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BIOSYNTHESIS OF PARAFFINS IN *BRASSICA OLERACEA*: FATTY ACID ELONGATION-DECARBOXYLATION AS A PLAUSIBLE PATHWAY

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Abstract—Incorporation of fatty acids of chain length C_2 to C_{14} into the C_{16} acid of *Brassica oleracea* was not inhibited by trichloroacetate at concentrations sufficient to inhibit their incorporation into paraffins almost completely. The sensitivity to trichloroacetate of incorporation of fatty acids into paraffins remained nearly the same when the chain length of the precursor fatty acid was increased from C_2 to C_{16}, but incorporation of C_{18} acid into paraffin was much less sensitive to trichloroacetate. Carbons 1 and 2 of stearic acid were incorporated into nonacosane at the same rate as the entire C_{18} molecule, showing that C_{18} acid does not undergo degradation of its carbon chain before being incorporated into the paraffin. These results support the hypothesis that the C_{16} chain is elongated to a C_{30} unit which on decarboxylation gives rise to the paraffin. Light and 3-(4-chlorophenyl)-1,1-dimethylurea (CMU) affect incorporation of C_{18} into neither paraffins nor very long fatty acids; trichloroacetate inhibits C_{18} incorporation into paraffins and to a lesser degree into very long fatty acids, suggesting that the formation of such acids is related to paraffin synthesis. The time course of incorporation of C_{18} acid into very long fatty acids and paraffins failed to show a precursor-product relationship, and the very long fatty acids were found to be esterified in the phospholipids and triglycerides of the particulate structures. On the basis of present knowledge it is proposed that C_{16} acid becomes the substrate for an elongation-decarboxylation enzyme complex which is situated somewhere outside the chloroplasts. This enzyme complex elongates C_{16} to C_{30}, decarboxylates this C_{30} unit, and releases the paraffin from the complex. During the elongation process dissociation of small amounts of fatty acids occurs at each stage. These are rapidly estfieried into phospholipids and triglycerides.

INTRODUCTION

THE cuticular wax of plants invariably contains long chain paraffins of length C_{21} to C_{33} with C_{29} or C_{31} usually predominating.[1] These paraffins can be utilized by microorganisms[2] and to a limited extent by animals.[3] Little is known about the biosynthesis of paraffins in spite of the widespread occurrence of paraffins in nature.[4–6] Glucose carbon atoms are incorporated into paraffins in yeast,[7] acetate units into the *n*-heptane of *Pinus jeffreyi*,[8] the long chain paraffins of beeswax,[9] and also into the *n*-nonacosane and its derivatives, 15-nonacosanone and 15-nonacosanol of the cuticular wax of *Brassica oleracea*.[10] Degradation of the ketone indicated that the carbonyl carbon originated from the methyl carbon of acetate.[10] Palmitic acid was shown to be incorporated as a unit into the paraffin, and stearic acid was found to be converted into paraffins much more efficiently than palmitic acid.[11] These observations made

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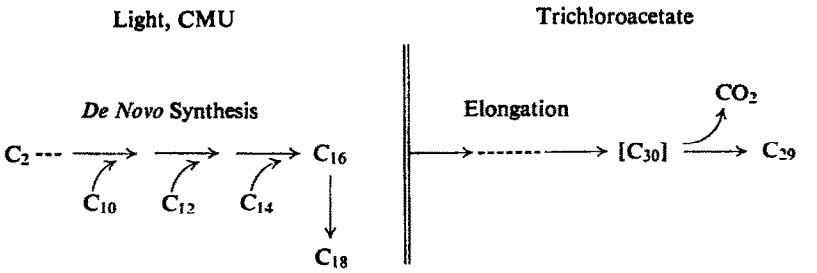
the previously suggested pathways[10, 13] for paraffin synthesis untenable and suggested the occurrence of an elongation-decarboxylation pathway[11] as shown in Scheme 1.



SCHEME 1.

Experiments on the effect of light, 3-(4-chlorophenyl)-1,1-dimethylurea (CMU) and trichloroacetate on the incorporation of precursors into paraffins and internal lipids suggested that the site of synthesis of paraffins is different from that of internal lipids. Medium to long chain fatty acids were incorporated into paraffins, the longer fatty acids being better precursors than the shorter ones. The longer fatty acids (such as C_{18}) were also apparently elongated into fatty acids of chain length C_{20}–C_{28}.[11] These observations raised the possibility that the very long fatty acids are intermediates of paraffin synthesis. This paper describes attempts to test the elongation-decarboxylation pathway and examines the possibility that the very long acids found in broccoli leaves may be intermediates of paraffin synthesis.

