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HYDROCARBONS IN SPINACH: TWO DISTINCTIVE CARBON RANGES OF ALIPHATIC HYDROCARBONS*

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Abstract—Hydrocarbons occurring in the external lipids and in the internal lipids of fresh spinach leaves were separately isolated by TLC and then were identified by gas–liquid chromatography in conjunction with Molecular Sieve 5A treatment and catalytic reduction. The external lipids contained 10% hydrocarbons which were normal paraffins: n-C_{27} to n-C_{33} showing a clear odd-number preference, and n-C_{31} predominating. On the other hand, the internal lipids contained only 0·1% hydrocarbons, principally hydrocarbons similar to those found in the external lipids, n-C_{31} predominating, but, in addition, a significant proportion of a new series of aliphatic paraffins, n-C_{16} to n-C_{28} not showing odd-number preference, was present. The presence of these two distinctive carbon ranges of aliphatic paraffins in spinach suggests that there are two different enzyme systems responsible for their synthesis.

INTRODUCTION

IN RECENT years long-chain hydrocarbons occurring in higher plants have been studied extensively,[1–3] in many cases the compositions of hydrocarbon fractions from certain species of plants are characteristic and can be used as a taxonomic aid.[4] These studies are mostly carried out with hydrocarbons of the cuticle waxes which are believed to be an important factor in maintaining water balance. Internal lipids also include a small but significant proportion of hydrocarbons which may have physiological functions other than water balance, perhaps maintaining inner cell structure. However, very little is known about their chemical composition.

This paper reports a study of the compositions of hydrocarbon fractions isolated from the external lipids and the internal lipids of spinach, *Spinacia oleracea*.

RESULTS AND DISCUSSION

Each experiment was carried out with four leaves of ordinary spinach, weighing 20–30 g. The schematic diagram illustrated in Fig. 1 shows the procedures used for the extraction of lipids from fresh spinach leaves. Extracts I–IV are designated as extracts of the external lipids and Extract V is designated as extract of the internal lipids. Table 1 shows the weight of the leaf samples and of each extract. The weight of the dry leaf samples amounted to 12·4–13·2 per cent of the weight of fresh leaf. The external lipids amounts to 0·6–1·0 per cent of dry leaf

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[3] R. C. CLARK, JR., Technical Report No. 66-34 (Woods Hole Oceanographic Institute, Woods Hole, Mass, 1966).

[4] G. EGLINTON and R. J. HAMILTON, in *Chemical Plant Taxonomy* (edited by T. SWAIN), Academic Press, London and New York (1963).

with 79–84 per cent contained in the first chloroform extract, whereas the internal lipids amounted to 0·4–1·4 per cent of dry leaf.

These lipid samples were fractionated by TLC, first with hexane and then with benzene. The chromatogram thus obtained showed that a major portion of the total external lipids,

