THE BIOSYNTHESIS OF WAXES IN PLANTS

by

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I hereby recommend that this dissertation prepared under my direction by Kaoru Matsuda

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be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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INTRODUCTION

The developments which have occurred in the past 20 years have given a truly phenomenal clarification of many metabolic processes which occur in both plants and animals. As a result of a great variety of studies, the step by step sequence in the formation and degradation of many compounds can now be presented. The energy relationships existing between catabolic and anabolic reactions are being clarified rapidly; studies of the functions of particulates in cells have given a partial basis for explaining the simultaneous occurrence of anabolic and catabolic reactions; also, progress in biochemical genetics is now yielding an integrated concept linking metabolic processes to gene control and enzyme action.

The understanding of metabolism, however, is by no means complete or approaching completion. This is true even in the case of compounds which have been studied extensively - new alternate pathways for both their synthesis and degradation are being presented continually. Furthermore, either because of lack of interest or analytical difficulties, the metabolism and functions of many other materials hardly have been investigated. In plants, the waxes can be considered to be among the least studied of all such substances.

Although waxes occur almost universally in higher plants, they are identified still by general physical (melting point, hardness, etc.) and chemical (iodine value, saponification numbers, etc.) characteristics. This is understandable since they are known to be a heterogeneous mixture of long-chain aliphatic keto and hydroxy acids, alcohols, esters, paraffins and many other materials soluble in lipide solvents. Thus, formidable analytical problems undoubtedly account for much of the present dearth of knowledge of their metabolism. The understanding of wax oxidation is now limited mostly to results obtained from studies using microorganisms, and little is known of the manner and extent of oxidation in higher plants. The knowledge of wax synthesis in higher plants has progressed very little beyond a hypothesis presented in 1934 by Chibnall and Piper (12).

The objectives of this study were to develop a system for the study of plant wax metabolism, to extend the knowledge of wax metabolism, and to learn something about the factors which affect wax synthesis and degradation in-vitro so that an explanation of the relationships of wax metabolism to other biological reactions can be achieved ultimately. Also, since the metabolism of paraffins, fatty alcohols and esters of all kinds have not been studied extensively, it was felt any added knowledge about their

metabolism would be a contribution to the understanding of the total life processes which occur in plants.

REVIEW OF THE LITERATURE

The chemistry of plant waxes has been studied quite extensively since 1850 but analytical difficulties often led to conflicting statements regarding their constitution. The elimination of much of the confusion which existed in the early literature and the clarification of the chemical nature of waxes has resulted largely from the efforts of Chibnall and his associates (11, 13, 14, 64). After first synthesizing suitable standards, these workers made extensive studies on a large number of waxes and showed that the major components of plant waxes are straight chain compounds of 23 or 24 to 36 carbon atoms in length. Furthermore, they showed that while waxes are mixtures of various homologs of fatty acids (or substituted acids), primary alcohols, esters, paraffins and secondary alcohols, there is uniformity in that all acids, primary alcohols and esters possess an even number of carbon atoms. On the other hand, all paraffins, secondary alcohols and ketones have an odd number of carbon atoms in their chain. While recent studies (54, 90) have shown that even-chain length paraffins and odd-chain length fatty acids do occur, the amounts detected have been small and the conclusions made by Chibnall et al, have not been disputed.

