Biosynthesis of Wax in *Brassica oleracea**

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ABSTRACT: The waxes deposited on the surface of leaves of several varieties of *Brassica oleracea* (cabbage, broccoli, and cauliflower) were found to be similar in chemical composition. Leaves that were rapidly growing assimilated [^{14}C]acetate into the wax most rapidly. Both carbons of acetate were incorporated into the various components of the wax at equal rates. Chemical degradation of the 15-nonacosanone of the surface wax showed that the carbonyl carbon originated predominantly from the methyl carbon of acetate and not from the carboxyl carbon. Other short-chain fatty acids such as pentanoate and hexanoate contributed carbon to the wax almost as efficiently as acetate. Trichloroacetate effectively inhibited the synthesis of wax from acetate, $1-2 \times 10^{-5}$ M being required for 50% inhibition. The wax components most severely inhibited were the paraffins, ketones, and secondary alcohols. The inhibitor produced much smaller effects on the synthesis of fatty acids, primary alcohols, and esters. Comparison of the incorporation of acetate in the presence of trichloroacetate into the paraffins within the leaf and those on the surface suggested that the effect is exerted mainly upon the biosynthesis rather than upon the excretion of wax from the leaf interior.

Trichloroacetate, at a concentration several times higher than that required to inhibit wax synthesis, did not inhibit synthesis of internal fatty acids. Accordingly either the site of synthesis of surface wax or its biosynthetic pathway is different from that of the internal lipids. Measurements of the specific activities of the wax components at different times of acetate incorporation suggest that the esters and the C_{29} compounds may be synthesized at different sites in the leaf.

The cuticle of plant leaves is believed to consist of a network of cross-esterified hydroxy fatty acids, cutin, embedded in wax[1] (Martin, 1964; SilvaFernandes *et al.*, 1964). Wax is also found on the surface of leaves of several species in the form of small particles often with characteristic shapes (Juniper, 1959). The cuticular wax is generally found to be a mixture of long-chain paraffins, esters, ketones, alcohols, and acids. Other types of compounds such as flavones and triterpenes are also present in the surface wax of certain plants (Horn *et al.*, 1964). Although the physical and chemical structure of the plant cuticle has been studied extensively, little is known about the biosynthesis of its components.

Analysis of the surface wax of cabbage leaves (*Brassica oleracea*) revealed that about 65% consists of seven straight-chain aliphatic compounds, all containing 29 carbon atoms (Purdy and Truter, 1963a,b,c). The rest consists of fatty acids of chain length C_{12} to C_{24}, primary alcohols (C_{12} to C_{28}), and esters containing acids and alcohols of similar chain length as those found in free alcohols and acids. Among the components are nonacosane and 15-nonacosanone, both of which had been isolated 35 years ago by Chibnall and co-workers from cabbage (Channon and Chibnall, 1929). Because of the structural similarity of the paraffin and the ketone they were assumed to be interrelated metabolically. Chibnall also suggested that a condensation of 2 molecules of pentadecanoic acid followed by a decarboxylation could give rise to 15-nonacosanone, which in turn could be reduced to the corresponding C_{29} paraffin. On the other hand, Purdy and Truter (1963c) suggested that 10,15-dioxononacosane is the precursor of all C_{29} compounds of the wax. However no direct experimental approach to the problem of biosynthesis of plant wax has previously been reported save for an attempt with apple fruit in which [1-^{14}C]-acetate failed to become incorporated into the paraffins (Mazliak, 1964). In this paper experiments relating to the problem of the biosynthesis of the cuticular wax of *Brassica oleracea*, with use of ^{14}C-labeled precursors, are reported.

Experimental Procedure

Plants. Cabbage and broccoli (*Brassica oleracea*) seeds were germinated in the greenhouse and were transplanted after 2 weeks into soil in pots resting in sand on a subirrigated bench. The seedlings were grown for about 4 to 6 weeks, and leaves were then removed as required until the plants began to form heads or to blossom.

Incorporation of Substrates. Excised leaves (about 8 g fresh weight) were kept in the dark with the petioles immersed in water for 1 or 2 hours, during which

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[1] Although the term "wax" in its strict chemical meaning is defined as esters of long-chain alcohols with long chain acids, the term has also been widely used in an operational sense which also includes a variety of other long-chain compounds. The term wax is used in this paper in this broader meaning. The references cited give further details about the various compounds that are usually included in the wax fraction.

time they became fully turgid. Radioactive acetate, pentanoate, and hexanoate, as their sodium salts, were dissolved in water and placed in a small polyethylene cup fixed at the center of a beaker. The petioles were immersed in the solution containing 10 μmoles of the substrate and placed under tungsten lights that provided approximately 2000 foot-candles. As the leaves absorbed the radioactive solution, more water was added. Usually within 5 minutes the tip of the leaf became radioactive. After about 30 minutes in the light the leaves showed some wilting. This was normally overcome by a short (30 minute) dark period. In longer experiments, the leaves were kept in the light at 600 to 800 foot-candles.

At the end of the culture period, the leaves (but not the cut surface) were dipped in 300 to 400 ml of chloroform for 30 seconds. Of all the radioactivity that can be washed into chloroform by immersing the leaf for a minute, about 99% was released in the first 10 seconds; after that little further radioactivity is released even after 1.5 minutes. The chloroform solution was washed with acidified water and evaporated to dryness under reduced pressure. The wax was dissolved in benzene or chloroform and the radioactivity was determined.

Trichloroacetate Experiments. Sodium trichloroacetate at the desired concentration was added to the solution containing 20 μmoles of radioactive acetate before the petiole of the leaf was introduced. After 4 hours of metabolism in the light, the wax was isolated as before and separated into 8 fractions by thin-layer chromatography. The spots were carefully scraped off and the radioactivity in each was determined.

To obtain the lipids other than those on the surface, that is the internal lipids, the leaf was dried overnight in an oven at 100° immediately after removal of the surface wax. The dried leaf was crushed and the lipids were extracted with ethyl ether for 24 hours in a soxhlet apparatus. The paraffins were then isolated by thin-layer chromatography.

Gas Chromatography. Gas chromatography was performed with a Perkin-Elmer Model 810 instrument with a flame ionization detector. Coiled copper columns (0.62 cm od) were always used. The methyl esters were analyzed on a 6-ft column with either 3% silicone rubber gum (SE-30) on siliconized 80–100 mesh Chromosorb W as the solid support or 15% stabilized diethylene glycol succinate on 60–80 mesh Anakrome as the packing material. Aliphatic amines were separated on a 6-ft 4% Versamid 900 column. The solid support, 80–100 mesh siliconized Chromosorb W, was first coated with 1.5% sodium hydroxide in methanol before being coated with the Versamid. Hydrocarbons were analyzed either on a 6-ft 2% SE-30 column with 80–100 mesh siliconized Chromosorb W as the solid support or on a 4-ft 4% SE-30 column with Chromosorb P as the solid support. The details of the conditions used for the gas chromatographic separations are shown with the diagrams. For identification purposes, samples of known pure compounds were always used.

