reagent (19 ml acetic anhydride + 1 ml conc. H_2SO_4). The absorption spectra were measured in a recording spectrophotometer (Perkin-Elmer Uvicord) at 3-min intervals.

Precipitation of Sterols

The digitonin-precipitable sterols were obtained by the method of Windaus.[15] They were regenerated by dissolving them in centrifuge tubes in a minimum of pyridine and adding ether until the free digitonin was precipitated. The precipitate was centrifuged off and the supernatant ether decanted. The precipitate was washed twice with ether and the combined ether extract and washings were treated with small volumes of N HCl in a separating funnel until no white precipitate (pyridine hydrochloride) was noted in the ether. The ether was finally washed free from acid with small volumes of water, taken to dryness over nitrogen, absolute ethanol being added at the end to remove the last traces of water.

Gas–liquid Chromatography

A Pye Argon Chromatography unit was used with a 4 ft × 4 mm column of 5% Dow Corning Silicone QF1 on 80–100 gas Chrom P solid support. The column temperature was 219° and voltage 1500. Gas flow was 14 ml/sec.

Acknowledgements—We thank the Carmarthenshire County Council for a Scholarship to B. L. Williams. This investigation was partly supported by a grant to the Department by the D.S.I.R.

[11] E. STAHL (Editor), *Dunnschicht Chromatographie*, Springer, Heidelberg (1962).
[12] H. BOLLINGER, in *Dünnschicht Chromatographie* (Edited by E. STAHL), Springer, Heidelberg (1962).
[13] R. NECKER and A. WETTSTEIN, *Helv. Chim. Acta* **35**, 276 (1952).
[14] E. I. MERCER, B. H. DAVIES and T. W. GOODWIN, *Biochem. J.* **87**, 326 (1963).
[15] A. WINDAUS, *Ber.* **42**, 238 (1909).