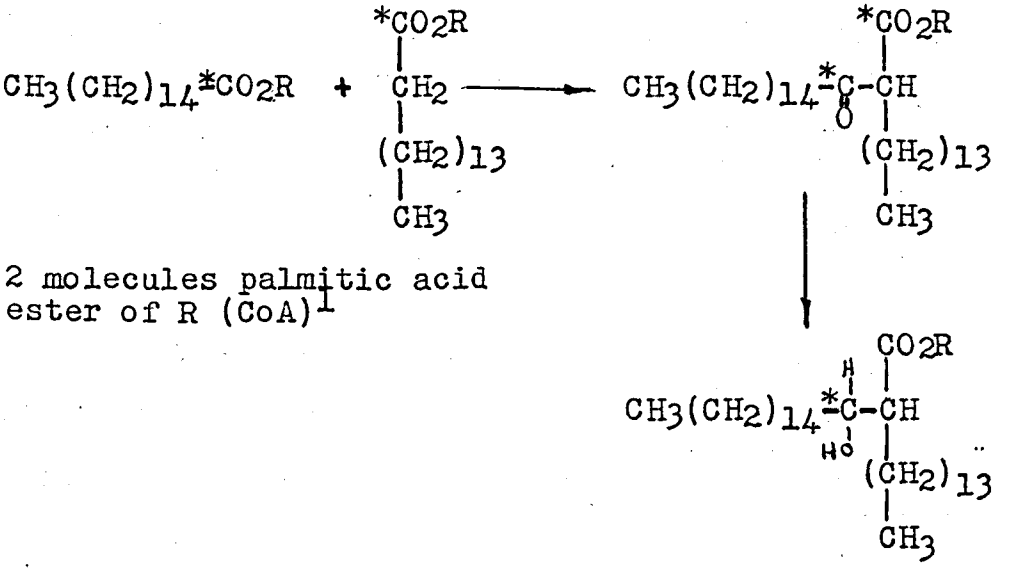
In addition to studying wax composition, Chibnall and his associates were interested in their metabolism. They postulated that wax acids were produced first by condensation of two-carbon fragments (12); primary alcohols were believed to be produced by reduction of the acids; paraffins, secondary alcohols and ketones were considered to be formed by decarboxylations of the corresponding n+1 acid. Wax oxidation was thought to occur in essentially the reverse manner whereby paraffins, alcohols and other wax compounds were changed into fatty acids which were then cleaved into two-carbon intermediates (32).

Recent studies on wax metabolism have given some support to the concept of a two-carbon intermediate in synthesis. In studies with flax and candelilla plants, Sutton (81) showed that radiocarbon from acetate-1-C^{14} definitely is incorporated into waxes of both plants. Fulco and Mead (19) demonstrated that rats which were given intraperitoneal injections of acetate-1-C^{14} incorporated the radiocarbon into 24-carbon fatty acids. Step-wise degradation of the acids showed a complete synthesis from acetate.

Although studies with acetate support the two-carbon intermediate concept, a recent study by Gastambide-Odier and Lederer (20), suggests ketones and possibly

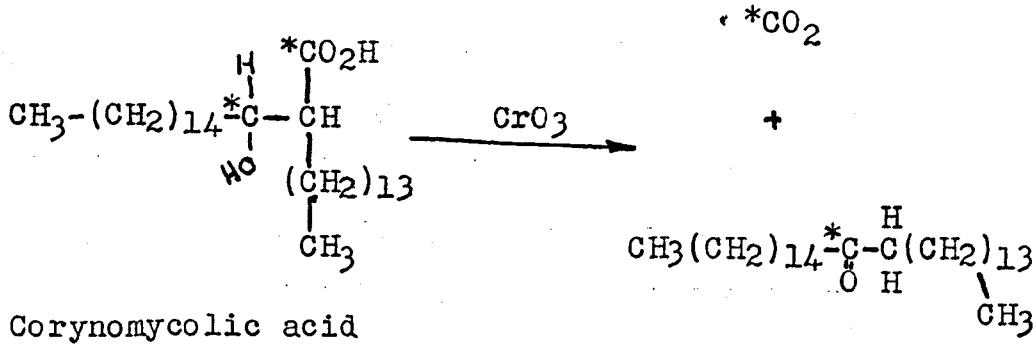
paraffins may be formed by reactions of two glyceride fatty acids (fatty acids possessing 14 to 18 carbon atoms). They cultured the bacterium, Corynobacterium diphtheriae, in potassium palmitate-1-C^{14} and determined the specific activity of the corynomycolic acid which was formed. They also oxidized the acid with chromic acid and obtained palmitone (a natural product) and CO_2. The CO_2, palmitone and corynomycolic acid were all radioactive but the specific activity of the acid was twice that of either palmitone or CO_2. From the results, they suggested the following reaction sequence:

Biosynthesis:



[1]The abbreviations used in this dissertation are presented and defined in the appendix.