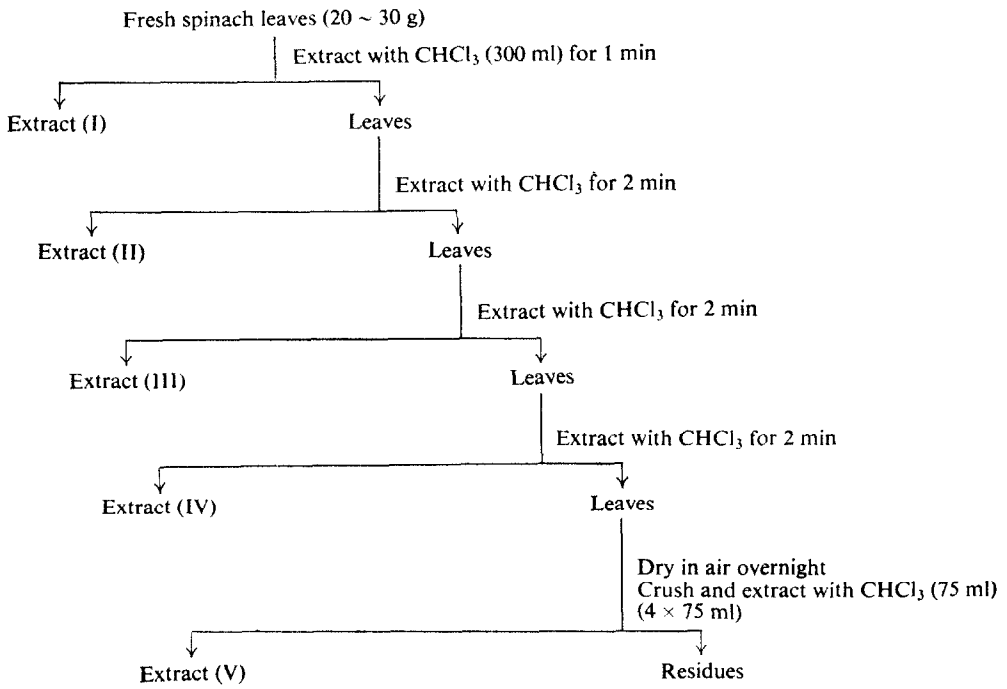

FIG. 1. SCHEMATIC DIAGRAM FOR EXTRACTING LIPIDS FROM SPINACH LEAVES.

TABLE 1. DISTRIBUTION OF LIPIDS ON THE SURFACE AND IN THE INSIDE OF SPINACH LEAVES

Exp. no.	Leaf sample (g)		External lipids* (mg)				Internal lipids† (mg)
	Fresh	Dry	1st	2nd	3rd	4th	
1	29·9	3·7	22·0	2·9	0·8	0·5	29·1
2	28·2	3·5	23·9	2·2	1·7	0·9	44·3
3	26·7	3·4	24·1	3·3	1·6	1·5	47·3
4	22·6	3·0	31·7	1·7	3·9	2·1	37·4

* Represents $CHCl_3$ extract from fresh spinach leaves.
† Represents $CHCl_3$ extract from dry spinach leaves which have been extracted for external lipids.

approximately 90 per cent, remained at the origin and this probably was a mixture of polar compounds; the 10 per cent that was mobile was nearly all aliphatic paraffins. Similarly, approximately 50 per cent of the total internal lipids remained near or at the origin and they were mainly pigments including chlorophylls; the 50 per cent that travelled was almost all material other than aliphatic paraffins which were only a very minor fraction, approximately

1 per cent. The hydrocarbon fraction (top band), isolated from the chromatogram with chloroform, lacked any functional group, according to its u.v. and i.r. spectra.

Figure 2 shows the gas–liquid chromatograms of the hydrocarbon fraction isolated from the external lipids (Extract I) and the internal lipids (Extract V). The peaks of the normal paraffin hydrocarbons are indicated by the carbon numbers shown above each peak. They were identified on the basis of: (i) identical peak position with the corresponding standard normal hydrocarbon when co-chromatographed on two columns, SE-30 and XE-60; (ii) disappearance of these peaks after Molecular Sieve 5A treatment (Fig. 3); (iii) no change

