BBA 4145

SEPARATION OF THE LIPIDS OF PHOTOSYNTHETIC TISSUES: IMPROVEMENTS IN ANALYSIS BY THIN-LAYER CHROMATOGRAPHY

B. W. NICHOLS*
Brunel College, London (Great Britain)
(Received March 13th, 1963)

SUMMARY

Thin-layer chromatography on silicic acid, using solvents of carefully controlled water content, has permitted the separation of the total lipids of lettuce and cabbage into at least 19 components.

Only a small proportion (chiefly phosphatidyl glycerol) of the leaf phospholipids was present in the chloroplast fraction, which contained a novel, unidentified lipid in addition to mono- and di-galactolipids. Free sterols, sterol esters and triglycerides were detected in extracts from the whole leaf, but were absent from the corresponding chloroplast preparation. Sterol glycosides were abundant both in the whole leaf and in the stalk, but the other glycolipids were concentrated mainly in the photosynthetic tissue.

INTRODUCTION

The work described in this paper represents part of a programme designed to elucidate the course of biosynthesis of the lipids in photosynthetic tissue[1,2]. The lipids from such tissues differ from those from other plant sources in having a high proportion of glycolipids and phospholipids, and a low triglyceride content[3-9]. Consequently, the polar lipid fractions of these tissues are often extremely complex and difficult to separate into their component lipid classes.

ZILL AND HARMON[5] attempted the fractionation of total leaf and chloroplast lipids of spinach on silicic acid columns of the HIRSCH AND AHRENS[10] type, but obtained only poor resolution of the phospholipids and galactolipids. WHEELDON[11] obtained an improved separation of the acetone-insoluble lipids (mainly phospholipids) of cabbage leaf by chromatography on silicic acid columns using chloroform–methanol mixtures as eluents, but there was still considerable overlapping of components. To date the most effective separations of leaf lipids have been achieved by KATES[6] employing chromatography on silicic acid-impregnated cellulose papers, but even under these conditions several important components were inseparable.

This paper describes the results obtained using an improved method for the separation of leaf lipids, namely thin-layer chromatography on silicic acid.

* D.S.I.R. Research Fellow.

MATERIALS AND METHODS

Preparation of lipid extracts

The leaves from half a lettuce were macerated at room temperature with 100 parts by weight of isopropanol, under which conditions phospholipase activity is slight[6]. The mixture was centrifuged, the residue extracted in a similar manner and finally shaken overnight with 100 parts (w/v) of chloroform–isopropanol (1:1). The combined extracts were evaporated almost to dryness under reduced pressure and the lipids partitioned into chloroform by the method of FOLCH, LEES AND SLOANE-STANLEY[12].

Flesh-free stalk from the same lettuce was extracted in a similar manner.

A chloroplast preparation was made from the remaining half lettuce by the method of JAMES AND STUMPF[2] and the chloroplast lipids extracted and purified as described above.

Similar extracts were prepared from cabbage tissue.

Preliminary fractionation of lipids

The lipid preparations, dissolved in diethyl ether, were applied to columns of Mallinckrodt silicic acid (250 parts by weight; activated at 110° overnight) and the "neutral" lipids (hydrocarbons, sterol esters, triglycerides, free fatty acids, diglycerides and sterols) and most of the pigments were eluted with diethyl ether. Polar lipids (glycolipids, phospholipids, sterol glycosides) and residual pigment were then displaced with ether–methanol (1:1, v/v) and methanol. Positive N_2 pressure was generally applied to the columns and the separation of neutral and polar lipids from a total of about 250 mg lipid took less than 3 h. Under these conditions, no apparent degradation of the pigments, as observed by other workers[5], was experienced.

Such a separation was not always necessary for qualitative studies on total extracts, but was an essential preliminary to the quantitative separation of the individual lipids, which will be reported elsewhere.

Preparation of chromatoplates

Thin layers of Merck "Silica Gel G"* were applied to glass plates with the Desaga equipment** as described by MANGOLD[13], and the coated plates routinely activated by heating at 110° for 20 min immediately prior to use.

Fractionation of neutral lipids

Neutral lipids and pigments were fractionated by thin-layer chromatography on silicic acid, using hexane–diethyl ether–acetic acid (70:30:1) as eluting solvent (Fig. 1).

Fractionation of phospholipids and glycolipids

Phospholipids and glycolipids were separated by thin-layer chromatography in diisobutyl ketone–acetic acid–water (40:25:3.7), chloroform–methanol–water (85:25:3), chloroform–methanol–7 N ammonium hydroxide (60:35:5), chloroform–methanol–acetic acid–water (65:25:8:4), and chloroform–methanol–acetic acid (65:25:8) (Fig. 2).

* British distributor: Anderman and Co. Ltd., Battlebridge House, Tooley Street, London, S.E.1.