RESULTS AND DISCUSSION

Effect of Trichloroacetate on Incorporation of Shorter Fatty Acids into C_{16} and C_{18} Acids

Incorporation of acetate into internal lipids has been shown to be unaffected by trichloroacetate.[10] However under the same conditions incorporation of acetate into paraffins was severely inhibited by trichloroacetate. For this and other reasons it was proposed[11] that paraffin synthesis takes place at a site different from that of *de novo* synthesis of fatty acids which is known to take place in the chloroplasts.[12, 16] According to this hypothesis the incorporation of any fatty acid shorter than C_{16} into C_{16} and C_{18} acids takes place inside the chloroplasts, a site not affected by trichloroacetate (see Scheme 1), and therefore incorporation of fatty acids shorter than C_{16} and C_{18} should not be affected by trichloroacetate. In order to test this possibility, the effect of various concentrations of this inhibitor on the incorporation of C_2 to C_{16} fatty acids into the leaf fatty acids was studied, and the results are summarized in Tables 1 to 4. It is obvious that trichloroacetate at concentrations sufficient to inhibit paraffin synthesis almost completely (see Fig. 1) did not affect the formation of fatty acids of chain length up to C_{16}. Neither the radioactivity in the total lipids nor the percentage distribution was affected by trichloroacetate. These results thus clearly demonstrate that as predicted by the hypothesis the various fatty acids shorter than C_{16} are in fact incorporated into C_{16} and the unsaturated C_{18} fatty acids by means that are not affected by trichloroacetate.

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TABLE 1. EFFECT OF TRICHLOROACETATE ON [1-^{14}C]ACETATE INCORPORATION INTO LIPIDS

Concentration of trichloroacetate (μmoles/flask)	Incorporation of radioactivity into				
	Total lipids (% of administered)	Paraffin (% of administered)	Fatty acids (% distribution)		
			C_{16}	C_{18}	C_{18U}
0	36	1·7	44	13	43
5	35	0·5	41	9	50
10	39	0·4	40	11	49
20	37	0·2	51	12	37

2·2 g Chopped broccoli leaves were incubated under light with 5 μmoles sodium [1-^{14}C]acetate (10 μc) for 4 hr with appropriate amounts of sodium trichloroacetate in 6 ml of distilled water.

TABLE 2. EFFECT OF TRICHLOROACETATE ON [1-^{14}C]DECANOIC ACID INCORPORATION INTO FATTY ACIDS

Concentration of trichloroacetate (μmoles/flask)	Radioactivity in leaf fatty acids (% distribution)					
	C_{10}	C_{12}	C_{14}	C_{16}	C_{18U}	C_{18}
0	5·2	2·0	4·4	40	36·5	12
5	4·2	2·1	2·2	51	31	10
10	6·9	3·3	—	35	42	13
20	4·5	1·8	2·0	42	39	11

Experimental conditions were the same as under Table 1 but 2·5 μc [^{14}C]decanoic acid was used instead of acetate.

TABLE 3. EFFECT OF TRICHLOROACETATE ON [1-^{14}C]LAURIC ACID INCORPORATION INTO FATTY ACIDS

Concentration of trichloroacetate (μmoles/flask)	Radioactivity in leaf fatty acids				
	C_{12}	C_{14}	C_{16}	C_{18U}	C_{18}
0	33	10·6	28	18	7·7
5	29	7·2	34	21	6·3
10	34	11	34·5	15	4·6
20	41	9·5	29·4	15	4·6

Experimental conditions were the same as under Table 1 but 2·5 μc [1-^{14}C] lauric acid was used in the place of acetate.

TABLE 4. EFFECT OF TRICHLOROACETATE ON [1-^{14}C]MYRISTIC ACID INCORPORATION INTO FATTY ACIDS

Concentration of trichloroacetate (μmoles/flask)	Radioactivity in leaf fatty acids (% distribution)			
	C_{14}	C_{16}	C_{18U}	C_{18}
0	52	37	6	6
5	52	38	6·5	4
10	53	34	8	4
20	55	35	6·6	3·5

Experimental conditions were the same as under Table 1, but 2·5 μc [1-^{14}C]myristic acid was used instead of acetate.

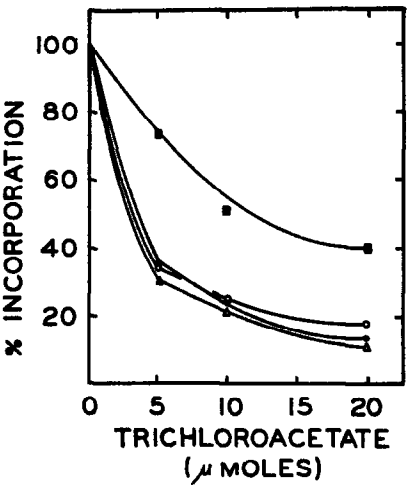


FIG. 1. ■ [U-^{14}C]STEARATE, ○ [1-^{14}C]ACETATE, ● [1-^{14}C]MYRISTATE, △ [U-^{14}C]PALMITATE. 2·2 G CHOPPED LEAVES WERE INCUBATED AT 30° FOR 4 HR WITH 2·5 μC OF THE APPROPRIATE SUBSTRATE IN A TOTAL VOLUME OF 6 ML. INCORPORATION IS PLOTTED AS PER CENT OF CONTROL WHICH DID NOT CONTAIN ANY TRICHLOROACETATE.

