THE BIOSYNTHESIS OF TRANS-Δ^3-HEXADECENOIC ACID BY CHLORELLA VULGARIS.

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Trans-Δ^3-hexadecenoic acid was first isolated from the leaves of spinach and antirrhinum by Debuch (1961) who also elucidated its structure. It has since been identified in the photosynthetic tissues of a variety of higher plants (Weenink and Shorland, 1964; Allen et al, 1964; Haverkate et al, 1964; Nichols et al, 1965a; Nichols, 1965b), and algae (Klenk et al, 1963; Nichols, 1965a; Haverkate, 1965). In these tissues, it occurs in significant amounts in only one lipid, namely phosphatidyl glycerol. Recently, however, trans-Δ^3-hexadecenoic acid has been found as a major component of the seed oil from Helenium bigelowii (Hopkins and Chisholm, 1964) where it presumably occurs on a triglyceride.

The very high turnover rate of phosphatidyl glycerol in light-grown systems (Miller, 1964; Nichols and James, unpublished observations) and the appearance of the trans-acid in this molecule only after light-stimulation of etiolated systems (Nichols et al, 1965a; Haverkate, 1965; Nichols, 1965a) directed our attention to the pathway of biosynthesis of this acid, and this communication describes our studies on its synthesis in the green alga Chlorella vulgaris.

MATERIALS AND METHODS

Chlorella vulgaris. An inoculum of light-grown cells of Chlorella vulgaris was cultured for six days in darkness on a tryptone-glucose nutrient medium (Harris et al, 1965).

^{14}C-labelled metabolites. [3-^{14}C]-β-ketopalmitic acid, [3-^{14}C]-β-hydroxy palmitic acid and [2-^{14}C]-αβ-trans-hexadecenoic acid were generously donated by Dr. D.H. Nugteren of the Unilever Research Laboratories, Vlaardingen, Holland. 2-^{14}C-acetic acid, 1-^{14}C-myristic acid and 1-^{14}C-palmitic acid were obtained from the Radiochemical Centre, Amersham, England. All labelled compounds were checked for mass and radioactive purity by conversion to their methyl esters which were analysed by radiochemical gas chromatography (radio-GLC) (James and Piper, 1963) using Apiezon L and ethylene glycol adipate polyester (EGA) as stationary phases.

Incubation of cells with metabolites. Dark-grown cells of Chlorella vulgaris were harvested by centrifugation and resuspended in phosphate buffer (0.2 molar, pH 7.4). Labelled palmitate (10 μC), myristate (10 μC), β-ketopalmitate (3 μC), β-hydroxy palmitate (3 μC) and αβ-trans-hexadecenoate (3 μC) were added to similar aliquots of this suspension and the mixtures aerated for 24 hours in a water bath (25^{o}C) over a 1 KW tungsten-iodide lamp.

Isolation of lipids. After incubation, algal cells were harvested by centrifugation and the pellets extracted overnight with chloroform-methanol (2:1 v/v) at room temperature. The lipid extracts were fractionated, and pure phosphatidyl glycerol isolated, by methods described elsewhere (Nichols and James, 1964). This fraction and the other lipids were transmethylated by refluxing with methanol-benzene-sulphuric acid (20:10:1 v/v) for 90 minutes. The resultant fatty acid methyl esters were analysed by radio-GLC on EGA columns.

Isolation and oxidation of trans-Δ^3-hexadecenoic acid. The fatty acid methyl esters prepared from the isolated phosphatidyl glycerol were fractionated by thin layer chromatography (TLC) on silicic acid impregnated with silver nitrate (Morris, 1962) using 10% diethyl ether in light petroleum as mobile phase. In this system, the methyl ester of the trans-acid migrates more slowly than methyl palmitate but faster than methyl oleate and methyl palmitoleate. The esters were located on the chromatogram with dichlorofluorescein, the relevant sector of adsorbent scraped from the chromatogram and the adsorbed