** British distributor: Camlab (Glass) Ltd., Cambridge.

Detection and identification of individual lipids

Lipids were detected on the chromatograms by spraying with 50% H_2SO_4 followed by charring at 250° for 20 min. During the first minutes of the charring operation free sterols (except some saturated sterols), sterol esters and sterol glycosides gave a typical Lieberman-Burchard colour reaction; at a later stage glycolipids turned deep purple and phospholipids pale brown. For the purpose of identifying the individual lipids, similar chromatograms were reacted with the following reagents: ninhydrin[14], molybdate–perchloric acid[14], diphenylamine[14], periodate–Schiff[15] and ferric chloride–sulphosalicylic acid[16].

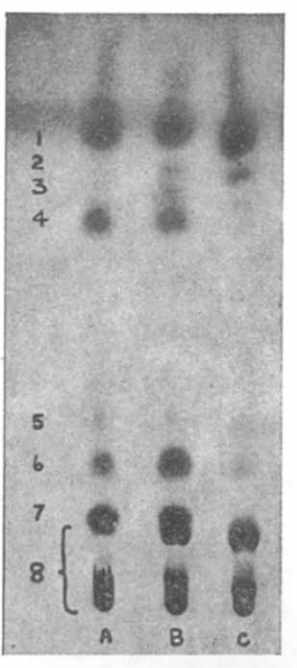

Fig. 1. Separation of neutral lipids of lettuce stalk (A), whole lettuce leaf (B) and lettuce-leaf chloroplasts (C). About 250 μg of each preparation were applied. Key to numbered components: 1 and 2, pigments; 3, sterol esters; 4, triglycerides; 5, free fatty acids; 6, sterols (and diglycerides); 7, sterols; 8, pigments, phospholipids and glycolipids.

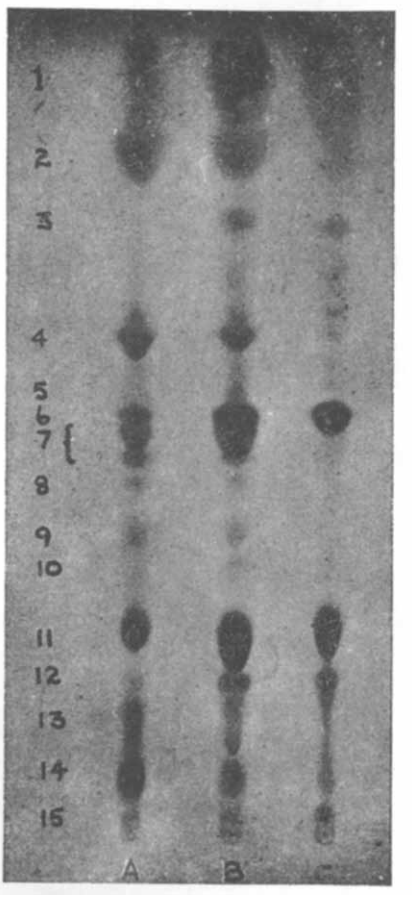

Fig. 2. Separation of phospholipids and glycolipids of lettuce stalk (A), whole lettuce leaf (B) and lettuce-leaf chloroplasts (C). About 100 μg of each preparation applied. Key to numbered components: 1, pigments and neutral lipids; 2, sterols; 3, pigments; 4, sterol glycoside; 5, phosphatidic acid; 6, monogalactolipid; 7, sterol glycoside; 8, unknown; 9, phosphatidyl ethanolamine; 10, phosphatidyl glycerol; 11, digalactolipid; 12, lipid A; 13, phosphatidyl choline; 14, phosphatidyl inositol and lipid B; 15, unknown. This component appeared in only a few preparations and is probably a galactolipid degradation product.

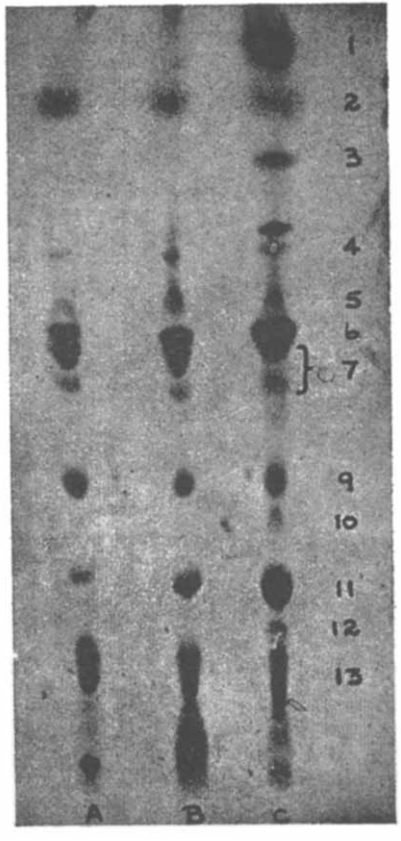

Fig. 3. Separation of phospholipids and glycolipids of cabbage stalk (A), cabbage inner leaf (B) and cabbage outer leaf (C). About 100 μg of each preparation applied. Numerals refer to key given with Fig. 2.

