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Note

Isolation of uniformly labelled fatty acids from *Chlorella pyrenoidosa* grown in an atmosphere of $^{14}CO_2$

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Fatty acids that are labelled in specific positions such as the carboxyl group can be obtained by chemical synthesis[1], but the preparation of uniformly labelled fatty acids is obviously much more difficult. Such compounds can easily be obtained biosynthetically. Several reports have appeared on the preparation of uniformly labelled fatty acids from intact higher plants[2,3], algae[4] and plant cell cultures[5] by growing them photoautotrophically in an atmosphere of $^{14}CO_2$. Since the fatty acid patterns of lipids in plant cells can be modified by controlling the factors prevailing during growth, such as illumination[6], temperature[7], growth regulators[8], as well as the cells age[9], plant cell cultures can be programmed for the production of particular fatty acids. Nevertheless, under any combination of conditions, mixtures of fatty acids rather than pure compounds are obtained. Hence, methods for separating individual compounds are required for in the biosynthetic preparation of labelled fatty acids. The processes of separation described so far involve relatively complicated techniques such as distillation and counter-current distribution of mixtures of labelled fatty acids.

The objective of the present communication is to demonstrate that simple and convenient chromatographic procedures can be used for isolating uniformly labelled fatty acids.

EXPERIMENTAL

Mixture of uniformly labelled fatty acids

The methyl esters of uniformly labelled fatty acids were prepared by methanolysis[10] of the total lipids of *Chlorella pyrenoidosa* (Stock culture No. 7516; American Type Culture Collection, Washington, D.C., U.S.A.) that had been grown in the presence of $^{14}CO_2$ under the conditions specified earlier[4].

Argentation chromatography

As far as possible, all processes were carried out in an oxygen-free nitrogen atmosphere.

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The methyl esters of uniformly labelled fatty acids were fractionated on 0.3-mm layers of silica gel G impregnated with 10% (w/w) silver nitrate, using hexane–diethyl ether (80:20, v/v) as a developing solvent. The plates were dried and scanned for radioactivity. Zones of the sorbent carrying the methyl esters of tri-, di, monoenoic and saturated fatty acids were marked on the layer during scanning. For the purpose of identification, "cold" methyl linolenate, methyl linoleate, methyl oleate and methyl stearate were simultaneously chromatographed on a separate plate and the fractions visualized by charring after spraying the layers with 50% sulphuric acid. The zones of sorbent carrying the various fractions of labelled methyl esters were scraped off and eluted with diethyl ether slightly acidified with concentrated hydrochloric acid (two drops of acid per 100 ml solvent) and washed with water. For this purpose, the sorbent zones were transferred into glass tubes fitted with screw caps, and acidified diethyl ether and water were added. The contents of the tubes were shaken vigorously and the diethyl ether phases containing the eluted methyl esters were withdrawn using Pasteur pipettes. Additional aliquots of diethyl ether were added to the aqueous phases containing the sorbent, and this process was repeated five times. The solutions were then combined and the ether removed by a stream of nitrogen. The methyl esters were dissolved in minimal amounts of hexane and subjected to chromatographic fractionation according to the chain lengths of the various fatty acids.

Reversed-phase chromatography

A solution of 15% (v/v) undecane in hexane was allowed to ascend a layer of silica gel G, 0.3 mm, until the solvent front had reached the upper edge of the plate. The plate can be used immediately after the hexane has been evaporated. For fractionating the methyl esters on such plates, mixtures of acetic acid and water in ratios of 85:15 or 90:10 (v/v) are suitable as developing solvents. It is important that the development is done in the same direction as the plate impregnation. After 4–5 h the front had reached a height of about 15 cm, and acetic acid was evaporated from the chromatograms for 40–60 min at room temperature with a stream of nitrogen. The plates were then scanned for radioactivity and the sorbent zones carrying the methyl esters of individual fatty acids were marked on the layer and then scraped off. The methyl esters were eluted with diethyl ether and washed with water as described above. Diethyl ether was removed by evaporation in a stream of nitrogen and the methyl esters of individual fatty acids were dissolved in small volumes of hexane and tested for purity by gas chromatography (GC)[11].