TABLE 1

Labelled Precursor		Labelled acids found in Phosphatidyl Glycerol	Labelled acids found in Other Lipids
1-^{14}C-myristate		14:0 16:0 $\Delta^3$16:1 18:1 18:2 18:3	14:0 16:0 $\Delta^9$16:1 16:2 18:1 18:2 18:3
1-^{14}C-palmitate	light, aerobic	16:0 $\Delta^3$16:1 18:1 18:2 18:3	16:0 $\Delta^9$16:1 16:2 16:3 18:1 18:2 18:3
	light, anaerobic	16:0 $\Delta^3$16:1(less) 18:0	16:0 $\Delta^9$16:1 16:2 18:0 18:1 18:2
	dark, aerobic	16:0 18:0	16:0 $\Delta^9$16:1 18:0 18:1
3-^{14}C-β-ketopalmitate		16:0 $\Delta^3$16:1	16:0 $\Delta^9$16:1 16:2 18:2
3-^{14}C-β-hydroxypalmitate		16:0 $\Delta^3$16:1	16:0 $\Delta^9$16:1 16:2 18:2
2-^{14}C-αβ-trans-hexadecenoate		16:0 $\Delta^3$16:1	16:0 $\Delta^2$16:1 $\Delta^9$16:1 18:2

substance eluted with diethyl ether. Residual traces of other methyl esters were removed from that of the trans-Δ^3-hexadecenoic acid by preparative GLC on EGA columns on which the trans-ester (RV_R 1.2) is well separated from that of palmitic acid (RV_R 1.0).

The purified ester was reduced to methyl palmitate by hydrogenation using Adams' catalyst, after which the reaction product was checked for homogeneity by GLC and then submitted to partial α-oxidation with acetone-permanganate according to the method of Murray (1959). The oxidation products were then reacted with diazomethane and the methyl esters analysed by radio-GLC.

RESULTS AND DISCUSSION

Myristic acid, β-ketopalmitic acid, β-hydroxy palmitic acid, αβ-trans-hexadecenoic acid and palmitic acid were all partially converted to trans-Δ^3-hexadecenoic acid by illuminated cells of Chlorella vulgaris (Table 1). However, myristic acid and the three palmitic acid derivatives were all also converted into palmitic acid itself, the specific activities of the samples of trans-Δ^3-acid obtained from these experiments being consistently close to those of the palmitic acid in the same preparation, indicating that palmitic acid is the immediate precursor of the trans-Δ^3-acid (Table 2).

TABLE 2. Relative Activities of Palmitic Acid and trans-Δ^3-hexadecenoic acid Moieties in Different Phosphatidyl Glycerol Preparations.

Precursor	Counts in palmitic acid / Counts in trans-Δ^3-hexadecenoic acid
1-^{14}C-myristate	1.3
1-^{14}C-palmitate	2.3 to 3.3 (in different experiments)
3-^{14}C-β-ketopalmitate	2.6
3-^{14}C-β-hydroxypalmitate	2.3
2-^{14}C-αβ-trans-hexadecenoate	3.0

This was confirmed in the studies with labelled palmitic acid which was converted only to unsaturated C_{16} acids, and C_{18} acids. That the trans-Δ^3-acid does not arise from the degradation of palmitic acid to fragments of lower carbon number, followed by resynthesis, is indicated by two pieces of evidence. Firstly, radio-GLC failed to detect the presence of radioactive compounds of shorter chain length. Secondly, α-oxidation of the reduced trans-Δ^3-acid to give acids of 15, 14, 13 and fewer carbon atoms showed that the labelled carbon was located exclusively in the 1-C position of the C_{16} acid.

As might be anticipated from the established absence of the trans-Δ^3-acid from etiolated tissue, its formation from palmitic acid is light-requiring and no Δ^3 desaturation of palmitate occurs in the dark, although some desaturation at the Δ^9 position does take place (Table 1). In requiring light for synthesis the trans-Δ^3-acid differs from all other known acids present in the photosynthetic tissues of algae and higher plants, for although the proportion of some acids (e.g. α-linolenic acid) increases markedly on changing the growth conditions from dark to light, they are nevertheless also synthesised in the dark to an appreciable extent.

