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BIOGENESIS OF NONACOSAN-15-ONE IN *BRASSICA OLERACEA*

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Abstract—The incorporation of [4-^{14}C]4-oxostearic acid, [4-^{14}C]stearic acid, and [1-^{14}C]eicosanoic acid, respectively, into the nonacosan-15-one of *Brassica oleracea* has been investigated. While the oxo-acid was found to be a poor precursor of nonacosan-15-one, the two *n*-fatty acids were efficiently incorporated as intact units into *n*-nonacosane and the related 15-ketone. In accordance with expectation, the label in the 4-position of stearic acid appeared in the carbonyl carbon of the ketone. These results suggest that the C_{29} hydrocarbon is first formed and subsequently oxidized to nonacosan-15-one.

INTRODUCTION

IN THE course of work[1] already published, it has been shown that palmitic acid (C_{16}) is utilized by the plant for the formation of nonacosane and its 15-oxo-derivative, without loss of any carbon atom from the chain. At that stage, and later, the study was motivated by the desire to identify a functional group in the chain which could survive as the 15-oxo-group, or specifically direct oxidations to this position. Successive attempts to find such a directing group have failed. Stearic acid (C_{18}) was found to be incorporated, intact,[2] in the synthesis of the C_{29} hydrocarbon and related 15-ketone. It is, in fact, a better precursor of these substances than is palmitic acid. It appeared possible that 4-oxostearic acid is the true progenitor of nonacosan-15-one. We now report that this substance, labelled for the purpose by ^{14}C in position-4, is a poor precursor of nonacosan-15-one, in spite of the fact that we have obtained direct evidence that C-4 of stearic acid becomes the carbonyl carbon of the ketone.

Furthermore, we have established that eicosanoic acid (C_{20}) is equally well incorporated into nonacosane and nonacosan-15-one as is stearic acid.

RESULTS AND DISCUSSION

Synthetic [4-^{14}C]4-oxostearic acid (sp. act. 1·4 mc/mmole) was dispersed in water with the aid of Tween-20 and sonication, and this solution was incubated with slices of young broccoli leaves, as previously described.[3] Very little radioactivity could be found in the fractions

* Scientific Paper 3478, Project 2001, College of Agriculture, Washington State University, Pullman, Washington, U.S.A.

[1] P. E. KOLATTUKUDY, *Biochem.* **4**, 1844 (1965).
[2] P. E. KOLATTUKUDY, R. H. JAEGER and R. ROBINSON, *Nature, Lond.* **219**, 1038 (1968).
[3] P. E. KOLATTUKUDY, *Phytochem.* **6**, 965 (1967).

(TLC) containing nonacosane and nonacosan-15-one. The labelled products detected were palmitic acid and pentadecaynoic acid. Young pea leaves also metabolized the labelled keto-acid, giving rise to labelled palmitic and pentadecanoic acid. We conclude that synthesis of fatty acids from the labelled fragments produced by degradation gave rise to the labelled palmitic acid. The labelled pentadecanoic acid probably originated from the cleavage at the carbonyl group. In any case, there was no evidence for any preferential incorporation of the keto-acid into nonacosan-15-one.

We have already provided evidence that the intact carbon chain of stearic acid is incorporated into nonacosane and nonacosan-15-one.[2] Therefore, the C-4 of stearic acid should end up as the carbonyl carbon of nonacosan-15-one, and this has now been experimentally confirmed.

[4-^{14}C]stearic acid was dispersed in water with the aid of Tween-20 and sonication (10 mg acid, $57{\cdot}6 \times 10^6$ counts/min in 7·5 ml H_2O and 5 drops Tween-20, sonication by Biosonic III needle probe for 3 min at maximum power). Young sliced broccoli leaves (8 g) were incubated in this radioactive solution (6 ml containing 8 mg stearic acid for 4 hr at 30°). After washing the slices with H_2O, the surface lipids were extracted by stirring the slices with 100 ml of $CHCl_3$–MeOH (2:1). The extract was shaken with 20% of its volume of H_2O, and the $CHCl_3$ layer separated, and evaporated to dryness. The surface lipid fraction was separated by column chromatography[1] into a hydrocarbon fraction containing $1{\cdot}5 \times 10^6$ counts/min (70% efficiency), and a fraction (eluted with benzene) containing ketones and other components which gave $2{\cdot}5 \times 10^6$ counts/min. From this latter fraction a chemically and radiochemically pure ketone fraction (1×10^6 counts/min) was isolated by repeated TLC on silica gel G in benzene. This fraction was diluted with 15 mg synthetic nonacosan-13-one and then converted into oximes, which were subjected to Beckmann