Thin-Layer Chromatography. Silica gel G (30 g) was made into a suspension with 60 ml water; glass plates, 20 × 20 cm, were coated to obtain a layer 250 μ thick. The gel was activated by heating the plates at 100° overnight, and these were stored in a desiccator until used. Samples of wax were applied as spots or bands, depending on the amount to be analyzed. The plates were developed in benzene for about 1 hour, during which time the solvent front reached the top of the plate. The spots were located by spraying the plate with 50% sulfuric acid and heating it at 180° for 30 min, or by the dextrin-iodine method (Mangold *et al.*, 1955). Internal lipids extracted from dried leaves could not be fractionated satisfactorily by the simple thin-layer chromatographic procedure described. However, development with hexane gave a pure paraffin fraction which could be isolated, the pigments being slower moving.

Column Chromatography. Alumina (Brockman activity I) and silica gel columns were used to separate the wax into fractions containing mixtures of certain components. Whenever paraffins alone were to be isolated, the crude wax was applied to an alumina column and the paraffins were eluted with *n*-hexane. The optimal ratio of alumina to wax required for the quantitative recovery of the paraffins, without contamination by other components, was determined in the following manner: Crude wax was mixed with a tracer amount of [16,17-^{14}C]dotriacontane and was placed on an alumina column. The hexane eluent was analyzed for functional groups by infrared spectroscopy and thin-layer chromatography, and the radioactivity in the eluent was also determined. The maximal load was thus found to be 100 mg of wax/10 g of alumina packed in a column 1.0 cm in diameter and 18 cm in height, and the recovery was found to be 95–100%. In actual practice a severalfold excess of alumina was always used. After eluting the paraffins with hexane, a mixture of the ketones and esters, sometimes with a little pigment, was obtained by elution with benzene. The ketones could be purified by rechromatographing the benzene eluent after saponification of the contaminating esters with alcoholic KOH.

Silica-Gel Column. The crude wax in a solvent was adsorbed on a small amount of gel and placed on a silica gel column 1.4 cm in diameter and 28 cm in height, and the column was developed with a rectilinear gradient elution technique (Bock and Ling, 1954). The mixing chamber contained 1000 ml of hexane and the reservoir 750 ml of a mixture of chloroform and benzene (6.5:1). The fractions eluted were detected by assaying the effluent for ^{14}C. Silica gel used for column chromatography was prepared from Mallinckrodt's silicic acid according to the method of Bulen *et al.* (1952).

Identification of Components. Thin-layer chromatography on silica gel G plates with known standards, comparison of the relative mobility of the components with that reported by Purdy and Truter (1963a), and the infrared spectra were used to identify the components.

Determination of Radioactivity. Aqueous and non-aqueous samples were mixed with 10 ml of a mixture

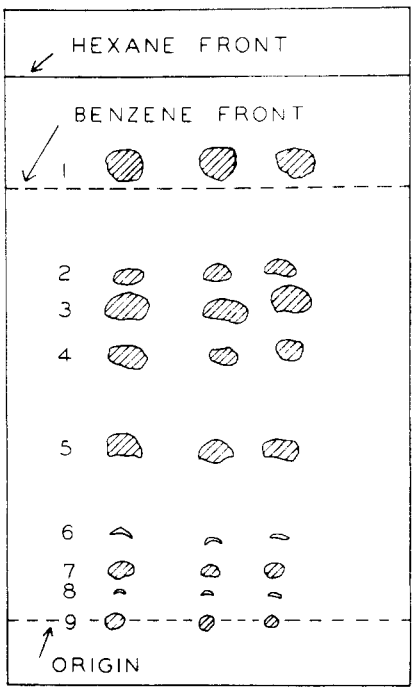

FIGURE 1: Thin-layer chromatography of leaf surface waxes of *Brassica oleracea* on silica gel G. Left to right: cabbage, broccoli, and cauliflower; (1) paraffins; (2) esters; (3) ketones; (4) unknown; (5) secondary alcohols; (6) unknown (probably ketols); (7) primary alcohols; (8) unknown; (9) fatty acids. Spots were located by spraying the plate with 50% sulfuric acid and heating it at 170° for 30 minutes. Identification by known samples of *n*-dotriacontane, 15-nonacosanone, 15-nonacosanol, and stearyl alcohol. The esters absorbed at 1735 cm^{-1} while the ketones absorbed at 1700 cm^{-1}.

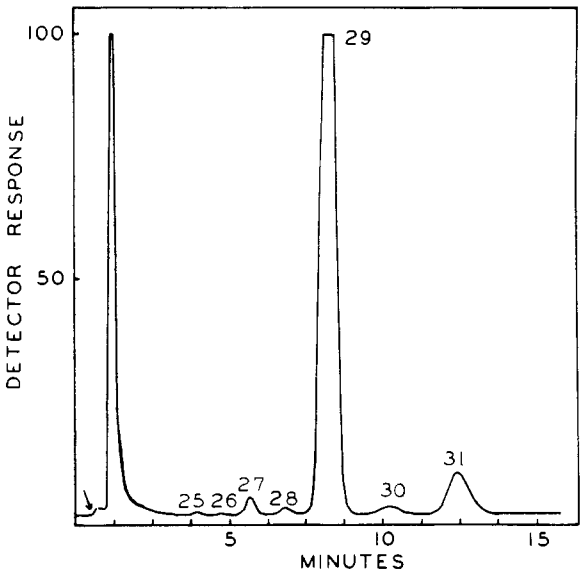

FIGURE 2: Gas-liquid chromatography of the paraffin fraction isolated from surface wax of cabbage leaves. Experimental conditions: 6 ft coiled copper column (0.25 in. od), 3% SE-30 on 80–100 mesh siliconized Chromosorb W; temperatures of column, injector, and detector 280, 360, and 310°, respectively; carrier gas He at 45 ml/min. Identification by authentic samples of C_{26}, C_{28}, C_{30}, and C_{32}. The number of eack peak represents the chain lengths.

of toluene and ethanol (2:1, v/v) containing 6 g of 2,5-diphenyloxazole and 100 mg *p*-bis[2-(5-phenyloxazolyl)]benzene/liter, and the radioactivity was determined in a Nuclear-Chicago liquid scintillation spectrometer. Spots from thin-layer chromatograms were carefully scraped into the counting vial and dispersed in the scintillation mixture with a mechanical shaker and were then assayed for ^{14}C directly. This method was found to be satisfactory, as 90–95% of the radioactivity applied at the origin could be recovered in the areas of the spots. Internal standards were always used to determine the efficiency of counting, which was usually 50–60%. Counting was always done with a standard deviation of <1%.