Oxidation studies:



Corynomycolic acid

Palmitone

Interestingly, Channon and Chibnall (11) originally believed that secondary alcohols and paraffins were produced from ketones which, in turn, were formed by reactions of the type indicated by Gastambide-Odier and Lederer. However, they abandoned the idea in favor of the wax fatty acid-decarboxylation pathway since they were unable to find any C_{15} glyceride fatty acids to explain the formation of C_{29} paraffins which are found in many waxes. In addition, they found symmetrical ketones only rarely in the waxes which they studied. Kreger (39), on the other hand, detected symmetrical ketones quite frequently in waxes and suggested reactions involving two glyceride

fatty acids may account for much of the synthesis of secondary alcohols and paraffins. The work of Gastambide-Odier and Lederer tends to strengthen this belief.

The literature on the oxidation of waxes, like that of wax synthesis, is practically non-existent. Furthermore, almost all of the present information on wax degradation has come from studies with microorganisms.

In 1932, Hopkins and Chibnall (32) attempted to study the intermediate products formed when paraffins were metabolized by Aspergillus versicolor. However, they were able to isolate only CO_2 and mold mycelia and could not study the intermediates formed in the oxidation process. Through use of different substrates, they showed that the organism grew well on either ketones or paraffins up to 34 carbon atoms in length, but not on secondary alcohols. They suggested paraffins were oxidized to ketones or polyketones which were then oxidized further to fatty acids. The acids, in turn, were thought to be broken down into two-carbon fragments.

Senez and Konovaltschikoff-Mazoyer (68) studied the volatile products formed by cultures of Pseudomonas aeruginosa grown on n-heptane as a carbon source. The products were almost all acids with hexanoic and heptanoic acids predominating (56%). In addition, they found 18% acetic, 10% propionic, 7% butyric and 4% valeric acids.

Heinen and Linskens (29) found a species of a Penicillium on rotten leaves which grew well on cutin. They found a cutinase which formed free fatty acids that were then acted upon by a dehydrogenase.

In 1958 Millman and Yotis (57) studied the metabolism of beeswax (largely myricyl palmitate) by various bacteria and molds. Extracts from Micrococcus urease, Aspergillus flavus, Bacillus macerans and Candida albicans usually had esterase activity as measured by liberation of CO_2 from solutions containing bicarbonate. De-esterification and formation of free acids were considered to be the first step in the degradation of waxes.

In considering all results from the oxidation studies, there is still no proof of formation of a ketone or two-carbon intermediate as suggested by Hopkins and Chibnall (32). However, there was a common suggestion that fatty acids were intermediates in the wax degradation process. This fact, combined with the results from the wax synthesis studies raises the possibility that acids may be intermediates in wax metabolism as a whole.

Although studies on wax metabolism have been limited, a great many workers have studied the metabolism of the closely related glyceride fatty acids. As a result, pathways for both their oxidation and synthesis have been presented and many relationships of these pathways to other

biochemical reactions are now known. Some of these data have been summarized in a series of recent reviews (24, 49, 55, 80). Since glyceride fatty acids bear similarities to the components of waxes, the extensive literature on their metabolism was used as a guide in the present study of wax metabolism.

The rapid progress in the field of glyceride fatty acid biochemistry has resulted largely from advances in analytical techniques and the use of radioisotopes as tracers. However, the understanding of the specific steps involved in fatty acid metabolism and the relationships of the steps to other biochemical pathways has resulted from the development of studies at the enzyme level. The key to the enzyme level studies, in turn, was the discovery (46, 61) and subsequent isolation (47) of CoA.

The role of CoA in plant and animal metabolism was clarified rapidly through a series of studies following its discovery. The "active" form of acetate long associated with the synthesis of acetoacetate, citric acid, and fatty acids, was soon shown to be acetyl CoA. Soodak and Lipmann (73) showed that in the presence of ATP, partially purified CoA and a suitable pigeon liver enzyme, acetate is condensed to form acetoacetate. At approximately the same time, Stern and Ochoa (77) found CoA to be a requirement for the enzymatic condensation of acetate with oxaloacetate to form

citrate - an intermediate in the citric acid cycle. In 1951, Lynen and Reichert (51) isolated "active" acetate from yeast cells and showed it is, in fact, acetyl-CoA. Furthermore, they showed that acetate is linked to CoA through a thiol bond and suggested the linkage is another type of high energy bond.