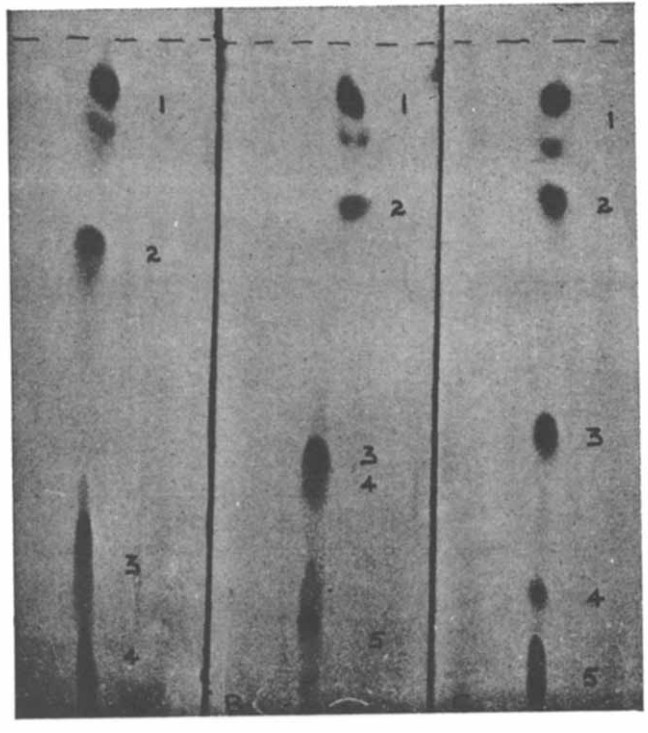

Fig. 4. Separation of galactolipids from other leaf lipids. Solvents: Plate A, chloroform–methanol (65:25, v/v); B, chloroform–methanol–water (65:25:3, v/v); C, chloroform–methanol–acetic acid (65:25:8). Key: 1, neutral lipid and pigment; 2, monogalactolipid; 3, digalactolipid; 4, phosphatidyl ethanolamine; 5, phosphatidyl choline and other lipids. If desired, neutral lipids and pigment may first be removed on a silicic acid column (see text).

Further structural evidence was provided by examination of the deacylated lipids as described by DAWSON[17]. For this, individual lipid components were isolated by preparative thin-layer chromatography; the separated bands were visualised under ultraviolet light after spraying with dichlorofluorescein, scraped from the plate, and the lipid displaced from the adsorbent with methanol–chloroform (2:1, v/v). The separated components were then subjected to alkaline hydrolysis and the deacylated products examined by paper chromatography.

RESULTS AND DISCUSSION

While KATES[6] has obtained good resolution of many leaf lipids by chromatography on silicic acid-impregnated cellulose papers, the application of the thin-layer technique, using slight variants of the solvent systems proposed by MARINETTI *et al.*[18], has given a greatly improved separation of all the major, and most of the minor components (Fig. 2).

As observed by workers employing silicic acid-impregnated papers for similar studies[19,20], the amount of water present in the developing solvent is extremely critical and must be adjusted to suit the requirements of each particular study. Solvents based on a 40:25 (v/v) mixture of diisobutyl ketone and acetic acid seldom give good over-all resolution if more than 4 parts water are present; the best general-purpose solvent was found to be that containing 3.6–3.8 parts water.

Phosphatidyl choline was readily separable from digalactolipid under these conditions, which also revealed the presence in cabbage and lettuce leaves of a novel, unidentified lipid component (lipid B) which may be comparable with the "glycoside a" detected by KATES in bean-leaf extracts[6]. The nature of lipid A is also uncertain, although its chromatographic behaviour indicates that it might be the sulfolipid described by BENSON *et al.*[3]. Preparation of pure samples of these unidentified lipids is at present being carried out, prior to a detailed examination of their structure.

Although solvents based on diisobutyl ketone give far better general resolution of leaf phospholipids and glycolipids than any other investigated, they have the disadvantages of being comparatively slow running (3.5 h to travel 14 cm) and of having a low volatility which makes them difficult to evaporate from the plate under conditions which will not cause degradation of the adsorbed lipids.

The chloroform–methanol–acetic acid–water solvent does not have these disadvantages and gives a similar type of separation to acidic diisobutyl ketone. However, although in some cases the chloroform solvent has proved of great value, as in the separation of lipid A from digalactolipid and phosphatidyl choline, in general it gives a far poorer overall resolution than the Marinetti solvent.