Materials. [1-^{14}C]Acetate, [2-^{14}C]acetate, [1-^{14}C]-pentanoate, and [1-^{14}C]hexanoate were purchased as their sodium salts from Volk Radiochemical Co. [16,17-^{14}C]Dotriacontane was bought from Calbiochem, Los Angeles, Calif. The *n*-hydrocarbons used as standards in gas chromatography were obtained as a gift from Dr. B. J. Humphrey, Humphrey Chemical Co., North Haven, Conn. Fatty acid ester standards were obtained as a gift from T. R. Lynn, Analabs, Hamden, Conn. The liquid phases and solid supports used for gas chromatography were purchased from Analabs. The 15-nonacosanone was prepared by dry distillation of barium pentadecanoate under reduced pressure, and 15-nonacosanol was obtained by reducing 15-nonacosanone with lithium aluminum hydride.

Degradations. The ketone (mp 80–81°), isolated by one of the methods described above, was converted to the oxime by refluxing it with hydroxylamine hydrochloride in alcohol-pyridine mixture (1:1). The oxime (mp 51°) was converted to the substituted amide by Beckmann rearrangement carried out in glacial acetic acid with concentrated sulfuric acid as the catalyst according to the method of Furukawa (1932). The amide thus obtained was hydrolyzed to give the acid and amine as described by Furukawa (1932). The acid fraction was analyzed as the methyl ester by gas chromatography, and the amines were also separated by gas chromatography. Since the acid fraction contained mainly pentadecanoic acid and only very small quantities of other acids, the acid fraction as such was subjected to degradation (Figure 4) according to the method of Dauben *et al.* (1953). Degradation was also done after further purification of the acid fraction by vapor-phase chromatography.

Determination of Specific Activity. Samples of crude wax were fractionated by thin-layer chromatography on silica gel G. The silica gel was carefully scraped off

from the regions containing paraffins, esters, ketones, and secondary alcohols, and transferred separately into disposable pipets fitted with a glass wool plug in the narrow portion of the pipet. The wax components were eluted with a few milliliters of ethyl ether. The ether solution was dried on small, round, weighed microscope cover slips which were again weighed. The cover slips were then transferred into counting vials, liquid scintillation mixture was added, and ^{14}C was determined. The quantity of wax to be fractionated was so chosen that the component present in least amount would be greater than 0.1 mg, in order to minimize the error in weighing. Usually 0.1 to 0.6 mg of compounds was handled in these experiments, and a microbalance was used for the weighing.

Results and Discussion

The constancy of the composition of the hydrocarbons of the surface wax in a given plant species has been well demonstrated by Eglinton and co-workers (Eglinton *et al.*, 1962). The other components of the surface wax may also be expected to show a species specificity. The composition of the surface wax of the leaves of three plants, cabbage, broccoli, and cauliflower, all belonging to the species *Brassica oleracea*, was examined by thin-layer chromatography (Figure 1). The chromatographic pattern suggests that the surface waxes of the three plants are similar in composition. Gas chromatography of the paraffin fractions isolated from the three waxes showed their composition also to be similar; a typical gas chromatographic pattern is shown in Figure 2. Nonacosane (C_{29}) is the major component (85–92%) of the paraffin fractions with much smaller quantities of C_{31} (6–10%) being present. Measurable quantities (0.5–2%) of C_{27}, C_{28}, and C_{30} are also found, together with traces of C_{24}, C_{25}, C_{26}, and C_{32}.

Presumably the same sequence of biochemical reactions involved in the synthesis of the various components of cabbage wax are also concerned in the synthesis of these substances in other members of the *Brassica* family.

Incorporation of [^{14}C]*Acetate into Wax.* The structural similarity between the wax components and the long-chain fatty acids suggests that the leaf wax of plants is synthesized from fatty acids which in turn can be synthesized from acetate. In fact acetate was earlier found to be incorporated into beeswax (Piek, 1964). Accordingly, acetate may be suspected to be a precursor of wax, and so radioactive acetate was administered to cabbage and broccoli leaves. After different periods in the light, the surface wax was isolated and the radioactivity extracted into the chloroform was determined. From 3 to 4% of the administered acetate was found to be incorporated into the wax in 24 hours. In these preliminary experiments, acetate was administered in 4 to 5 ml of solution, and so the entry of radioactivity into the leaf was gradual. However, when the substrate was administered to the leaves in 0.2–0.5 ml solution, the entry of ^{14}C into the leaf was quite rapid, and consequently 4–5% of the administered radioactivity was found in the surface wax in 3–4 hours. Broccoli leaves incorporated acetate into the surface wax even more rapidly, 5–7% in about 4 hours. Although light stimulated acetate utilization by as much as 60%, no absolute requirement for light could be demonstrated. Stimulation of uptake of the substrate by the transpiration stream in light complicates matters when light and dark incorporation are compared.

In order to make sure that [^{14}C]acetate is converted into the wax and that the radioactivity released into the chloroform did not originate from a component other than wax, the chloroform extract was fractionated by thin-layer chromatography. Radioactivity distribution among the various components showed clearly that all components of wax became radioactive.

Although every fraction obtained by thin-layer chromatography had some radioactivity, it was necessary to demonstrate that the major components of the wax, namely the C_{29} compounds, became radioactive. To examine this, the paraffin fraction was further separated into individual hydrocarbons by gas chromatography, and the radioactivity in each of these components was determined. Table I shows clearly that the nonacosane, which is the major component, has most of the radioactivity and that the radioactivity is distributed in proportion to the concentration of the several components. Obviously nonacosane is derived from [^{14}C]-acetate.

TABLE I: Distribution of Radioactivity among Various Paraffins of Cabbage Wax.[a]

Components	Radioactivity (% of total activity in the paraffins)	Composition (%)
n-Paraffins of chain length below C_{29}	1.8	1.8
Nonacosane	92.6	91.0
n-Paraffin of chain length above C_{29}	5.7	7.1

[a] Each fraction was collected in the capillary portion of the disposable pipets extending into the effluent tube of the gas chromatograph which was equipped with an effluent splitter, and the radioactivity in each fraction was determined after washing the capillary tube into a counting vial. Recovery was determined with [16,17-^{14}C]-dotriacontane as a standard and was essentially complete.