Other studies extended the knowledge of CoA participation to include fatty acid reactions. The 1950-1955 efforts to elucidate the mechanism of fatty acid oxidation in extracts of animal tissue showed that CoA activates glyceride fatty acids as well as acetate (38, 53) and acetyl-CoA is the final product of oxidation (50). In addition, other studies with animal tissues showed CoA to be a necessary cofactor and acetyl-CoA an intermediate (8, 41, 87) in fatty acid synthesis. The participation of CoA in plant metabolism was suggested in 1954 when Millerd and Bonner (56) proved that the enzymes which catalyze activation of acetate to acetyl-CoA are present in wheat, spinach, and other plants. Later, Stumpf and Barber showed CoA is required for oxidation (78) as well as synthesis (79) of plant fatty acids. Thus, CoA has come to be regarded as the factor which gives biological activity to nearly all fatty acids, and acetyl-CoA is the link existing between fatty acid metabolism and the citric acid cycle.

Biochemists now feel that many of the basic

problems of fatty acid oxidation have been solved. It is now accepted, for example, that the enzymes required for the most common form of fatty acid degradation, β-oxidation from the carboxyl end of the molecule, are localized in the mitochondria of both animal (35, 37, 60) and plant (5, 59, 78) cells. Furthermore, the sequence of steps and the cofactors and enzymes involved in the oxidation process in some animals are known, and there is evidence that the sequences are the same in plants.

In the early 1950's, the first step in the oxidation process was found to be the activation, in the presence of ATP and Mg^{++}, of the fatty acid by the formation of the acyl-CoA derivative (38, 53). In the presence of DPN^{+}, water and another molecule of CoA, the activated molecule is then cleaved to an acyl-CoA derivative of an acid two carbon atoms shorter, and acetyl-CoA. The shortened fatty acid-CoA derivatives are then degraded further through a successive series of such reactions until all the original fatty acid is converted into acetyl-CoA. The specific steps and enzyme and cofactor requirements involved in the complete oxidation scheme are outlined in Lynen and Ochoa's original publication (50).

As yet, the enzymes which carry on fatty acid oxidation in plants have not been isolated but the available data suggest the steps are the same or similar to those

involved in animal systems. Evidence from both in vivo (18, 23) and in vitro (83, 85) studies indicate β-oxidation is a common phenomenon in plants. Studies with mitochondrial systems (6, 78) have shown that cofactors required for oxidation in plants include those required for oxidation in animal systems. In addition, Stumpf and Barber (78) have shown that plant mitochondria, like animal mitochondria, require the addition of a citric acid cycle intermediate to spark fatty acid oxidation.

The understanding of the steps involved in fatty acid synthesis also has undergone rapid expansion. Studies with labeled acetate have furnished convincing proof of fatty acid synthesis by condensation of two carbon or apparent two carbon fragments. Furthermore, there is no longer any doubt that the pathway from acetate to fatty acids does not include sugars as intermediates. However, there is considerable disagreement on the specific steps and the pathway of formation of the acids.

One of the earliest suggestions of a two carbon intermediate in fatty acid formation was made by Raper (66) in 1907. He postulated that fatty acids could be formed as the result of aldol condensations of acetaldehyde, reductions in the chain, and oxidation of the terminal carbon. In 1926, Smedley-MacLean and Hoffert (72) showed that fatty acids accumulate in yeast cells grown on acetate

or ethanol as the carbon source. This study supported Raper's two-carbon fragment hypothesis, but Smedley-MacLean and Hoffert felt the pathway of formation was acetaldehyde ⟶ carbohydrate ⟶ fatty acid. The clarification of the general pathway of formation and the conclusive proof of a two-carbon building block resulted from a number of studies using labeled compounds.