GC

Gas chromatography was done on a Perkin-Elmer F22 instrument (Perkin-Elmer, Norwalk, CT, U.S.A.) using a glass column (6 ft. × 1/4 in.) packed with 5 CP Silar, 10% on Gas-Chrom Q (80–100 mesh), at a temperature of 220°C with nitrogen as carrier gas.

Measurement of radioactivity

The radioactivity of fractions separated on chromatoplates was determined directly by gas flow counting using a Berthold LB 2760 TLC-Scanner (BF-Vertriebsgesellschaft, Wildbad, G.F.R.). The radioactivity of isolated methyl esters was as-

TABLE I

UNIFORMLY LABELLED FATTY ACIDS FROM *CHLORELLA PYRENOIDOSA*

Chain length: number of double bonds	*Amount*		
	*%**	*mg***	*μCi***
16:0	23.1	46	96
16:1	4.7	10	
16:2	5.2	10	7
16:3	2.9	5	4
18:0	1.0	2	
18:1	37.7	76	201
18:2	15.5	30	27
18:3	8.2	15	10

* Determined by GC analysis.

** Fatty acids were isolated by argentation chromatography followed by reversed-phase chromatography; radioactivity determined by liquid scintillation spectrometry.

sayed by suspending aliquots of these compounds in Aquasol-2 scintillation fluid (New England Nuclear, Boston, MA, U.S.A.) and counting in a Packard Model 2425 Tri.Carb liquid scintillation spectrometer.

Quantitation of the peaks obtained by scanning of chromatoplates and in GC was done by triangulation.

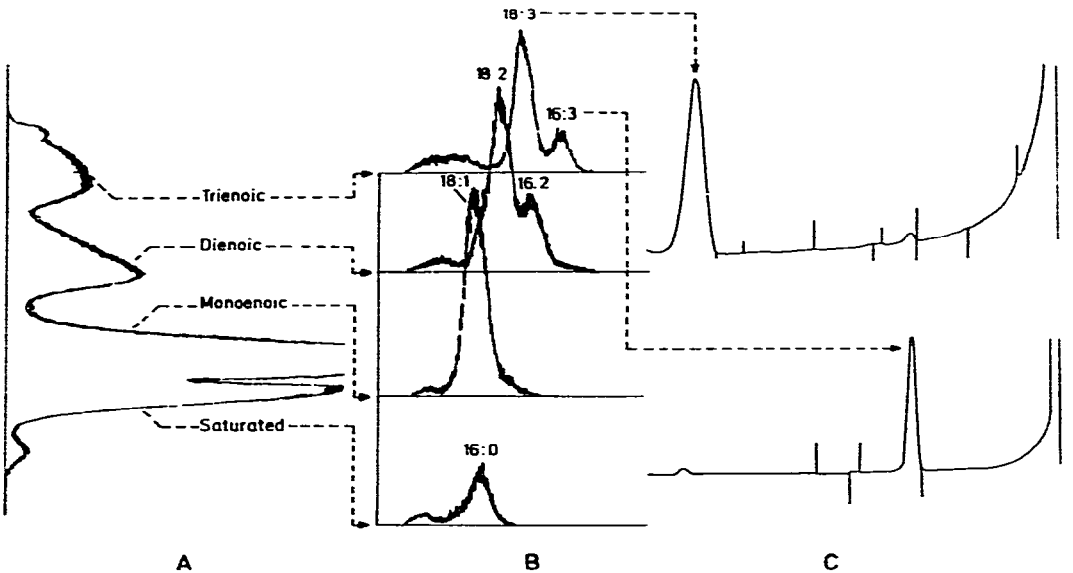

Fig. 1. Separation of uniformly labelled fatty acids by TLC. A, Typical radiochromatogram scan showing the fractionation of the methyl esters into groups with different degrees of unsaturation. Silica gel G containing 10% silver nitrate; hexane–diethyl ether (80:20, v/v). B, Typical radiochromatogram scan showing the fractionation of groups of methyl esters with the same degree of unsaturation into individual compounds. Silica gel G impregnated with undecane; acetic acid–water (90:10, v/v). C, Typical gas chromatograms showing the purity of individual compounds isolated by consecutive TLC in systems A and B.