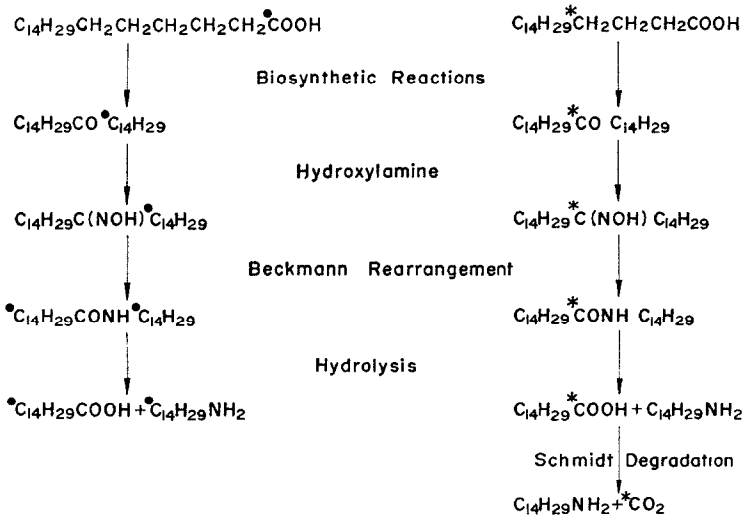

FIG. 1. HYPOTHETICAL FATE OF C-1 (●) OF EICOSANOIC ACID AND C-4 (*) OF STEARIC ACID AND CHEMICAL DEGRADATION OF NONACOSAN-15-ONE DERIVED FROM THESE ACIDS.

If the intact chain of eicosanoic acid-1-^{14}C is incorporated into nonacosan-15-one the label would be at the C-10 position of the ketone and hence the label is indicated to be on the alkyl moiety. If the intact carbon chain of stearic acid-4-^{14}C is incorporated into nonacosan-15-one all the label should be at the carbonyl carbon.

rearrangement as described before.[2] After hydrolysis of the resulting substituted amides, essentially all the radioactivity was contained in the fatty acid fraction, with very little ^{14}C in the amines (Fig. 1). Analysis of the fatty acids (as methyl esters) by radio GLC showed the expected C_{13} and C_{17} acids (derived from the carrier nonacosan-13-one), and pentadecanoic acid which contained essentially all the radioactivity (Fig. 2). This proves that the labelled ketone was nonacosan-15-one. When the fatty acid fraction was subjected to micro-Schmidt degradation,[4] 93% of the ^{14}C was released as $^{14}CO_2$, showing that essentially all

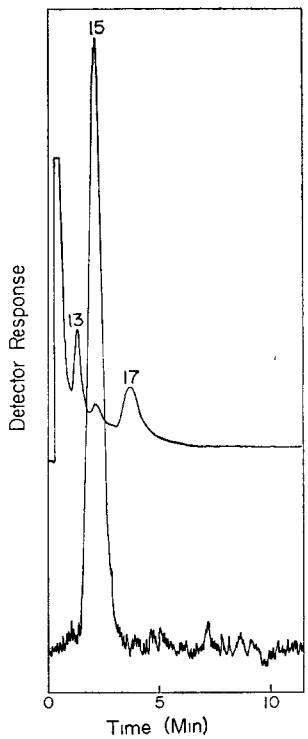

FIG. 2. RADIO GAS–LIQUID CHROMATOGRAM OF THE METHYL ESTERS DERIVED FROM THE DEGRADATION OF THE LABELLED KETONE ISOLATED FROM *B. oleracea* LEAVES WHICH METABOLIZED [4-^{14}C]-STEARIC ACID.

Coiled copper column (25 × 0·64 cm); 4% silicone rubber SE-30 on 80–100 mesh 'Gas Chrom Q'; column temp. 178°; flow rate 100 ml Argon/min. Top tracing: flame ionization detector response. Bottom tracing: radioactivity as monitored by a Barber–Colman radioactivity monitor. The effluent from the gas chromatograph was split; about 80% was diverted into the combustion furnace (660°) for radioactivity determination while the rest was being monitored by the flame ionization detector. Under the present conditions 3300 dpm in C_{15} acid injected into the gas chromatograph gave a radioactivity peak with about full scale height at 1K setting.