In 1947, White and Werkman (94) compared the fatty acid and sugar contents of cultures of the yeast, Saccharomyces cerevisiae, when cultured for 46 hours. Treatments included cultures with no carbon source, with added non-labeled acetate, and with acetate-1-C^{13}. Cultures which were not given acetate did not change appreciably in fatty acid content but the reducing sugars decreased to about 1/3 of the initial amount. Cultures which were given either form of acetate increased in fat content about 110%. The reducing sugars also decreased in both the latter cultures, but the decrease in either case was less than that obtained from cultures which had no acetate. This suggested either a "sparing" of sugars on acetate addition or a synthesis of carbohydrates from acetate. Since the amount of C^{13} isotope in the fatty acids was about 12 times that in the reducing sugars, they tended to discount the possibility of much sugar synthesis and felt acetate was passed directly to fatty acids.

Further support for a direct acetate to fatty acid pathway was presented by Popjak (65). His report was based on a series of studies made on the fatty acids of milk from goats and rabbits that were given C^{14}-labeled acetate, pyruvate or glucose. As a starting point, he based part of his argument on the prior knowledge that pyruvate arises from glucose through the glycolytic scheme. Rabbits given either carboxyl-labeled acetate or α-labeled pyruvate produced octanoic acid labeled in the odd-numbered positions. Methyl-labeled acetate, β-labeled pyruvate and glucose-1-C^{14} produced labeling at the even-numbered positions. The results were in accord with the idea that the pathway of formation was:

glucose ⟶ pyruvate ⟶ acetate + CO_2 ⟶ fatty acid.

Popjak's work showing the alternate labeling of fatty acids supported another concept of fatty acid synthesis - that of head-to-tail condensation of acetate or some other two-carbon fragment, and subsequent reduction of the chain to form the fatty acid.

The first studies on the method of condensation were made by Wood et al. (95) using Clostridium butylium. When cultures of the organism were given acetate-1-C^{13}, the heavy isotope was found in the 1 and 3 position of butanol. Cultures made with 1, 3 - labeled butyric acid showed 85% conversion into 1, 3 -labeled butanol. This

indicated a head-to-tail condensation of acetate to form butyrate and a subsequent reduction to butanol.

In a study with animals, Rittenberg and Bloch (67) found that when rats were given CD_3-$C^{13}OOH$, the fatty acids isolated from tissues were labeled with both deuterium and the heavy carbon atom. This showed both carbons of acetate were used by the rat to make fatty acids. Decarboxylation of the first carbon of the fatty acid showed the C^{13} percentage of the trapped CO_2 to be about twice that of the average of the rest of the molecule. They felt that only alternate carbon atoms could contain the heavy isotope and such a compound would arise from a series of head-to-tail condensations of acetate or some other two-carbon fragment.

Gibble and Kurtz (21), in 1956, presented the first proof of a head-to-tail condensation of acetate fragments in the formation of plant fatty acids. The fatty acids obtained from flax embryos cultured in vitro in sodium acetate-1-C^{14} were found to be labeled in odd-numbered positions of the carbon chain.

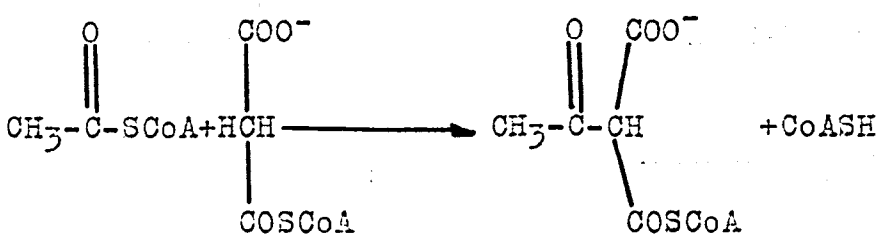
Studies at the sub-cellular level have given further support to the concept of head-to-tail condensation of two-carbon or apparent two-carbon fragments. In addition, these studies indicated that synthesis can occur in several ways instead of the single pathway usually envisioned

for fatty acid oxidation.