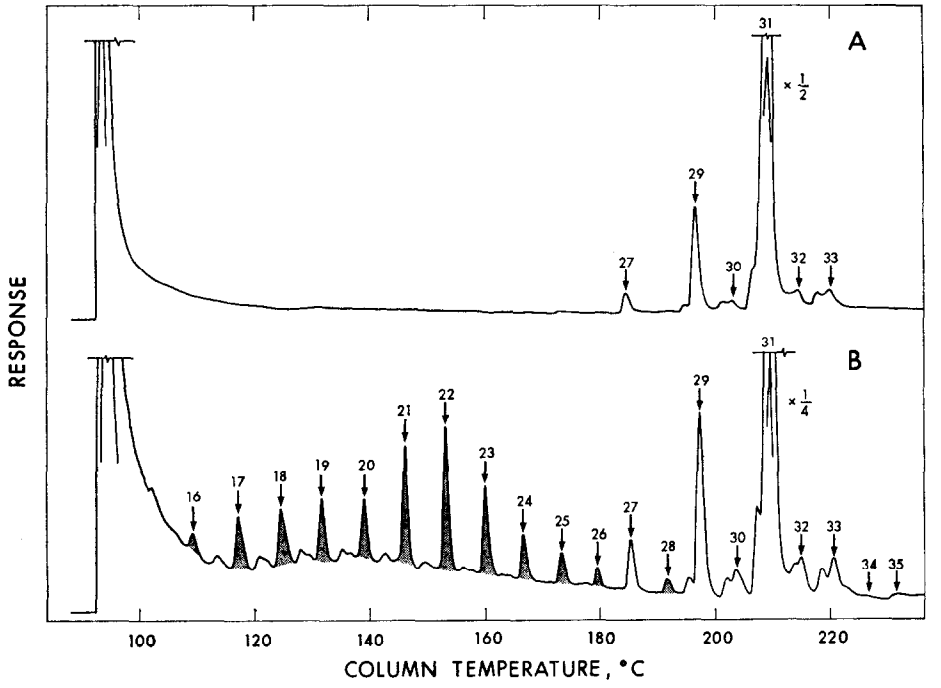

FIG. 2. GAS–LIQUID CHROMATOGRAM OF THE INTERNAL AND EXTERNAL HYDROCARBONS ISOLATED FROM SPINACH LEAVES

Numbers indicate normal paraffins. A: external hydrocarbons contained in $\frac{1}{2000}$ of Extract I (see text) isolated from 28·2 g of fresh spinach leaves. B: internal hydrocarbons contained in $\frac{1}{20}$ of Extract V isolated from 28·2 g of fresh spinach leaves. A column of 1% SE-30 on chromosorb W was used with a Micro-Tek model 2000 R-GC chromatograph. For detail see Experimental. Shaded peaks occurred only in the internal hydrocarbons in significant portions and n-C_{27} was present in both samples.

in the peak position before and after catalytic reduction of the hydrocarbon fraction; (iv) absence of olefinic compounds as indicated by identical TLC on silica gel G and on silica gel G impregnated with $AgNO_3$. Small peaks appeared between the numbered peaks and were presumed to be branched-chain or, perhaps, cyclic paraffins since Molecular Sieve 5A treatment did not remove these compounds but their exact chemical structure had not been determined.

The external hydrocarbon fraction was 10 per cent of the total external lipids (Fig. 2A) and accounted for 0·06 per cent of the total dry weight of the leaf. The external hydrocarbons consisted mainly of normal paraffins (n-$C_{27} \sim n$-C_{33}). Hentriacontane, n-C_{31}, was

found to be the major component (58 ~ 75 per cent) of the total external hydrocarbons. There were paraffinic hydrocarbons other than the normal paraffins but their proportion was very small ($\leqslant$ 5 per cent). The internal hydrocarbon fraction was 0·1 per cent of the total internal lipids (Fig. 2B) and accounted for 0·0004 per cent of the total dry weight of the leaf. As can be seen on the chromatograms, the composition of the internal hydrocarbon was, in part, identical with that of the external hydrocarbons but in addition the internal hydrocarbons included 25–34 per cent of C_{16} to C_{28} normal hydrocarbons. This lower group had a rather broad carbon-number distribution—C_{22} being the most prominent in the chromatogram of Fig. 2B but other leaf samples gave C_{21} or C_{18} as the most plentiful.

The hydrocarbon samples from the external lipids, Extract I and Extract II, did not contain the range of hydrocarbons with 16 to 28 carbon atoms which are characteristic of the internal hydrocarbons. As chloroform extraction continued, however, this range of