As the concentration of trichloroacetate is increased there is noticeable inhibition of incorporation of C_{12} and C_{14} into saturated C_{18} (Tables 3 and 4). Similar concentrations of trichloroacetate also inhibit incorporation of C_{16} into C_{18} (results not shown). These observations may be explained by assuming a dual site of synthesis of saturated C_{18} acids. It is known that C_{18} is synthesized in the chloroplasts[12, 16] and this synthesis would not be affected by trichloroacetate. However C_{18} synthesis is presumably also involved during wax synthesis, and this process being outside the chloroplasts would be affected by trichloroacetate. As a result the higher concentrations of trichloroacetate show some inhibition of incorporation of radioactivity into saturated C_{18}.

Effect of Trichloroacetate on Incorporation of Fatty Acids into Paraffin

According to the hypothesis shown in Scheme 1, in the presence of trichloroacetate any fatty acid shorter than C_{16} would proceed up to C_{16} uninhibited. The next sequence of

reactions, involving elongation of the C_{16} chain becomes limiting for paraffin synthesis. Therefore incorporation of fatty acids of chain length C_2 to C_{16} into paraffin should be equally sensitive to trichloroacetate. If C_{16} is the starting point for the elongation system responsible for paraffin synthesis, C_{18} would be the product of the first elongation step which is sensitive to trichloroacetate. Therefore exogenous C_{18} acid could enter the reaction sequence at a point which is beyond part of the trichloroacetate-sensitive steps involved in paraffin synthesis, and so incorporation of C_{18} acid into the paraffin may be less sensitive to trichloroacetate than that of C_{16} acid. Results of experiments to test these possibilities are summarized in Fig. 1. As predicted from the hypothesis, incorporations of C_2, C_{14} and C_{16} acids into the paraffin are equally sensitive to trichloroacetate and incorporation of C_{18} acid into the paraffin is much less sensitive to trichloroacetate. Sensitivity to trichloroacetate of incorporation of C_{10} and C_{12} into the paraffins was similar to that of C_{16} (data not shown). These results clearly support the pathway shown in Scheme 1 and suggest that C_{16} acid is the substrate for the elongation system responsible for paraffin synthesis. This is a reasonable suggestion since C_{16} seems to be the end product of *de novo* synthesis of fatty acids.[12]

Incorporation of the Carbon Chain of Stearic Acid into Paraffins

Stearic acid was found to be a much more efficient precursor of nonacosane than any of the shorter fatty acids.[11] Fatty acids isolated from leaves which had metabolized stearic acid always contained some radioactive palmitic acid. This observation raises the possibility that stearic acid is incorporated into paraffins after prior conversion into palmitic acid. However, one would then have to assume that the palmitic acid thus produced is somehow more easily available for incorporation into paraffins than the exogenous palmitic acid. This possibility had to be explored especially because the other pathways proposed for paraffin synthesis involve palmitic acid.[10, 13] Results with 1-, 2-, and U-^{14}C-stearate showed that radioactivity is incorporated equally into the paraffin fraction from C_{18} irrespective of the labeling position in the C_{18} acid. Examination of the product by GLC showed that the major radioactive paraffin was nonacosane in all cases. Since the incorporation time was only 30 min, during which the rate of incorporation is linear (as shown later in this paper), the results indicate that the rate of incorporation of carbons 1 and 2 is equal to that of the entire C_{18} molecule. Therefore the C_{18} molecule is incorporated as a unit into nonacosane as predicted by the hypothesis.

In all cases essentially the only radioactive product was nonacosane as shown by radio GLC.

Time-course of Incorporation of Fatty Acids into Paraffins

In previous experiments, no attempts were made to study the time course of C_{18} incorporation. In Fig. 2 are summarized results of such experiments which show that C_{18} incorporation was remarkably rapid, leveling off in less than 30 min. The early linear portion can be extended somewhat by increasing the concentration of substrates. These observations are true for other fatty acids as well, as is shown in Fig. 2. Furthermore the relationship between chain length and efficiency of incorporation into paraffins found previously is now demonstrated in more detail. These results eliminate the possibility that the differences in incorporation between C_{18} and other fatty acids shown previously[11] were a result of the much longer time period studied. It must be pointed out that the rate of uptake of fatty acid is obviously a complicating factor in the interpretation of these data. However, as indicated before[11] shorter fatty acids were taken up faster than longer fatty acids, so this factor probably does not affect the conclusions. Fatty acids of different chain lengths undergo different metabolic transformations; significant portions of the shorter ones such as C_{10} are incorporated into

unsaturated fatty acids, whereas longer ones such as C_{16} and C_{18} are not.[11,17] Results of experiments with whole tissues may be influenced by such complicating factors. Therefore such results may be taken as only tentative, even though in drawing conclusions attempts have been made to take into consideration such possible complicating factors.