It would be desirable in biosynthetic studies to use leaves at the stage when they are synthesizing surface wax most rapidly. In order to determine at what stage of growth this occurs, five pairs of leaves representing

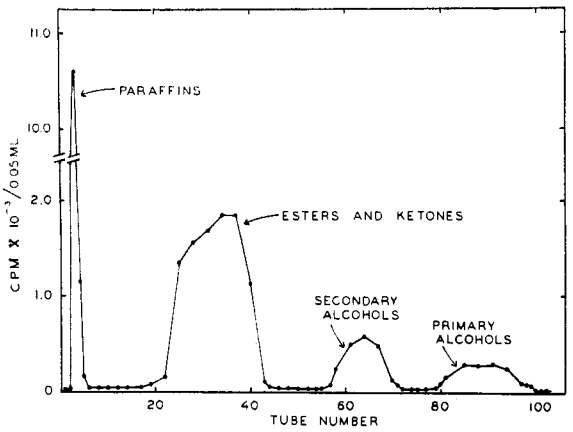

FIGURE 3: Column chromatography of the ^{14}C-labeled surface wax of cabbage leaves on 1.4- × 28-cm silica gel column by rectilinear gradient elution. Reservoir contained a mixture of 600 ml of benzene and 150 ml of chloroform and the mixing chamber 1 liter of *n*-hexane. Each tube contained 10 ml. Identification of components was by thin-layer chromatography on silica gel G plates and by infrared spectra.

leaves of different ages were removed from a cabbage plant. Each pair was fed ^{14}C-labeled acetate, and after 48 hours of metabolism under the light the surface wax was isolated and the radioactivity in the wax was determined (see Table II). The percentage incorporation of ^{14}C into the surface wax may be taken as a measure of the rate of synthesis. Leaf pairs 2 and 3, which were expanding most rapidly, produced surface wax at the fastest rate. Leaf pair 5 was almost fully mature and synthesized surface wax least rapidly. These results agree well with the observation that the young leaves of

TABLE II: Effect of Leaf Age on the Incorporation of [^{14}C]Acetate into the Surface Wax of Cabbage.[a]

Leaf Position	Incorporation into Surface Wax (% of administered)	Radioactivity[b] (% of total in wax)	
		Paraffins	Benzene Effluent
1 (youngest)	3.2	31	48
2	7.0	37	40
3	6.3	34	45
4	4.9	33	40
5 (oldest)	2.5	23	28

[a] [2-^{14}C]Acetate (19 × 10^6 cpm), 1 μmole/g fresh weight, was administered in 4.5 ml of water. [b] Wax isolated from each leaf after 48 hours of metabolism in light was put on alumina columns. After eluting the paraffin with hexane, benzene eluted the esters, ketones, and a pigment.

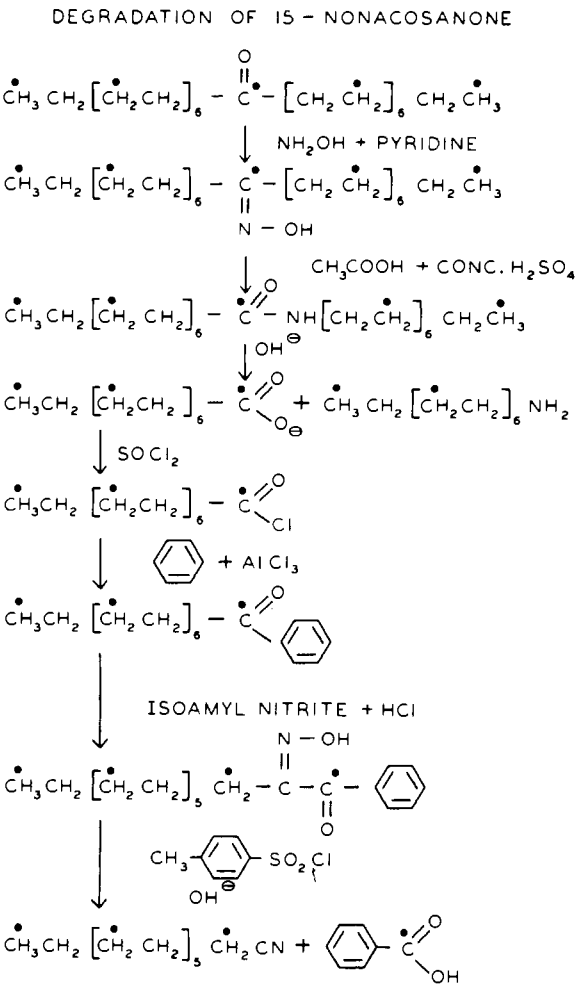

FIGURE 4: Schematic representation of the sequence of reactions used for isolating the carbonyl carbon of 15-nonacosanone. The hypothetical positions of ^{14}C in the nonacosanone derived from [2-^{14}C]acetate is shown by the dots.

cabbage renew surface wax bloom that has been removed mechanically, but old leaves do not (Schieferstein and Loomis, 1956). Similarly, in *Nicotiana* the amount of surface wax per unit area of the leaf increases with the age of the leaf until it reaches maturity, and then remains constant (Skoss, 1955). The outer leaves of cabbage are found to have the heaviest wax coating and the inner leaves the least (Purdy and Truter, 1963a). Table II also indicates that the composition of the wax synthesized by the leaves is probably independent of the age of the leaf, at least until maturity.

If, in fact, acetate serves as a precursor of wax in some fashion similar to the synthesis of fatty acids, then both carbons of acetate would be expected to serve equally as precursors of wax. This possibility was examined by studying the extent of incorporation of ^{14}C from [1-^{14}C]acetate and [2-^{14}C]acetate into the surface wax of cabbage. The results are shown in Table III. Clearly both carbons of acetate are incorporated into wax to an

TABLE III: Incorporation of the Individual Carbons of Acetate into Wax.[a]

Period of Metabolism (hr)	[1-^{14}C]-Acetate (% of administered)	[2-^{14}C]-Acetate (% of administered)
12	3.9	3.7
12	3.0	3.4
24	5.2	6.2
48	9.1	9.0

[a] Radioactive acetate (19×10^6 cpm), 10 μmoles, was administered in 4 ml of water, and uptake was complete in a few hours.

equal extent, and acetate apparently enters synthesis as a unit.

A further substantiation would be provided if the various components of the wax were also labeled to the same extent by either [1-^{14}C]acetate or [2-^{14}C]acetate. To examine this, the crude wax from experiment 4 in Table III was fractionated on a silica gel column by the gradient elution technique described in the previous section. Distribution of the radioactivity is shown in Figure 3, and the extent to which [1-^{14}C]acetate and [2-^{14}C]acetate labeled these fractions is shown in Table IV. Both carbon atoms of acetate were incorporated equally into the various fractions.

Evidence thus far obtained suggests strongly that acetate is the precursor of most components of the surface wax. If acetate as a unit is incorporated into the wax, alternate labeling of the wax by specifically labeled acetate would be expected. Accordingly, it was thought desirable to subject a pure wax component to a reaction by means of which a particular carbon atom could be isolated. Most of the wax is made up of C_{29} compounds, with the straight-chain paraffin predominating. However, a method of determining radioactivity in a specific carbon atom of a paraffin is not readily available. Hence attention was turned to the C_{29} ketone, which may be the precursor of the corresponding paraffin. The reaction sequence to which the ketone was subjected is shown in Figure 4. The acid and amine fractions obtained by the hydrolysis of the amide were analyzed by gas chromatography. Pentadecanoic acid was the major component of the acid fraction; 80% of the ^{14}C was found in this major component and the rest in acids longer than pentadecanoic acid (Figure 5). Tetradecylamine was the major compound of the amine fraction (Figure 6). More than 70% of the radioactivity in the amine fraction was found in tetradecylamine. These results show that 15-nonacosanone is the major component of the ketone fraction, thus confirming the results previously reported by other workers (Channon and Chibnall, 1929; Horn *et al.*, 1964; Purdy and Truter, 1963c). The acid fraction was subjected to the degradation scheme shown in Figure 4, and from the specific activity of isolated benzoic acid the per cent of radioactivity present in the carboxyl carbon of penta-