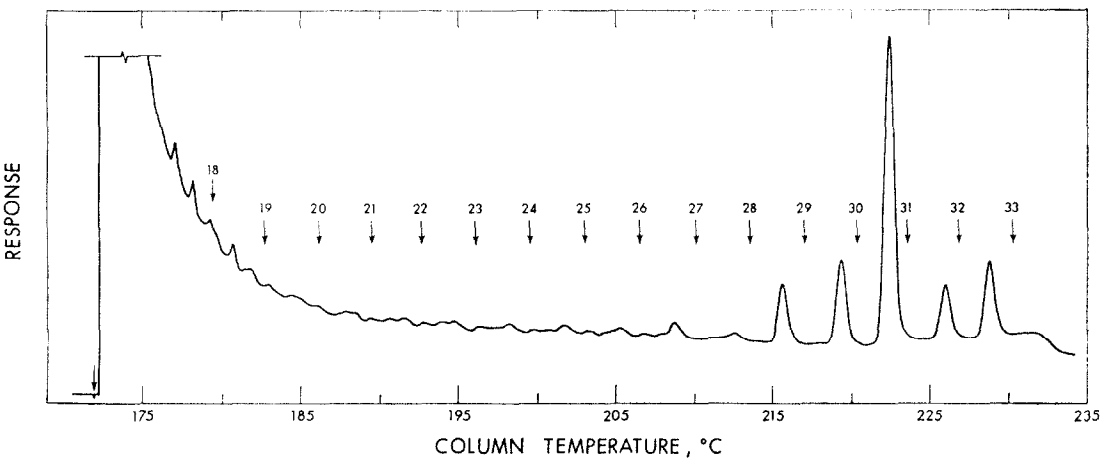

FIG. 3. GAS–LIQUID CHROMATOGRAM OF THE EXTERNAL HYDROCARBONS THAT HAD BEEN TREATED WITH MOLECULAR SIEVE 5Å.

The sample was the same as one used for Fig. 2 but was treated with Molecular Sieve 5Å in isooctane to remove normal hydrocarbons.

hydrocarbons tended to be significant and the hydrocarbon samples from Extracts II and III contained the C_{16} to C_{28} hydrocarbons in a significant proportion, 2–5 per cent of the total hydrocarbons. This suggests that chloroform tends to extract the internal hydrocarbons in addition to the external hydrocarbons from fresh leaves if a prolonged extraction is applied.

It is noteworthy that the C_{27} to C_{33} hydrocarbons show a clear odd-carbon-number preference regardless of whether found on the surface or inside of the leaf. On the other hand, the C_{16} to C_{28} hydrocarbons are only present in a significant proportion in the inside of the leaf and show no odd-number preference. In this respect they are different from most biogenic hydrocarbon samples and resemble fossil hydrocarbons.[5, 6]

A similar lack of any odd-number preference has been demonstrated for C_{16} to C_{28} hydrocarbons in lower plants (liverworts, algae and lichen),[7] lower organisms (marine sponge

[5] E. E. BRAY and E. D. EVANS, *Geochim. Cosmochim. Acta* **22**, 2 (1961).
[6] R. L. MARTIN, J. C. WINGERS and J. A. WILLIAMS, *Nature* **199**, 110 (1963).
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and horny coral),[8] and wool grease.[9] The most reasonable explanation for the presence of these two classes of hydrocarbons in spinach leaves is that they are produced by two different pathways. This has been postulated by Mold *et al.*[9] in the case of wool wax. At present it is too early to discuss probable synthetic routes in detail but for the synthesis of the external hydrocarbons, the "condensation pathway" postulated for the synthesis of tobacco hydrocarbons[10] is highly appropriate since the carbon-number distribution of the external hydrocarbons isolated from spinach and tobacco are very similar. The pathway,[10] which has been discussed in detail, involves condensation of two fatty acids followed by elimination of one carbon atom (decarboxylation) and reduction to yield the hydrocarbon with one carbon less than the total carbon atoms of the two fatty acids.

Lower paraffinic hydrocarbons with fewer than 27 carbon atoms (similar to the group of hydrocarbons found in the internal lipids of spinach) are also present in the inner heartwood of *Garcinia eugeniifolia* and of *Calyphyllum scriblitifolium*[11] and in the acetone extract of red clover, *Trifolium pratense*[12] (presumably a mixture of the external and the internal lipids), and in the internal lipids of tobacco leaf (T. Kaneda, unpublished observations). They all give a broad carbon-number distribution and a slight or no odd-number preference. It is difficult to suggest a synthetic route to these hydrocarbons. Fatty acids or their derivatives are generally considered to be closely related to the direct precursor for the hydrocarbons produced in plants.[2, 10, 13] Therefore, odd-numbered fatty acids, presumably produced by the α-oxidation scheme, should participate in the hydrocarbon synthesis along with the common even-numbered fatty acids in order to produce the hydrocarbon distribution mentioned above.

It would be desirable to know whether a range of normal alkanes, C_{16} to C_{28}, with a broad carbon-number distribution showing an odd-number preference is characteristic of the internal hydrocarbons of most higher plants. The extraction scheme reported here should be a useful technique to carry out such investigation.