After elucidation of the oxidation cycle, it was felt generally that synthesis of fatty acids was merely the reverse of oxidation. This was logical since each of the steps in the oxidation cycle was shown to be reversible (50). Also, a number of studies with extracts from animal tissues showed cofactors or enzymes required for synthesis were similar or identical to those required for oxidation (30, 42, 69, 88). Thus, a substantial case exists for a pathway of synthesis which is more or less the reverse of β-oxidation.

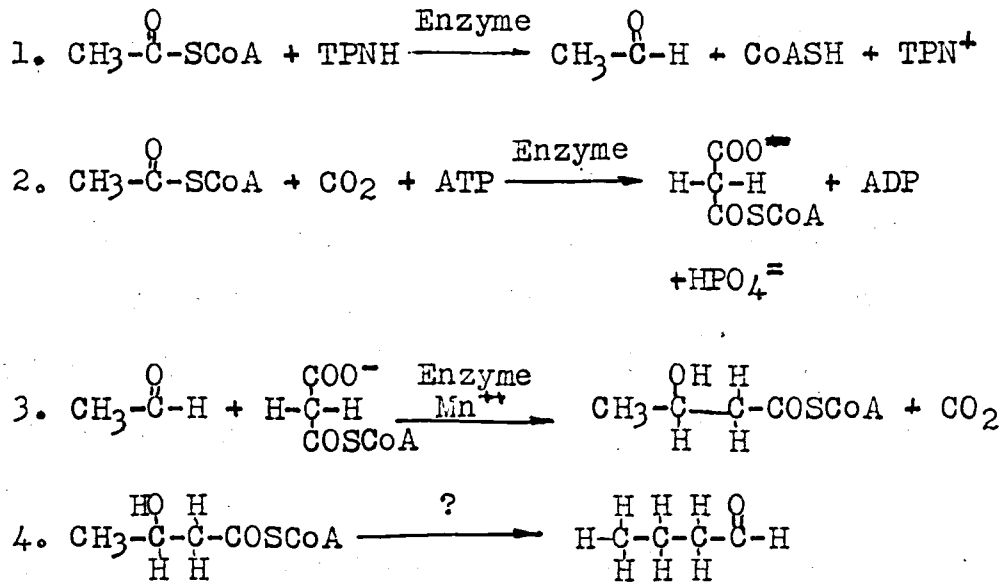
Starting in 1955, Green and his associates at Wisconsin (24) began a systematic study to determine if other pathways for fatty acid formation existed. Using a pigeon liver system previously studied by Brady and Gurin (8), they found synthesis of fats occurred outside the mitochondria (site of oxidation) and required Mn^{++}, TPNH, ATP, biotin (89) and CO_2(22), although CO_2 was not incorporated. In addition, malonic acid was produced as an intermediate (86). Since cofactor requirements, intermediates, and site of synthesis differed from the β-oxidation scheme, Wakil and Ganguly (87) concluded fatty acid synthesis was not the reverse of oxidation. As a pathway for synthesis, they suggested CO_2 became linked to acetyl-CoA on the methylene carbon, yielding malonyl-CoA.

The malonyl-CoA reacted then with a molecule of acetyl-CoA, as shown below:



CO_2 was then split off and the five-carbon derivative was reduced - leaving a four-carbon fatty acid derivative of CoA. The four-carbon derivative was then thought to react with another molecule of malonyl-CoA and the same series of steps was repeated until a six-carbon derivative was produced, and so on.

Using the same extra-mitochondrial enzymes, Brady (7) found acetaldehyde and malonyl-CoA could react to form fatty acids. TPNH and Mn^{++} were required but ATP was not. Acetyl-CoA did not replace malonyl-CoA unless ATP also was added. When acetyl-CoA was used rather than malonyl-CoA, he, as well as the Wisconsin group, felt that ATP was required to activate CO_2 to allow the formation of malonyl-CoA. He believed TPNH was required to effect the reduction of acyl-CoA derivatives to the corresponding free aldehydes which then reacted with malonyl-CoA molecules. The reaction was considered to be an aldol condensation of the Knoevenagel type which united aldehydes with the active α-carbon of malonyl-CoA. The sequence which he proposed is as follows:



The differences existing between the schemes proposed by Brady and the Wisconsin group are due probably more to differences in interpretation rather than changes in metabolic pathway brought about by the use of different substrates. Further work will be required before the sequence of formation of fatty acids by the pigeon liver can be resolved.