TABLE IV: Per Cent Radioactivity Distribution in Various Wax Fractions.[a]

Fraction	[1-^{14}C]-Acetate	[2-^{14}C]-Acetate
1. Paraffins	33.0	32.0
2. Esters, ketones	45.0	43.0
3. Secondary alcohols	9.6	9.7
4. Primary alcohols	6.1	5.2

[a] Wax fractionated in this table was from experiment 4 in Table III. The compounds contained in each fraction were tentatively identified by infrared spectroscopy and thin-layer chromatography on silica gel G with octacosane, 15-nonacosanone, 15-nonacosanol, and stearyl alcohol as standards. Esters and ketones could easily be identified by their peaks at 1735 and 1700 cm.$^{-1}$, respectively. Fraction 2 contained a pigment in addition to ketones and esters. Fraction 4 showed a carbonyl peak in addition to the OH peak, and this is believed to arise from ketols.

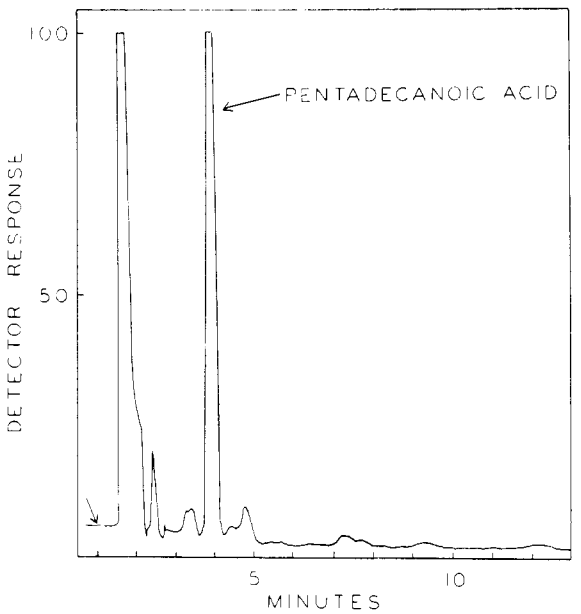

FIGURE 5: Gas-liquid chromatography of the methyl esters of fatty acids derived from the degradation of ketone fraction which was isolated from the surface wax of cabbage leaves. Experimental conditions: 6-ft coiled copper column (0.25 in. od), 3% SE-30 on 80–100 mesh siliconized Chromosorb W; temperatures of the column, injector, and detector 210, 330, and 250°, respectively. Carrier gas He at 45 ml/min.

TABLE V: Incorporation of Individual Carbons of Acetate into the Carboxyl Carbon of Pentadecanoic Acid Derived from 15-Nonacosanone.

Precursor	Specific Activity (cpm × 10^{-4}/mmole) Pentadecanoic Acid	Carboxyl Carbon[a]	Radioactivity in the Carboxyl Carbon of Pentadecanoic Acid (%)
[1-^{14}C]Acetate	37.9 (216 mg)	1.16	3.06
[2-^{14}C]Acetate	79.8 (300 mg)	7.32	9.20
	13.5[b] (807 mg)	1.30	9.60

[a] The carboxyl carbon was isolated as benzoic acid which was purified by sublimation; often an additional crystallization after charcoal treatment was required to obtain pure acid, mp 121–122°. [b] The pentadecanoic acid fraction was purified by gas chromatography and better than 90% of the radioactivity was in pentadecanoic acid. The amount of the acid used for each degradation is shown in parentheses.

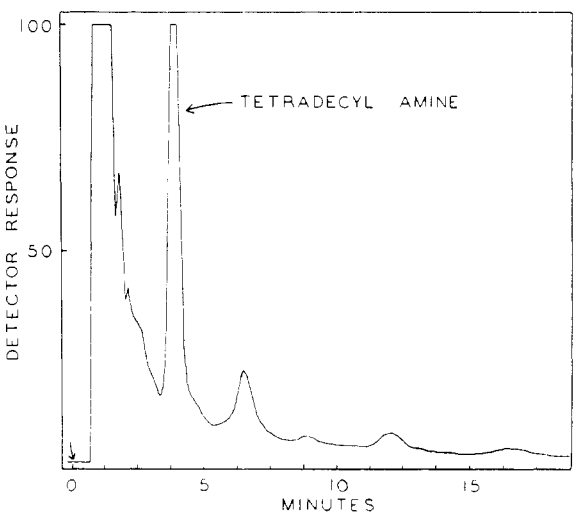

FIGURE 6: Gas-liquid chromatography of the amines derived from the degradation of the ketone fraction which was isolated from the surface wax of cabbage leaves. Experimental conditions: 6-ft. (0.25 in. od) coiled copper column, 4% Versamid 900 on siliconized 80–100 mesh Chromosorb W. The solid support was first coated with 1.5% NaOH in methanol before coating with the liquid phase. Temperatures of the column, injector, and detector were 230, 325, and 250°, respectively; carrier gas He at 35 ml/min. The amine sample shown in this figure was obtained from a sample of ketone which was isolated with carrier which contained some impurity; the C_{16} amine peak (the one after C_{14} amine) in this figure is primarily derived from this impurity.

decanoic acid was calculated (see Table V). If acetate were to be incorporated as a unit into the ketone, the carbonyl carbon could be labeled by either [1-^{14}C]-acetate or [2-^{14}C]acetate, depending on the pathway of synthesis, but not by both. Table V shows the extent to which [1-^{14}C]acetate and [2-^{14}C]acetate labeled the carbonyl carbon atom of the nonacosanone isolated from cabbage leaf to which a specifically labeled acetate was administered. If no randomization of label had occurred, no radioactivity from one carbon of acetate and 12.5% from the other would have been expected in the carboxyl carbon of the pentadecanoic acid derived from the ketone. In this experiment, leaves metabolized radioactive acetate for 48 hours, and this length of time is likely to have caused some randomization of label which would explain the deviation from the theoretical value. Trace quantities of even numbered, labeled fatty acids that could have been present in the C_{15} acid also would give some labeling by [1-^{14}C]-acetate and would show up as a decrease in the percentage of ^{14}C from [2-^{14}C]acetate. However, even when the pentadecanoic acid was further purified by gas chromatography, the labeling pattern did not change. So the difference between the measured and the theoretical labeling pattern is believed to arise from minor randomization of the ^{14}C during the 48-hour period of metabolism. In any case it is quite clear that acetate was incorporated as a unit, and that the carbonyl carbon of the ketone originated predominantly from methyl carbon of acetate. If the nonacosanone is made from 2 molecules of pentadecanoic acid, as suggested by Channon and Chibnall (1929), and if 6 molecules of acetate were added to a C_3 unit to give the pentadecanoic acid, then one would expect the carbonyl carbon of 15-nonacosanone to be labeled by [1-^{14}C]-acetate and not by [2-^{14}C]acetate. From the results shown in Table V this possibility may be ruled out. However, if such a pathway exists and operates to a small extent, the small amount of activity in the carbonyl carbon that comes from [1-^{14}C]acetate would be expected. The labeling pattern presented here is still in agreement with the pathway suggested by Channon and Chibnall (1929) because, if the pentadecanoic acid, which may be the precursor of the ketone, were pro-

duced by an α-oxidation of a C_{16} unit, one would anticipate finding ^{14}C in the carbonyl carbon of the ketone when [2-^{14}C]acetate is supplied. A direct synthesis of a C_{30} fatty acid from acetate, with subsequent decarboxylation of the end carbon, also would result in the labeling pattern shown in Table V. If the latter mechanism were operating, the carbonyl group in the 15 position would have to be either conserved during synthesis or introduced into the paraffin resulting from a decarboxylation.