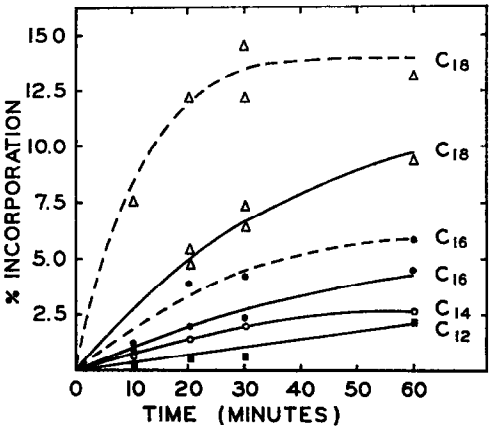


FIG. 2. BROKEN LINE REPRESENTS SUBSTRATE OF HIGH SPECIFIC ACTIVITY—27 MμMOLE OF SUBSTRATE PER FLASK. THE SOLID LINES REPRESENT SUBSTRATES OF LOWER SPECIFIC ACTIVITY—170 MμMOLES OF SUBSTRATE PER FLASK. IN ALL CASES TOTAL VOLUME WAS 6 ML AND INCUBATION WAS DONE AT 30° UNDER LIGHT.

Time-course of Incorporation of Stearic Acid into Lipids

The remarkably rapid rate of incorporation of stearate into wax, and the slowing down of the rate after 30 min raised a question about the limiting factor responsible for the leveling off. When the total fatty acid fraction of the leaf (after saponification) was examined after a 4 hr incubation with [^{14}C] stearic acid, the C_{18} contained about 75 per cent of the radioactivity of the fatty acids, and yet incorporation into paraffin leveled off in about 30 min. This observation suggested that either the C_{18} of the total lipids is mostly bound (esterified) and hence not free to become incorporated into paraffins, or that some factor other than the availability of substrate is limiting. The observation that free fatty acids are seldom found in plant cells in appreciable quantities, and that active esterification systems exist in plants makes it more likely that the availability of the substrate is limiting. To examine this possibility, a time course of stearate incorporation into various classes of lipids in the leaf was carried out by means of thin-layer chromatography as described in the experimental procedure, and the results are summarized in Fig. 3. The rapid incorporation of radioactivity into the wax is accompanied by a rapid disappearance of radioactivity in the free fatty acid fraction. Although only about 15 per cent of the radioactivity is incorporated into paraffins, more than 30 per cent of the ^{14}C is in the total wax fraction and, in the meantime, over 30 per cent of the fatty acid is esterified into triglycerides and phospholipids. Thus it seems that unavailability of the substrate is the main reason for the slowing of the incorporation after 30 min. This conclusion is consistent with the observation that with increasing amounts of the substrate the linear portion of incorporation can be extended beyond 30 min (see Fig. 2). The effect of concentration on the rate of incorporation is further illustrated in Table 5. The experimental period

[17] A. T. JAMES, *Biochem. Biophys. Acta* **70**, 9 (1963).

was kept short (30 min) so that the rate of incorporation of externally administered stearate could be measured. As the concentration of exogenous stearate increased, the amount incorporated into paraffin increased and reached a maximum of almost 20 mμmoles per g per hr at a concentration of $4{\cdot}3 \times 10^{-5}$ M stearate.

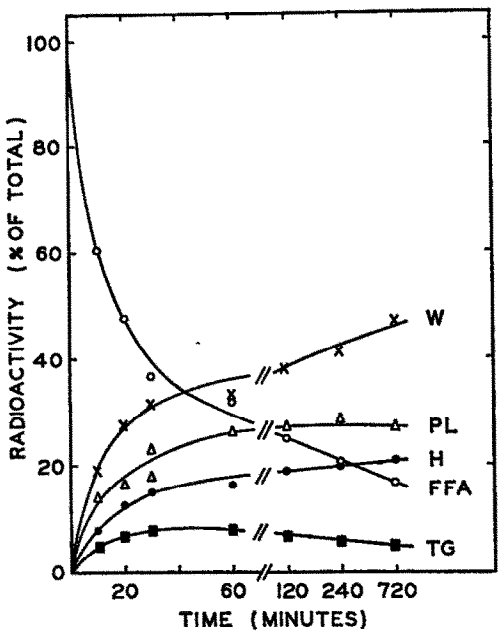


FIG. 3. W—WAX, PL—POLAR LIPID, H—HYDROCARBONS, FFA—FREE FATTY ACIDS, TG—TRIGLYCERIDES. WAX FRACTION REFERS TO HYDROCARBONS, ESTERS, KETONES AND UNKNOWN FRACTIONS OF BENZENE SYSTEM OF THIN-LAYER CHROMATOGRAPHY[10] AND DOES NOT INCLUDE SECONDARY ALCOHOL AND PRIMARY ALCOHOL FRACTIONS. THIS FRACTIONATION WAS DONE WITH THIN-LAYER CHROMATOGRAPHY WITH HEXANE, ETHYL ETHER, FORMIC ACID (40:10:1) AND THE ASSAYS FOR HYDROCARBON WERE DONE BY COLUMN CHROMATOGRAPHY.