If the anhydrous chloroform–methanol–acetic acid system is employed, then a good separation is achieved of the two galactolipids from the other phospholipids and glycolipids, which move only slowly in this solvent (Fig. 4). Attempts were made to separate the galactolipids from the phospholipids by the fractional crystallisation method of WEENINK[8], but a significant quantity of digalactolipid was invariably found in the acetone-insoluble fraction. Lipid A also appeared in both fractions, whereas sterol glucosides were acetone-soluble under these conditions.

The "glycoside C" reported by Kates in bean-leaf lipids, and by ZILL AND HARMON[5] in spinach-leaf lipids would seem to correspond with our substance 7 (Fig. 2),

which we have identified as sterol glucoside. This material was inseparable, on our chromatograms, from authentic sterol glucoside from peanut, gave a positive Lieberman–Burchard reaction with H_2SO_4 on the chromatoplate and on hydrolysis gave glucose and a sterol, but no glycerol. The sterol glucosides are abundant in both the photosynthetic and non-photosynthetic tissue of lettuce and cabbage, but are completely absent from leaf-chloroplast preparations.

Triglycerides, free sterols and sterol esters were present in small quantities in the total lipids of lettuce and cabbage leaf but none of these compounds could be detected in the corresponding chloroplast preparation (see Fig. 1). Very small amounts of diglyceride (only separable with difficulty from free sterol) were present in a few of our preparations but the concentration of this lipid was appreciable only when ether was incorporated in the extraction solvent, implying that this compound is present only as an artifact arising from autolysis of the lipids during extraction[6].

The major phospholipid in the chloroplast was phosphatidyl glycerol; presumably most of the phosphatidyl choline and phosphatidyl ethanolamine present in the total leaf were concentrated in the cell nuclei or mitochondria.

Finally, our findings are in accordance with the observations of other workers that there is probably a relationship between the occurrence of chlorophyll and of the galactolipids; thus the dark green outer leaves of cabbage contain a higher proportion of galactolipids than the pale inner leaves, which in turn have a higher glycolipid content than the stalk (Fig. 3)

ACKNOWLEDGEMENTS

This work was supported by a grant from the Department of Scientific and Industrial Research.

The author wishes to thank Dr. J. H. SKELLON for his interest and encouragement during the course of this work, and Dr. A. T. JAMES for many helpful suggestions. He is also indebted to Professor H. E. CARTER, Professor A. A. BENSON, Dr. G. HÜBSCHER and Dr. A. DAWSON for generous gifts of reference compounds.

REFERENCES

1 A. T. JAMES, *Biochim. Biophys. Acta*, 70 (1963) 9.
2 A. T. JAMES AND P. K. STUMPF, *Biochim. Biophys. Acta*, 70 (1963) 20.
3 A. A. BENSON, R. WISER, R. A. FERRARI AND J. A. MILLER, *J. Am. Chem. Soc.*, 80 (1958) 4740.
4 R. A. FERRARI AND A. A. BENSON, *Arch. Biochem. Biophys.*, 93 (1961) 185.
5 L. P. ZILL AND E. A. HARMON, *Biochim. Biophys. Acta*, 57 (1962) 573.
6 M. KATES, *Biochim. Biophys. Acta*, 41 (1960) 315.
7 R. O. WEENINK, *Biochem. J.*, 82 (1962) 523.
8 R. O. WEENINK, *J. Sci. Food Agr.*, 12 (1961) 34.
9 F. B. SHORLAND, *J. Sci. Food Agr.*, 12 (1961) 39.
10 J. HIRSCH AND E. H. AHRENS, *J. Biol. Chem.*, 233 (1958) 311.
11 L. W. WHEELDON, *J. Lipid Res.*, 1 (1960) 439.
12 J. FOLCH, M. LEES AND G. H. SLOANE-STANLEY, *J. Biol. Chem.*, 226 (1957) 487.
13 H. MANGOLD, *J. Amer. Oil Chemists' Soc.*, 38 (1961) 708.
14 H. WAGNER, L. HÖRHAMMER AND P. WOLFF, *Biochem. Z.*, 334 (1961) 175.
15 J. BADDILEY, J. G. BUCHANAN, R. E. HANSCHUMAKER AND J. F. PRESCOTT, *J. Chem. Soc.*, (1956) 2818.
16 W. D. SKIDMORE AND C. ENTENMAN, *J. Lipid Res.*, 3 (1962) 471.
17 R. M. C. DAWSON, *Biochem. J.*, 75 (1960) 45.
18 G. V. MARINETTI, J. ERBLAND AND E. STOTZ, *J. Biol. Chem.*, 233 (1958) 562.
19 M. H. HACK AND C. W. LEATHERMAN, *J. Chromatog.*, 5 (1961) 531.
20 J. E. MULDREY, O. N. MILLER AND J. G. HAMILTON, *J. Lipid Res.*, 1 (1959) 48.