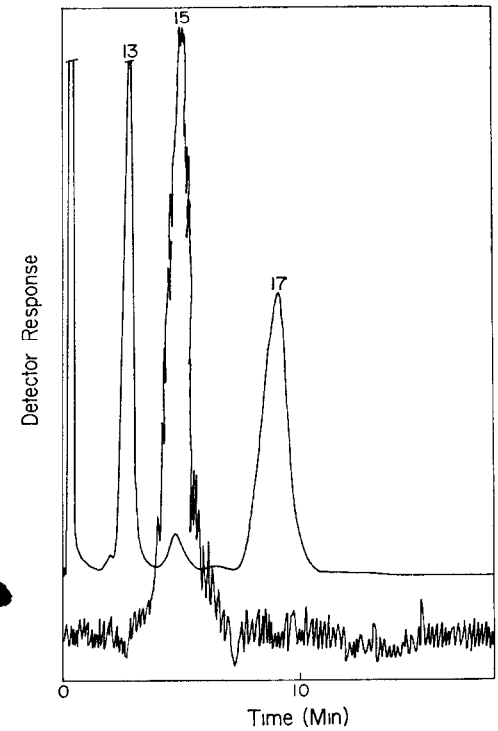

FIG. 3. RADIO GAS–LIQUID CHROMATOGRAM OF THE METHYL ESTERS DERIVED FROM THE DEGRADATION OF THE LABELLED KETONE ISOLATED FROM *B. oleracea* LEAVES WHICH METABOLIZED [1-^{14}C]-EICOSANOIC ACID.

Coiled copper column (183 × 0·64 cm); 12% diethylene glycol succinate on 80–100 mesh 'Gas Chrom Q'; column temp. 182°; flow rate 90 ml argon/min. Top tracing: flame ionization detector response. Bottom tracing: radioactivity as monitored by a Barber Colman radioactivity monitor.

[4] R. O. BRADY, R. M. BRADLEY and E. G. TRAMS, *J. Biol. Chem.* **235**, 3093 (1960).

the ^{14}C of the nonacosan-15-one was in the carbonyl carbon. These results demonstrate clearly that the carbonyl carbon of the ketone originated from C-4 of stearic acid.

We have further shown that [1-^{14}C]eicosanoic acid and [1-^{14}C]stearic acid were about equally well incorporated into *n*-nonacosane and nonacosan-15-one. The labelled acids, each with a sp. act. of 0·89 mc/m-mole, were dispersed in H_2O (34×10^6 counts/min at 60% efficiency in 8 ml H_2O) with the aid of Tween-20 and sonication (2 min at 95% power setting on a Biosonik II). Each substrate solution was incubated at 30° for 2 hr with 2·5 g of sliced young broccoli leaves. After washing the leaves with H_2O, the surface lipids were extracted and components isolated as described in the previous experiment. Thus, [1-^{14}C]eicosanoic acid and [1-^{14}C]stearic acid gave 225,000 and 229,000 counts/min respectively in the hydrocarbon fraction and 107,000 and 100,000 counts/min respectively in the ketone fraction. Examination of the hydrocarbon fraction by radio GLC showed only one radioactive component, the retention time of which coincided in both cases with that of *n*-nonacosane.

The ketone fraction (from the eicosanoic acid experiment), isolated by repeated TLC, showed only one component, by TLC and GLC. Since these chromatographic techniques would not allow us to locate the carbonyl group in the chain, we resorted to a chemical degradation described in the previous experiment. In this case both the amine fraction and the acid fraction contained approximately equal amounts of ^{14}C. The labelled fatty acid fraction isolated contained C_{13} and C_{17} chains expected from the unlabelled synthetic nonacosan-13-one which was added before degradation. However, only pentadecanoic acid was labelled (Fig. 3), showing that [1-^{14}C]eicosanoic acid gave rise to nonacosan-15-one, probably without loss of any carbon (Fig. 1).