TABLE 5. EFFECT OF CONCENTRATION OF STEARATE ON ITS RATE OF INCORPORATION INTO PARAFFIN

Concentration of stearate ($M \times 10^{-5}$)	Rate of incorporation (μmoles g^{-1}, hr^{-1})
0·4	4·3
2·3	12·8
4·3	18·4
6·2	19·8

2·2 g Chopped broccoli leaves were incubated at 30° under light for 30 min with appropriate amounts of [$U^{14}C$]stearate in a total volume of 6·5 ml, which contained 0·05 M phosphate buffer, pH 6·8.

Effect of Light and CMU on Incorporation of Stearic Acid into Very Long Fatty Acids

I have previously demonstrated that fatty acids of intermediate chain length are elongated in broccoli leaves into very long fatty acids up to at least C_{26}. The longer percursors such as C_{18} were converted more efficiently into very long fatty acids, and also they were incorporated into paraffin more efficiently than shorter ones. These observations, together with the evidence that paraffins are also produced by an elongation process, made it necessary to find out whether the very long fatty acids detected in these tissues are in fact precursors of paraffins. For this purpose the effects of light, of CMU and of trichloroacetate on stearate elongation were studied. The results shown in Table 6 demonstrate that incorporation of stearate into very long fatty acids does not require light, just as paraffin synthesis is insensitive to light,[11] whereas it has been clearly demonstrated that the *de novo* synthesis of intermediate chain length fatty acids is stimulated by light. CMU at a concentration sufficient to inhibit acetate incorporation into fatty acids almost completely did not affect incorporation of stearate into very long fatty acids just as CMU did not affect paraffin synthesis from stearate.[11] These similarities between stearate incorporation into paraffins and very long fatty acids suggest that these acids are biosynthetically related to paraffins.

TABLE 6. EFFECT OF LIGHT AND CMU ON THE INCORPORATION OF STEARIC ACID INTO VERY LONG FATTY ACIDS

Experimental condition	Incorporation into: Very long fatty acids (% of radioactivity recovered in fatty acids)				Incorporation into: Paraffins (% of administered)
	C_{20}	C_{22}	C_{24}	C_{26}	
Light	7·7	2·6	4·9	3·9	13·1
Dark	8·4	2·8	3·5	3·8	12·5
CMU $8 \cdot 10^{-5}$ M and light	8·1	2·2	5·1	4·0	13·4

2·2 g Chopped young broccoli leaves were incubated for 2 hr with 2·5 μc stearic acid (27 mμmoles) in a total volume of 6 ml. Other details are described under Experimental section.

Effect of Trichloroacetate on Incorporation of Stearic Acid into Very Long Fatty Acids

As shown in Scheme 1, trichloroacetate inhibits the incorporation of fatty acids into the paraffins by inhibiting the elongation steps. If the formation of the very long fatty acids detected in this tissue is related to the paraffin synthesis, trichloroacetate would be expected to inhibit elongation of stearate into these very long fatty acids. Results shown in Table 7 demonstrate that the concentration of trichloroacetate required to inhibit the incorporation of stearic acid into very long fatty acids is much higher than that necessary to inhibit paraffin synthesis (see Fig. 1). Furthermore C_{18} incorporation into longer fatty acids such as C_{24} is more easily inhibited than into shorter ones such as C_{20}. In fact the concentrations of trichloroacetate used in these experiments, although sufficient to inhibit paraffin synthesis almost completely, hardly inhibited formation of C_{20} fatty acids. As indicated by the rapid transformations of administered C_{18} acid (see Fig. 3), these experiments may be considered as "pulse" labeling. Then if the sequential conversion, two carbon atoms at a time from C_{18} to C_{30} acids, occurs followed by decarboxylation to C_{29} paraffin, each step being sensitive to

trichloroacetate, the incorporation of ^{14}C into later intermediates and into the final product (paraffin) would be expected to be more severely inhibited by trichloroacetate than it is into the earlier intermediates such as C_{20} acid. The results in Table 7 and Fig. 1 show that this is the case and are therefore consistent with the elongation pathway as shown in Scheme 1. However it is possible that the very long fatty acids are related to the non-C_{29} compounds of the wax because both the synthesis of non-C_{29} compounds[10] and the formation of very long fatty acids are less sensitive to trichloroacetate. Still another possibility would be an unknown pathway for incorporation of C_{18} acid into the paraffin independent of the very long fatty acids, provided that such a pathway were more sensitive to trichloroacetate than the elongation of C_{18} acid into very long fatty acids. Attempts to determine whether very long chain fatty acids give rise to paraffins were made by administering C_{28} and C_{30} fatty acids but without success. These acids were not taken up when given to leaf discs or chopped leaves.