The incubation of dark-grown Chlorella cells with palmitate in the light but in the absence of exogenous O_2, results in an inhibition of synthesis of the trans-acid (Tables 1 and 3), the trans-acid content of the phosphatidyl glycerol reaching less than 50% of the normal level. This indicates that the conversion of palmitate to the trans-acid is an O_2-requiring reaction, a requirement which has already been demonstrated for the desaturation of palmitate in the Δ^9 position in Chlorella (Harris et al, 1965).

To summarise, the trans-Δ^3-hexadecenoic acid present in light-grown cells of Chlorella vulgaris is derived from palmitic acid by a dehydrogenation reaction which requires light and probably oxygen. The fact that the phosphatidyl glycerol molecule is the only one in which this acid is combined suggests that perhaps this molecule might be involved in this conversion. The elegant studies of Haverkate and van Deenen (1965) have shown that in

spinach leaves most of the trans-Δ^3-hexadecenoic acid is specifically located at the 2-position on the glycerol residue, and that palmitic acid is attached at the 1-position. This suggests that even if the presence of a phosphatidyl glycerol molecule is obligatory then the desaturation reaction does not occur when the palmitate is combined in this lipid, assuming that the same mechanism applies to the formation of this fatty acid in spinach as it does in Chlorella.

TABLE 3. Fatty Acid Composition of the Phosphatidyl Glycerol Fractions from Cells of Chlorella vulgaris (initially dark-grown) incubated under various conditions.

	Fatty Acid Composition (%)					
Conditions of incubation	16:0	Δ^3 16:1	18:0	18:1	18:2	18:3
Aerobic, in the light	37.6	20.5	1.2	3.7	29.9	6.6
Anaerobic, in the light	42.5	11.9	6.0	11.3	24.6	4.1
Aerobic, in the dark	60.0	1.1	4.3	13.1	16.0	1.3

Hopkins and Chisholm (1964) have shown trans-Δ^3-hexadecenoic acid to comprise some 10% of the total fatty acids present in the seed oil from Helenium bigelowii. It seems improbable that the acid can be specifically located in a phosphatidyl glycerol fraction in such a tissue and it is also possible that it is formed by a mechanism differing from that operative in Chlorella. Alternatively, illumination of the photosynthetic tissues of higher plants and green algae might produce some cofactor which is required for the synthesis of the trans-acid and which is already present in the germinating seed.

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REFERENCES

Allen, C.F., Good, P., Davis, H.F. and Fowler, S.D., Biochem. Biophys. Res. Comm., 15 424 (1964)

Debuch, H., Z. Naturforschung, 16 561 (1961)

Harris, R.V., Harris, P. and James, A.T., Biochim. Biophys. Acta (in Press) (1965)

Haverkate, F., Thesis "Phosphatidyl glycerol from Photosynthetic Tissues", Univ. of Utrecht, 1965

Haverkate, F., de Gier, J. and van Deenen, L.L.M., Experientia, 20 511 (1964)

Haverkate, F. and van Deenen, L.L.M., Biochim. Biophys. Acta, 106 78 (1965)

Hopkins, C.Y. and Chisholm, J., Can. J. Chem., 42 2224 (1964)

James, A.T., Harris, R.V. and Harris, P., Biochem. J., 95 6p (1965)

James, A.T. and Piper, E.A., Analyt. Chem., 35 515 (1963)

Klenk, E., Knipprath, W., Eberhagen, D. and Koof, H.P., Z. Physiol. Chem., 334 44 (1963)

Miller, J.A., quoted by A.A. Benson in "Mechanism of Photosynthesis", Ed. Tamiya, Pergamon Press London 1963 p.341

Morris, L.J., Chem. and Ind., 1238 (1962)

Murray, K.E., Aust. J. Chem., 12 657 (1959)

Nichols, B.W., Biochim. Biophys. Acta, 106 274 (1965a)

Nichols, B.W., Phytochemistry, 4 769 (1965b)

Nichols, B.W. and James, A.T., Fette Seifen Anstrichmittel, 66 1003 (1964)

Nichols, B.W., Wood, B.J.B. and James, A.T., Biochem. J., 95 6p (1965a)

Weenink, R.O. and Shorland, F.B., Biochim. Biophys. Acta, 84 613 (1964)