RESULTS AND DISCUSSION

Table I presents the results of analyses of the total fatty acids in the lipids of *Chlorella pyrenoidosa*. The data are similar to those reported by earlier investigators for the same alga[4,12,13].

Since the basic study by Mangold and Schlenk[4], over two decades ago, on the preparation of uniformly labelled fatty acids, work in this field has been concentrated on improving the methods of analysis for the labelled acids produced[12,13–15]. This has not been paralleled by an improvement in the methods for isolating such fatty acids in pure form. Earlier methods of isolation are tedious and time-consuming. In contrast, conventional thin-layer chromatographic (TLC) methods are simple and fast. Fig. 1 illustrates the successive steps of separation by TLC.

In the first step, A, the mixture of methyl esters was fractioned according to the degree of unsaturation of the components by argentation chromatography. Hexane–diethyl ether (80:20, v/v) gave good resolution of methyl esters with up to three double bonds, although in some cases the separation of methyl esters of saturated and monoenoic fatty acids was not satisfactory. Such compounds were then separated in a consecutive step using hexane–diethyl ether (90:10 or 95:5 v/v) as solvent. Mixtures that contain higher polyunsaturated esters should be resolved by solvents containing higher proportions of diethyl ether, *e.g.*, hexane-diethyl ether (40:60, v/v) or, better, by developing the chromatograms twice with hexane–diethyl ether–acetic acid (94:4:2, v/v).

In the second step, B, methyl esters having the same degree of unsaturation were separated according to the chain lengths of the individual fatty acids by reversed-phase chromatography. Satisfactory separations were obtained with undecane as the stationary phase and acetic acid–water (90:10, v/v) as the developing solvent. An even sharper separation, as shown in Fig. 2, was obtained by slightly increasing the proportion of water in the solvent. However, the quality of separation using the same solvent ratio showed some day to day variation. In general, the most suitable ratio of acetic acid to water lies between 85:15 and 90:10 (v/v).

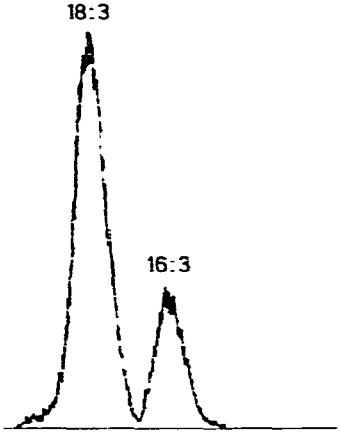

Fig. 2. Typical radiochromatogram scan showing the fractionation of methyl esters of trienoic fatty acids. Silica gel G impregnated with undecane; acetic acid–water (87:13, v/v).

The fractions obtained by reversed-phase chromatography usually contain methyl esters of fatty acids of up to 98% purity as shown by GC analysis, C. Impure fractions are purified either by argentation or reversed-phase chromatography, depending on the identity of the contaminants.

The results of the present study show how convenient conventional TLC techniques are in isolating labelled fatty acids. The entire process of separation is achieved within hours instead of weeks or even months. Thus, not only are time and effort saved but also the labelled fatty acids, especially the unsaturated ones, are less subject to autoxidation due to prolonged processing. Complicated equipment is not needed; for the isolation of the amounts of labelled fatty acids listed in Table I, only 10 silver nitrate-impregnated plates and 30 undecane-impregnated plates of silica gel G were employed.

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