TABLE 7. EFFECT OF TRICHLOROACETATE ON THE INCORPORATION OF STEARATE INTO VERY LONG FATTY ACIDS

Concentration of trichloroacetate (μmoles/flask)	Incorporation (% of radioactivity in recovered fatty acids)		
	C_{20}	C_{22}	C_{24}
0	7	5	6
2·5	10	5	10
5·0	8	3	7
10	8·5	3	5
20	8	4	3
40	8	2	2

2·2 g Chopped young broccoli leaves were incubated under light for 4 hr with the appropriate amounts of sodium trichloroacetate and 2·5 μc (27 mμmoles) [U-^{14}C]stearic acid.

TABLE 8. TIME COURSE OF STEARIC INCORPORATION INTO VERY LONG FATTY ACIDS AND PARAFFINS

Time (min)	Incorporation into: Very long fatty acids (% of radioactivity recovered in fatty acids)				Paraffins (% of administered)
	C_{20}	C_{22}	C_{24}	C_{26}	
10	6·2	—	—	—	7·6
20	5·8	0·7	1·3	1·9	12·1
30	7·3	1·6	1·4	1·9	14·4
60	8·8	2·1	3·2	3·5	15·6
120	7·3	2·4	3·9	3·2	18·7
240	8·4	2·4	4·7	3·9	19·5
360	9·5	2·8	5·1	4·0	20·5

2·2 g Chopped broccoli leaves were incubated under light for the specified period of time with 2·5 μc (27 mμmoles) [U-^{14}C]stearic acid in 6 ml water. At the end of the experimental period reaction was stopped by pouring 200 ml of a mixture (2:1) of $CHCl_3$–CH_3OH into the tissue suspension.

Time-course of Incorporation of Stearic Acid into Very Long Fatty Acids

The possibility that the very long fatty acids are intermediates in paraffin biosynthesis was further tested by studying the time-course of stearate incorporation into them and comparing

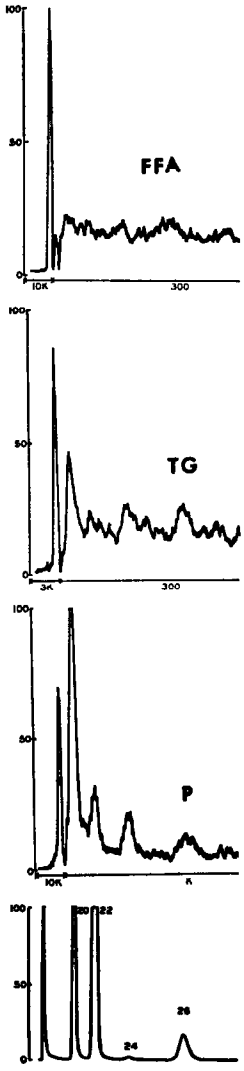


FIG. 4. FFA—FREE FATTY ACID, TG—TRIGLYCERIDES, P—POLAR LIPIDS (PRESUMABLY PHOSPHOLIPIDS); THE TRACING IN THE BOTTOM SHOWS THE FLAME-IONIZATION DETECTOR RESPONSE OF KNOWN FATTY ACIDS. NOTE THE DIFFERENCE IN ATTENUATION OF THE RADIOACTIVITY TRACING; THE FREE FATTY ACID FRACTION DOES NOT SHOW THE PRESENCE OF VERY LONG FATTY ACIDS EVEN WHEN THE QUANTITY INJECTED WAS GREATLY INCREASED AND THE SENSITIVITY OF RADIOACTIVITY DETECTOR WAS KEPT AT A MAXIMUM DURING THE ELUTION OF THE VERY LONG FATTY ACIDS.

it to that of paraffin synthesis to see whether a precursor-product relationship could be demonstrated. The results are summarized in Table 8. If these very long fatty acids were intermediates one would expect their radioactivity to decrease before paraffin synthesis

levels off. However the radioactivity in very long fatty acids was hardly measurable during the most rapid incorporation of ^{14}C into paraffins and the ^{14}C in very long fatty acids kept on increasing parallel to ^{14}C incorporation into paraffin. Even several hours after the incorporation of radioactivity into the paraffin leveled off the radioactivity in the very long fatty acids did not decrease. These data suggest that the very long fatty acids examined here are not intermediates of paraffin synthesis although they are probably derived from the elongation complex. This is perhaps not surprising because the existence of free intermediates would make the system thermodynamically very inefficient.

Location and Chemical Form of Very Long Fatty Acids

The very long fatty acids detected in the total lipids have been previously shown not to occur in the surface wax but are located within the leaf.[11] However it has not been established whether these very long fatty acids are free or esterified, and this information would be of interest especially in view of the possibility that they could be intermediates in paraffin biosynthesis. Therefore total lipids extracted from broccoli leaves which had metabolized [U-^{14}C]stearic acid were fractionated by thin-layer chromatography. The wax fraction, triglycerides, free fatty acids, and phospholipids were separately analyzed for very long fatty acids and the results are shown in Fig. 4. The major portion of the radioactivity incorporated into the internal lipids was in the more polar fractions (see Fig. 3 also), and this fraction contained most of the radioactive very long fatty acids. However the triglyceride fraction also contained a moderate proportion whereas the free fatty acid fraction contained none. The very long fatty acids in barley seedlings have been reported to be associated with neutral lipids rather than phospholipids.[14] The wax-ester fraction did not contain enough ^{14}C for proper analysis. This observation that the very long fatty acids are found esterified into phospholipids and triglycerides is consistent with the results of the time-course studies which failed to show a precursor-product relationship between very long fatty acids and paraffins.