Incorporation of Short-Chain Fatty Acids into Wax. Purdy and Truter (1963b), basing their views on the composition of the wax, noted that some components were missing which would be necessary if chain lengthening occurred by systematic additions of C_2 units. They implied that there could be synthesis of long-chain compounds from C_5 or C_6 units. Supposing that chain lengthening takes place by condensation of short-chain fatty acids, since carbonyl groups are found in the C_{10} and C_{15} positions of some C_{29} components of the wax (Purdy and Truter, 1963c), a logical precursor might be a C_5 unit. If C_5 units were the immediate precursor of the C_{29} carbonyl compounds, and these were converted to the paraffins according to the pathway proposed by Purdy and Truter, then [1-^{14}C]-pentanoic acid might be expected to contribute the ^{14}C to the wax more efficiently than either [^{14}C]acetate or [1-^{14}C]hexanoic acid. In order to test this possibility, incorporation of ^{14}C from the above three compounds into cabbage wax was compared, and the results in Table VI clearly show that pentanoic acid did not serve better as a precursor of wax than either acetate or hexanoate. Moreover, if the extent of ^{14}C incorporation into the paraffins is taken as an indication of the ability of the substrate to serve as a precursor of the C_{29} compounds, then pentanoic acid was not superior to the other substrates. In fact [1-^{14}C]pentanoate and [1-^{14}C]hexanoate contributed equal amounts of ^{14}C to the C_{29} compounds. Unless one assumes that hexanoate is rapidly converted into pentanoate and that activation of acetate and formation of the C_5 unit from acetate are much faster than the conversion of the C_5 unit into the C_{29} compounds, these results do not favor the view that the C_{29} compounds are synthesized by successive additions of C_5 units.

TABLE VI: Incorporation of Short-Chain Fatty Acids into Cabbage Wax.[a]

	Administered ^{14}C (%)			
Substrate	[1-^{14}C]-Hexanoic Acid	[1-^{14}C]-Pentanoic Acid	[1-^{14}C]-Acetate	[2-^{14}C]-Acetate
Crude Wax	6.3	6.0	5.7	6.9
Paraffins	2.1	1.9	1.9	2.4

[a] Each leaf of approximate fresh weight 9 g received 10 μmoles of the substrate; 23 $\times$ 10^6 cpm [1-^{14}C]-pentanoic acid; 25 $\times$ 10^6 cpm [1-^{14}C] hexanoic acid; 9.5 $\times$ 10^6 cpm [1-^{14}C]acetate; 19 $\times$ 10^6 cpm [2-^{14}C]-acetate. Sodium salt was used in each case; the substrate was administered in 4.5 ml. of water and was taken up in a few hours. Wax was extracted after 48 hours of metabolism in light, and paraffins were isolated by column chromatography on alumina.

Long-chain fatty acids of barley seedlings such as hexacosanoic acid (C_{26}) have been reported to be synthesized by the lengthening of preformed long-chain fatty acids by the systematic addition of C_2 units (Hawke and Stumpf, 1965). It is noteworthy that short-chain fatty acids other than acetate were not incorporated into such long-chain fatty acids, in contrast to the present results on wax synthesis. This suggests that the C_{29} compounds of the wax may not be synthesized by the kind of chain elongation system observed for fatty acids in barley (Hawke and Stumpf, 1965). If pentanoic acid and hexanoic acid contributed their carbons to the wax via acetate, then the cabbage leaf system differs from the barley system of Hawke and Stumpf in the ability to convert short-chain fatty acids into acetate units rapidly.

Trichloroacetate Inhibition. The amount of surface wax of certain plants has been reported to be diminished by treatment of the soil with trichloroacetate, which has been used as a selective weed killer (Dewey *et al.*, 1962). It was also indicated that the reduction in wax was most pronounced in those fractions of the wax which were most easily dissolved by organic solvents. However, the effect of trichloroacetate on the individual components of the wax has not yet been reported. Moreover, it has not been possible to tell whether the reduction in surface wax is attributable to an inhibition of excretion of the wax or to a true inhibition of the biosynthesis of wax. If trichloroacetate is an inhibitor of the biosynthesis per se of wax, this inhibitor may be useful in elucidating the biosynthetic pathway of various wax components. For these reasons the effect of trichloroacetate on the incorporation of acetate into wax was studied with excised leaves of *Brassica*. Since the effect of trichloroacetate on the wax synthesis was found to be similar in both broccoli and cabbage leaves, detailed analysis of the effects of trichloroacetate was studied only in broccoli, and the results of a series of experiments are summarized in Figure 7. It is clear that 0.05 to 0.1 μmole of trichloroacetate supplied to a leaf, approximately 5 g in fresh weight, inhibits surface wax synthesis by 50%. If all of the trichloroacetate administered is assumed to be evenly distributed throughout the leaf, then 1 to 2 $\times$ 10^{-5} M trichloroacetate inhibits surface wax synthesis by 50%. Dewey *et al.* (1962) reported a striking reduction of leaf wax content in kale and peas by treatment of the soil with trichloroacetate. However, similar reductions could not be demonstrated in nettle where the surface wax is not particulate. Pfeiffer *et al.* (1959) showed that the amount of wax extracted from leaves with hydrocarbon solvents was diminished considerably in peas and kale

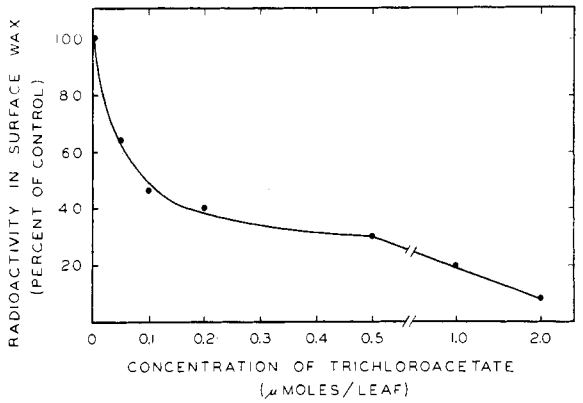

FIGURE 7: The effect of trichloroacetate on the incorporation of acetate into the surface wax of broccoli leaves. Each leaf, approximately 5 g fresh weight, received 20 μmoles of [2-^{14}C]acetate, 25.7 × 10^6 cpm, and the appropriate amount of sodium trichloroacetate; the acetate was metabolized in light for 4 hours, after which time the wax was isolated and ^{14}C determined. Each experiment included control leaves which did not receive trichloroacetate.