EXPERIMENTAL

Isolation of the External Lipids

Each experiment was carried out with four leaves of ordinary spinach, weighing 20–30 g. Fresh leaves were successively dipped in 300-ml portions of $CHCl_3$ for 1–2 min and the extracts were designated Extracts I, II, III and IV (Fig. 1).

Isolation of the Internal Lipids

The leaves that had been treated with $CHCl_3$ four times were dried in air and left overnight. Then they were crushed to small pieces, and extracted with $CHCl_3$ (4×75 ml). These extracts were combined and evaporated in N_2 (Extract V; Fig. 1).

Isolation of the Hydrocarbon Fraction from Lipid Extracts

The five extracts I–V in $CHCl_3$ were placed separately on the $CHCl_3$-washed silica gel G (Brinkman) plates (21×21 cm). The plates were developed with benzene until the solvent front advanced to 2 in. from the top. After drying, the plates were once more developed with *n*-hexane until the solvent front reached to 0·5 in. below the top. Pre-development with benzene was essential for the samples of the internal lipids but less important for the samples of the external lipids. This procedure separates aliphatic hydrocarbons from isoprenoids,

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[9] J. D. MOLD, R. E. MEANS, R. K. STEVENS and J. M. RUTH, *Biochem.* **3**, 1293 (1964).
[10] T. KANEDA, *Biochem.* **7**, 1192 (1968).
[11] R. E. GRICE, H. D. LOCKSLEY and F. SCHEINMANN, *Nature* **218**, 892 (1968).
[12] R. O. WEENINCK, *Biochem. J.* **82**, 523 (1962).
[13] P. E. KOLATTUKUDY, *Phytochem.* **6**, 963 (1967).

carotenoids, aliphatic ketones and fatty acid methyl esters.[14] Rhodamine 6G (0·002% in water) was used to locate each lipid fraction, and the top band was scraped off the plate and extracted with $CHCl_3$ to yield the hydrocarbon fraction. TLC on silver nitrate impregnated plates was used to separate paraffinic compounds from olefinic compounds. The plates were prepared by spraying a saturated solution of $AgNO_3$ on to Silica Gel G plates. The solvent system used was 10% ethyl ether in *n*-hexane. 1-Docosene was separated clearly from a standard *n*-C_{32} hydrocarbon on the impregnated plate.

Standard Hydrocarbons

The standards used were chemically synthesized hydrocarbons: normal series, *n*-C_{23} to *n*-C_{37} inclusive, and branched-chain series, i-C_{31} and a-C_{30},[14] commercial hydrocarbons: *n*-C_{16}, *n*-C_{18}, *n*-C_{20}, and 1-docosene, supplied by Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, U.S.A., and tobacco hydrocarbons which had been identified previously.[14]

Identification of the Hydrocarbons Isolated from Spinach

Spinach hydrocarbons were identified by co-chromatography with the standard hydrocarbons on 1% SE-30 and 3% XE-60 (an Aeropack 30 packing prepared by Varian Aerograph, Walnut Creek, California, U.S.A.) columns, both packed in $\frac{1}{8}$ in. × 6 ft. regular stainless-steel tube. Temperature of the SE-30 column was held at 100° for 2 min and then was programmed at a rate of 4° per min. In some cases the initial temperature of 175° and a programming rate of 3° per min were used with the same sequence as above. The XE-60 column was used isothermally at 175° for hydrocarbons with 16–25 carbon atoms and 220° for hydrocarbons with 25–35 carbon atoms. Molecular Sieve 5A[15] was used to subtract all normal hydrocarbons from the spinach hydrocarbon fraction and to confirm identification carried out by GLC .[14] To examine the possible occurrence of unsaturated hydrocarbons in the spinach hydrocarbon fraction, the sample was reduced catalytically[16] and was chromatographed on the SE-30 column.

Estimation of the Hydrocarbons

Each peak area on the chromatogram was measured by triangulation and the amount of the hydrocarbon corresponding to the peak was calculated based on the *n*-C_{32} hydrocarbon standard.

Spectrophotometric Examination of the Hydrocarbons

The hydrocarbon fraction isolated by TLC was examined for the possible presence of functional groups by means of spectroscopy. U.v. spectra were measured in *n*-hexane (0·05%) and i.r. spectra in KBr (0·2%).

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