After having established that the very long fatty acids are present in the leaf only in the esterified form, an attempt was made to determine whether they are associated with a particular organelle in the leaf. After incubating 5 g of chopped broccoli leaves for 2 hr with 5 μc [U-^{14}C]stearic acid, the leaf fragments were homogenized in 0·1 M phosphate buffer at pH 7 containing 0·4 M sucrose. The debris sedimented at 500 *g* (which undoubtedly contained small leaf fragments), the particulate fraction sedimented at 12,000 *g* and the microsomal fraction sedimented at 105,000 *g* all contained radioactive very long fatty acids, but the final supernatant fluid did not. These results are similar to those of Hawke and Stumpf.[14] However a chloroplast fraction sedimented at 1000 *g* was relatively free from very long fatty acids. Although in such experiments cross contamination cannot be ruled out, these results are consistent with the contention that very long fatty acids are synthesized outside the chloroplast.

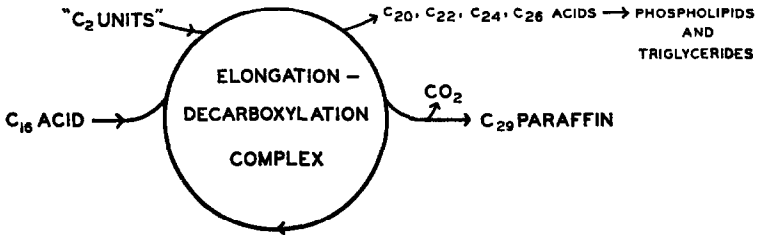
CONCLUSIONS

All the results thus far obtained make the other pathways already suggested for C_{29} biosynthesis untenable and strongly support the elongation-decarboxylation pathway.[11] According to this hypothesis (see Scheme 2) palmitic acid, which is the product of *de novo* fatty acid synthesis, becomes the substrate for an elongation-decarboxylation complex. This enzyme complex presumably elongates it to C_{30} which is then decarboxylated to the C_{29} compound and finally released. The elongation system will have to add 7 C_2 units; such successive addition of C_2 units could be very efficiently done with an enzyme complex somewhat like the fatty acid synthetase.[18] In addition to the data presented here and elsewhere[10, 11]

[18] F. LYNEM, *Fed. Proc.* **20**, 941 (1962).

the following observations also support this pathway. Just as in the case of fatty acid synthesis, no free intermediates have been detected during paraffin synthesis. Even when radioactive very long fatty acids were formed from [U-^{14}C]stearic acid, no C_{30} could be detected. Externally administered tritiated C_{28} and C_{30} acids were not converted into paraffins, and this failure could not be explained entirely by the slowness of uptake. The mechanistic problems involved in the decarboxylation of a saturated fatty acid may be resolved by assuming an enzyme-bound intermediate which would be more easily decarboxylated than a saturated C_{30} acid. It is possible for example to imagine an enzyme-bound β-keto C_{30} acid which could undergo decarboxylation and reduction to give the C_{29} compound.

The time course of C_{18} incorporation into very long fatty acids and the effect of CMU, light and trichloroacetate on the incorporation of C_{18} into paraffins and very long fatty acids suggest that these acids are biosynthetically related to the paraffins, but they do not show precursor-product relationship. The simplest explanation seems to be that the very long fatty acids are derived from the same (or similar) elongation complex which synthesizes the paraffins (see Scheme 2). Minor amounts of fatty acids are released from the enzyme complex at successive stages and these are rapidly esterified into the tissue lipids, primarily phospholipids, where such very long fatty acids are found. This process makes the dissociation



SCHEME 2.

irreversible, and therefore these acids are not available for incorporation into the paraffins. Consequently the radioactivity incorporated into such very long fatty acids does not decrease even hours after the incorporation of ^{14}C into the paraffin has leveled off. This hypothesis explains not only why a typical precursor-product relationship between the very long fatty acids and paraffins was not observed, but also why the very long fatty acids do not show a precursor-product relationship among themselves; instead the proportion of radioactivity in each acid remains rather independent of time.