Similar experiments with [2-^{14}C]palmitic acid showed that the major part of the radioactivity in the chemical degradation products of the ketone was in the fatty acid fraction, in which the major labelled component was C_{15} acid.[5] Micro-Schmidt degradation of this acid showed that most of the ^{14}C was in the carboxyl carbon, showing that C-2 of palmitate did indeed end up as the carbonyl carbon of the ketone. These results, together with the results discussed in the present communication, show that C-2, C-4 and C-6 positions of palmitic, stearic and eicosanoic acids, respectively, become the carbonyl carbon of the ketone. Therefore it is unlikely that a group in the fatty acid chain activates the fifteenth carbon from the methyl end of the acid for oxidation to a carbonyl group. Furthermore, if we assume that the fatty acids fed to the leaves were first oxidized to the corresponding keto-acids prior to the chain building, then we have to assume the existence of an oxidative enzyme system which is capable of differentiating between C_{16}-, C_{18}- and C_{20}-fatty acids; this enzyme system would have to have such a specificity that it can oxidize these acids to 2-oxopalmitic, 4-oxostearic, or 6-oxoeicosanoic acid respectively. Such a possibility appears unlikely. Our observation that the 4-ketostearic acid is a poor precursor of the ketone suggests that the carbonyl group is introduced into a prebuilt chain of carbon atoms.

Admittedly the hypothesis of enzymic oxidation of the hydrocarbon (C_{29}) or a long-chain acid (e.g. C_{28} or C_{30}) has not yet been rigidly proved, but it has certainly become the best working hypothesis. Bio-oxidation of aliphatic chains is commonly terminal and position-15 becomes virtually terminal if the chain is predicated to be folded at the centre (elongated U). It may be noted in parenthesis that a double fold (~) confers terminal character on position-10 (cf. Ginnol[6]), and this idea of a folded chain has many other

[5] P. E. KOLATTUKUDY, *Science* **159,** 498 (1968).
[6] S. FURUKAWA, *Sc. Pap. I.P.C.R.* **19,** 27 (1932).

applications, one of which is the mid-chain dehydrogenation of stearic acid, with the formation of oleic acid.[7]

Macey and Barber[8] have, *inter alia*, found that the normal type of *Brassica oleracea*, which produces nonacosan-15-one, also forms a mixture of almost equal amounts of tetradecanoic and pentadecanoic acids. In our view this intriguing result plainly indicates that the plant oxidizes the ketone to the normal products, the aforesaid mixture of acids.

EXPERIMENTAL

Methods. Details about the preparation of the plant material and substrate solutions, and the procedures for the isolation, identification, and measurement of radioactivity of the metabolic products have been previously described.[2,3]

Preparation of substrates. [4-^{14}C]4-Oxostearic acid, m.p. 97·5–99·5° (sp. act. 1·4 mc/m-mole) was obtained in 54% chemical yield by condensation of [1-^{14}C]pentadecanoyl chloride (prepared from the corresponding acid, sp. act. 1·5 mc/m-mole, supplied by the Radiochemical Centre, Amersham, England) with ethyl sodioacetosuccinate in ethereal solution, and subsequent hydrolytic fission of the resulting crude ethyl 3-acetyl-3-ethoxycarbonyl-[4-^{14}C]4-oxostearate by means of 1·3% aq. ethanolic KOH (H_2O–EtOH = 1:2; 18 hr at room temp., 1 hr under reflux). A quantity of the labelled pentadecanoic acid, equivalent to 37% of the input, was regenerated during hydrolysis. The above reactions were carried out in an inert atmosphere. This procedure is a modification of the G. M. Robinson method of synthesis of long-chain aliphatic keto-acids.[9]

[4-^{14}C]Stearic acid, m.p. 68–70° (sp. act. 1·3 mc/m-mole) was obtained by Huang–Minlon reduction of the above oxo-acid.

[4-^{14}C]Eicosanoic acid, m.p. 75–76° (sp. act. 0·89 mc/m-mole) was prepared in 79% chemical yield by the nitrile synthesis from 1-bromononadecane and K^{14}CN, using experimental conditions essentially as described by W. Stoffel.[10] The acidic material obtained in this manner was dissolved in *n*-hexane and chromatographed on silica gel (Bio-Sil A, 100–200 mesh). *n*-Hexane eluted some neutral material; [1-^{14}C]eicosanoic acid was eluted with hexane–ether (2:1), and crystallized from *n*-hexane.

The methyl esters of the labelled acids had the same retention time of (GLC) as standard specimens of the corresponding unlabelled esters.

[7] D. K. Bloomfield and K. Bloch, *J. Biol. Chem.* **235**, 337 (1960).
[8] M. Macey and H. N. Barber, *Nature, Lond.* **222**, 789 (1969).
[9] G. M. Robinson, *J. Chem. Soc.* 745 (1930).
[10] W. Stoffel, *Annalen* **673**, 26 (1964).