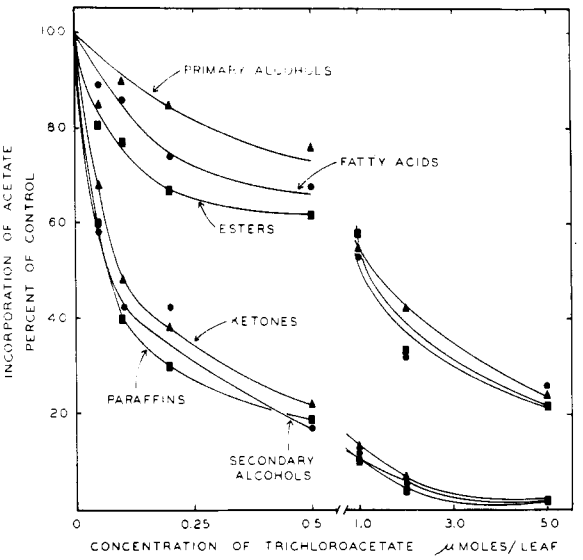

FIGURE 8: The effect of trichloroacetate on the incorporation of acetate into the various components of the surface wax of broccoli leaves. The wax samples represented in Figure 7 were fractionated by thin-layer chromatography on silica gel G with benzene as the developing solvent, and the effect of different concentrations of trichloroacetate on each fraction was determined.

TABLE VII: Thin-Layer Fractionation of the Surface Wax of Broccoli Leaves.[a]

Fraction	R_F	Radioactivity (% of total applied at origin)
1. Paraffins	0.90	36.7
2. Esters	0.77	4.7
3. Ketones	0.70	21.2
4. Unknown	0.59	8.7
5. Secondary alcohols	0.36	10.1
6. Unknown	0.17	0.98
7. Primary alcohols	0.10	6.1
8. Fatty acids	0.00	7.0

[a] Broccoli wax, isolated from a young leaf which had metabolized [2-^{14}C]acetate for 4 hours under light, was applied on a silica gel G plate and was developed for 50 minutes in benzene. The results represent the mean of 2 separate experiments which served as controls in the inhibition experiments shown in Figure 7.

and rather less so in nettles after trichloroacetate treatment. Juniper (1959) demonstrated a remarkable diminution in visible wax as a result of trichloroacetate treatment. Thus the observed inhibition of acetate incorporation into the surface wax agrees well with these reports of other workers.

It would be of interest to know whether trichloroacetate inhibits the synthesis of all of the components of the wax or of some of its components in particular. Such information may also shed light on the problem of the biogenetic relationship between the various substances of the wax. To examine this, the wax samples represented in Figure 7 were fractionated by thin-layer chromatography. A typical distribution pattern of radioactivity in the absence of trichloroacetate is shown in Table VII. The per cent inhibition was calculated from the distribution of radioactivity, and the results are shown in Figure 8.

Inhibition was most severe in the case of hydrocarbons, ketones, and secondary alcohols with much less inhibition of primary alcohols, acids, and esters. At a concentration of trichloroacetate which did not significantly inhibit the synthesis of fatty acids, alcohols, and esters of the wax, the paraffins, ketones, and secondary alcohols were inhibited approximately 40%. This suggests that either the trichloroacetate inhibition affects a step beyond the fatty acid stage in the pathway of the biosynthesis of the C_{29} compounds, or the biosynthesis of C_{29} compounds occurs independently of the synthesis of primary alcohols, acids, and esters, either by a different sequence of reactions or at different sites. If the former suggestion were to be true, an accumulation of fatty acids would have been found in the wax which is synthesized in the presence of trichloroacetate. On the contrary, the biosynthesis of fatty acids, primary alcohols, and esters in the wax was slightly inhibited by trichloroacetate. Hence it is more likely that the biosynthesis of the three C_{29} compounds

occurs at a different site from that of fatty acids, primary alcohols, and esters.

It has been suggested that the surface wax of leaves is synthesized at sites different from its final location (Chibnall and Piper, 1934). Hall and Donaldson (1962) have presented evidence that pores exist in the cuticle through which the wax is thought to be secreted. If trichloroacetate inhibition of the incorporation of acetate into surface wax is attributable to an inhibition of the excretion of wax, then the conclusions about the biosynthetic relationships among the wax components drawn from the inhibition experiments become invalid. Hence attempts were made to determine whether trichloroacetate inhibits secretion of wax or biosynthesis of wax. If the secretion alone was inhibited, then the wax that was not excreted should be found within the leaf. However it was difficult to isolate all of the components that are normally present in the surface wax from the subcutaneous tissue, because other compounds present within the leaf interfered with the chromatographic purification of the components. However the hydrocarbon fraction could easily be isolated by column or thin-layer chromatography. The incorporation of [^{14}C]acetate into paraffins inside the leaf was measured, and the effect of trichloroacetate on this fraction could be compared with its effect on the surface wax (Table VIII). Inhibition of surface wax synthesis is clearly reflected in a corresponding inhibition in the acetate incorporation into paraffins inside the leaf. It is evident from the table that significant radioactivity is not found in the paraffins inside the leaf when compared to surface paraffins. The quantity of paraffins inside the leaf was only a few per cent of that found on the surface of the leaf. Thus the results in Table VIII show that the inhibition of acetate conversion into the paraffins of the surface wax is not a reflection of inhibition on the secretion of the paraffins to the surface, but an inhibition of paraffin biosynthesis. This may also be true of the other components of the wax.

TABLE VIII: Effect of Trichloroacetate on the Incorporation of Acetate into Internal Lipids, Internal Paraffins, and Surface Paraffins of Broccoli Leaves.[a]

Concentration of Inhibitor (μmoles/leaf)	Radioactivity (cpm $\times$ 10^{-4}/leaf)		
	Ether-extractable Internal Lipids	Paraffins	
		Inside the Leaf	Outside the Leaf
0.0[b]	157	0.84	45.0
0.05	145	0.78	20.4
0.1	120	0.42	14.6
0.2	146	0.22	11.4
0.5[c]	150	0.25	8.1
1.0	180	0.20	6.2
2.0	146	0.09	1.5
5.0[c]	192	0.04	0.8
10	170		0.56
20	140		0.3

[a] Development by hexane (12 cm) clearly separated the paraffins (R_F 0.82); the composition of the paraffins inside the leaf was identical to that outside the leaf. The analyses shown in this table were done on the leaves represented in Figure 7. [b] Mean of three separate experiments. [c] Mean of two separate experiments. Paraffins inside the leaf were determined by thin-layer chromatography of the total ether extract on silica gel G plates.