The proposed elongation decarboxylation complex is probably located somewhere outside the chloroplasts. The observation that light and CMU affect only fatty acid synthesis and not the paraffin synthesis can be easily explained by a difference in site of synthesis. It is well known that the chloroplast is the site of *de novo* synthesis of fatty acids and therefore it is suggested that wax synthesis takes place elsewhere in the cell. Such a proposal also explains why trichloroacetate strongly inhibits the C_{29} synthesis without affecting *de novo* fatty acid synthesis, if one only assumes that chloroplasts are impermeable to trichloroacetate. From what is known about the permeability of chloroplast membranes this assumption seems to be reasonable. In support of the present hypothesis is the observation that the chloroplast fraction was free from radioactive very long fatty acids. Admittedly the hypothesis proposed here is speculative, but it explains the results thus far obtained and serves as a working hypothesis for further investigation in progress in this laboratory.

EXPERIMENTAL

Plants. Young leaves from brococli plants grown in the greenhouse were used as described previously.[11]

Substrates and Inhibitors. [U-^{14}C]Palmitic acid (93 mc/mmole), [U-^{14}C]stearic acid (93 mc/mmole), [1-^{14}C]stearic acid (37·1 mc/mmole), [2-^{14}C]stearic acid (14·2 mc/mmole), [1-^{14}C]myristic acid (15·4 mc/mmole), [1-^{14}C]lauric acid (21·0 mc/mmole), and [1-^{14}C]acetate were purchased from Nuclear-Chicago Corporation. Substrates and inhibitors were administered as described before.[11] Whenever non-radioactive fatty acids were used, they were dissolved with the aid of Tween 20 as were radioactive substrates, and all samples in the same experiment contained the same amounts of Tween 20 or phosphate in order to avoid effects caused by variations in concentration.

Incubation. Chopped leaves were used in all experiments, and incubation with or without inhibitors was done as described previously.[11] At the end of the incubation period the reaction mixture was transferred into 200 ml of a 2:1 mixture of chloroform and methanol, shaken well and allowed to remain for a few hours to permit complete extraction of the lipid components. After filtering the leaf residue, the total lipids were isolated as described earlier.[11] An aliquot of the lipid solution was analyzed by column chromatography for paraffins. The paraffin fraction was further analyzed by gas–liquid chromatography. Another portion of the total lipid solution was analyzed for various classes of lipids by thin-layer chromatography as described below. Still another portion was saponified, fatty acids were extracted, and the methyl esters of the fatty acids prepared as already described.[11]

Chromatography. Column chromatography for isolating paraffins was done as described before.[11] However, silica gel prepared as described previously,[11] SilicAR cc-4 100–200 mesh (Mallinckrodt Chemical Works, St. Louis, Mo.), and silica gel, 140–200 mesh (Analabs, Hamden, Conn.) were all used from time to time. These materials all gave the same results as long as they were first activated overnight at 110°.

Thin-layer chromatography on silica gel G for the purification of methyl esters was done on 0·5 mm thick layers with benzene as the solvent as described before.[11] Total lipids were separated into various classes by means of thin-layer chromatography on 0·3 mm thick layers of silica gel G with hexane:ethyl ether:formic acid (40:10:1) as the developing solvent. With this solvent system the major wax components paraffins, esters, ketones, and the less polar unknown compound of the benzene system[10] appeared near the solvent front in the same order as with the benzene system, and these components were taken together for radioactivity measurements and are referred to as the wax fraction. Just behind the unknown were, in the order of decreasing R_f values, the triglycerides, secondary alcohols, free fatty acids, primary alcohols and the polar lipids (presumably phospholipids) which did not migrate from the origin. The same thin-layer method was used with a 0·5 mm thick layer of silica gel G to isolate classes of lipids in order to detect the occurrence of radioactivity in the very long fatty acids in each class.

After locating the lipids on the analytical thin-layer plates with 2,7-dichlorofluorescein, the radioactivity in each fraction was determined directly on the silica gel as described before;[10] autoradiograms were also prepared from these plates on medical X-ray film to confirm the location of the radioactivity on the thin-layer plate. Mono, di-, and tripalmitins, stearic acid, methyl palmitate, cholesteryl palmitate, 15-nonacosanone, 15-nonacosanol, stearyl alcohol, and nonacosane were used as standards.

Gas–liquid chromatography was carried out on a Perkin–Elmer gas chromatograph equipped with a flame-ionization detector and effluent splitter. The paraffins were separated on 5% silicone gum rubber (SE30) and methyl esters of fatty acids on 12% Apiezon L, 15% diethylene glycol succinate and 5% silicone gum rubber columns. Authentic samples were always used as standards and, when trace components such as very long fatty acids contained significant radioactivity, the sample was rechromatographed after being mixed with non-radioactive standards. The identification of the very long fatty acids was based only on retention time on the three columns.

Determination of radioactivity. Radioactivity in samples of lipid solutions, thin-layer chromatograms and gas chromatographic fractions was determined as described previously;[10,11] duplicate or triplicate analyses were always made. Internal standards of [^{14}C]toluene were always used and corrections for quenching applied; in fractions containing pigments, such as the polar lipids fraction or total lipids, quenching was as high as 60–70 per cent. Counting was done with a standard deviation not greater than 3 per cent and with an efficiency of about 60 per cent in colorless samples.

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