At a concentration of trichloroacetate which inhibits surface wax synthesis completely, or even with a severalfold greater concentration of trichloroacetate, the incorporation of radioactivity from acetate into total ether-extractable lipid was not inhibited (see Table VIII). Similarly Dewey *et al.* (1962) found that treatment of plant roots with trichloroacetate markedly reduced those fractions of the wax which could be quickly dissolved in organic solvents, although at the same time "oils and internal fats" were not affected. This evidence suggests that wax synthesis occurs independently of internal fatty acid synthesis. On the other hand, if the wax were to be synthesized from the general fatty acid pool the inhibition must affect a step beyond fatty acid stage. If the latter explanation were correct, however, an accumulation of the fatty acids that would otherwise have formed wax should have been found in the leaves treated with trichloroacetate. Since this was not found, it is more likely that wax synthesis occurs independently of general fatty acid synthesis and involves either a different sequence of reactions or occurs at a different locus. It has been suggested that trichloroacetate interferes with the biosynthesis of coenzyme A and thus produces the toxicity which has been observed in yeasts and plants (Hilton *et al.*, 1958). Trichloroacetate was shown to compete with pantoic acid in the enzymatic synthesis of pantothenic acid (Hilton *et al.*, 1959). Such a mechanism for the inhibition of synthesis of the C_{29} compounds of *Brassica* leaves cannot explain the lack of inhibition of the internal lipid synthesis because both systems presumably involve reactions mediated by coenzyme A. One obvious explanation would be that compartmentation may allow trichloroacetate to have greater access to the locus of the synthesis of C_{29} compounds in the leaf than to the site of synthesis of other lipid components which has been found to occur in chloroplasts (Mudd and McManus, 1962; Stumpf and James, 1963). One other possibility would be a biosynthetic pathway for wax that does not involve coenzyme A, but this is unlikely. Moreover, the inhibition of acetate incorporation into the surface wax could be at least partially reversed by administering pantothenic acid with the substrate (results of preliminary experiments). This indicates that trichloroacetate is inhibiting the wax synthesis by interfering with the biosynthesis of pantothenic acid in a manner similar to that previously described by Hilton *et al.* (1959). Thus the explanation

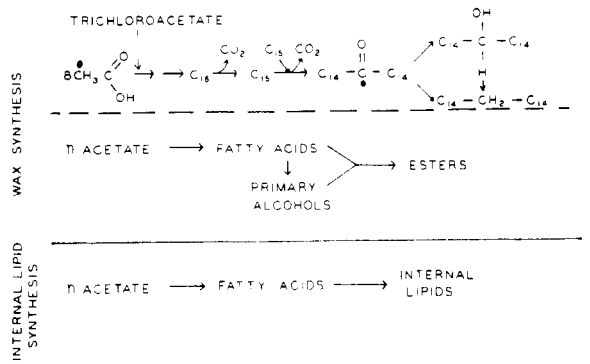

FIGURE 9: Schematic representation of the biosynthesis of lipids of *Brassica*.

which invokes compartmentation seems to be the correct one.

Specific Activity of Wax Components. From the experimental results discussed thus far no information as to the biogenetic relationship among the various components of the wax is available except for the suggestions that the alcohols, acids, and esters may be synthesized at a different site from synthesis of ketones, secondary alcohols, and paraffins. The order in which the various components become labeled from acetate may give some additional information about the biosynthetic interrelationships of the various wax components. For this reason the change in specific activity of some wax components with time was determined, and the results are shown in Table IX. The hydrocarbon seems to be the most radioactive component even at as short a time as 30 minutes, the ketones being almost as radioactive as the hydrocarbons. Such a specific activity pattern may be regarded as consistent with the hypothesis that the ketone is the precursor of the paraffin because these C_{29} compounds show similar specific activities. However, the 30-minute period used in such experiments may be too long to show clearly any precursor-product relationships. Hence shorter periods of incorporation were also used, and the results are shown in Table X. Even after as short an incorporation time as 5 minutes, hydrocarbon was the most radioactive component of the wax. Thus the attempts to detect which C_{29} compound becomes labeled first did not give conclusive results. Permeability or the rate of equilibration of the metabolically active pools with the less active surface pools may affect the results. At present no information as to the differences in the rates of equilibration of the various wax components inside the leaf with those that are outside is available. In any case, it is clear from Tables IX and X that the esters become radioactive very much slower than the ketones and paraffins. This agrees with the hypothesis that the esters may be synthesized at a different site from the locus of synthesis of the C_{29} compounds. Thus the results of the experiments with trichloroacetate and the specific activity comparisons converge strongly, suggesting that the C_{29} components of the wax are synthesized independently of the synthesis of primary alcohols, fatty acids, and esters. Similar conclusions have also been drawn from a study of wax synthesis in the honey bee (Piek, 1964).

The results discussed above may be incorporated into a schematic representation of the biosynthesis of the lipids of *Brassica* as shown in Figure 9. It must be pointed out that the details of the pathway for the biosynthesis of the C_{29} compounds indicated in the scheme is only speculative but is in accordance with the observed labeling pattern and the suggestions of

TABLE IX: Effect of Time on the Specific Activity of Surface Wax Components of Broccoli Leaves.[a]

Time	Specific Activity (cpm $\times 10^{-4}$/mg)		
	Hydrocarbons	Ketones	Esters
30 min	5.6	4.1	0.87
2 hr	10.4	6.7	2.2
4 hr	18.0	18.6	7.2
4 days	23.6	19.4	6.0

[a] Tracer amount of radioactive acetate, 19×10^6 cpm, was administered in 0.2 ml of water; uptake was over in 5–10 minutes. The wax was separated into fractions by thin-layer chromatography on silica gel G with benzene as the developing solvent. Components were eluted, weight (0.1–0.5 mg) was determined on a microbalance, and ^{14}C was assayed in a liquid scintillation spectrometer.

TABLE X: Distribution of Radioactivity among the Wax Components after a Short Period of Incorporation of [1-^{14}C]Acetate.[a]

	5 min	10 min
Administered, cpm	19×10^6	19×10^6
Uptake, cpm	9.4×10^6	14.4×10^6
Total ^{14}C in wax, cpm	8400	43400
Fraction	Radioactivity (% of total in the wax)	
Paraffins	39	43
Esters	2.1	2.5
Ketones	9.0	17
Unknown	21	16
Secondary alcohols	6.0	7.0
Unknown	1.8	0.6
Primary alcohols	8.1	6.3
Fatty acids	13	8.0

[a] Wax was fractionated by thin-layer chromatography on silica gel G and the ^{14}C in each spot was determined. The fractions are shown in the order of their mobility on the thin layer.

Channon and Chibnall (1929). The significant aspect of the scheme is the postulation of different sites for the synthesis of different classes of compounds which originate from the same precursor, namely acetate. Attempts to elucidate the biosynthetic pathway for the C_{29} compounds are in progress.

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