In plants, studies made thus far have suggested that fatty acid synthesis occurs through a malonyl intermediate pathway rather than through a reversal of the β-oxidation scheme (5, 59, 76). However, the site of synthesis has been shown to be the mitochondria rather than the cytoplasm (5, 59, 76, 79), as is the case for the malonyl intermediate pathway in animal systems. Thus,

this method of synthesis in plants is not entirely analogous to that of animals, or the differences in site of manufacture may have resulted from variations in sample preparation.

In summary, the results from all studies on glyceride fatty acid metabolism have shown that oxidation occurs mostly through the β-oxidation pathway in both plants and animals. Synthesis, on the other hand, appears to occur in a number of ways depending on interpretation and sample preparation as well as inherent differences in metabolism. In plants, however, all evidence indicates synthesis involves a malonyl intermediate and the process is localized in the mitochondria. Although much conflicting information now exists and much remains to be done before all aspects of fatty acid metabolism are understood, the progress in recent years has been remarkable and there is a suggestion that the understanding of the metabolism of these compounds is at the threshold of rapid expansion. Furthermore, similarities in chemical and physical behavior indicate that data from the fatty acid studies can be used to clarify the metabolism of the waxes.

Wax acids, like glyceride fatty acids, are predominantly saturated monocarboxylic acids possessing even numbers of carbon atoms. By analogy, it seem possible

that two-carbon fragments also would be involved in the synthesis and degradation of wax acids. This is strengthened by the work of Sutton (81) and Fulco and Mead (19) showing acetate to be incorporated into compounds which can be classified as waxes. Other suggestions can be derived from the fatty acid studies: the activation of normally water-insoluble glyceride acids by CoA indicates wax acids may be activated in a similar manner; the occurrence of CoA-fatty acid derivatives suggests acyl-CoA compounds may be the intermediates involved in the formation of the other components of waxes.

Since more relevant data for the study of waxes were not available, the results and some of the techniques used in the study of glyceride fatty acids were used as the primary guides in the attempt to understand the metabolism of waxes in plants.

MATERIALS AND METHODS

I. PLANT MATERIAL

Two wax producing plants were used for this study. Candelilla, Euphorbia antisyphilitica Zucc., a plant that contains long-chain paraffins, alcohols, acids and esters (14), was obtained in 1957 from the Big Bend area of Texas by Mr. H. O. Mann. The large clones from the reed-like plant were subdivided into three sub-groups, each of which was potted, labeled and grown in the greenhouse.

Fruits from jojoba, Simmondsia chinensis (Link) Schneider, which contains mono-unsaturated fatty acids and alcohols of 20 and 22 carbon atom lengths (26), were obtained in May and June of 1960 and 1961. The collections were made in the Saguaro National Monument and the Arizona-Sonora Desert Museum, Tucson, Arizona.

II. CULTURE METHODS

With the exception of special cases which are indicated in the results, the culture methods for both candelilla and jojoba were similar; two types of culture chambers were used. The initial studies of acetate incorporation into candelilla and jojoba were made with chambers of the type used by Abdul Wahab (1). These chambers consisted of a deep petri dish (90 x 150 mm)

which held 3 ml acetate culture solution (0.12 mg sodium acetate/ml) in a 12 x 57 mm petri dish top. A vial containing 2 ml 1 N NaOH was placed in the main chamber but outside the culture dish and used as a CO_2 trap. The lid of the main chamber was sealed with Dow Corning high vacuum silicone grease to prevent gas exchange with the atmosphere.

Later studies were made using square, 1 pint Mason jars which were placed in a horizontal position and stabilized with flat boards which were glued to the lower side. The jars were either sealed with a standard #12 rubber stopper or were fitted with a CO_2 -generation apparatus or other special equipment. Although the narrow neck of the jars allowed the use of only a 15 x 50 mm petri dish which contained 2 ml of acetate culture solution, the results were similar to those obtained using 3 ml of medium.

In studies with candelilla, only the green stems were used for the culture material. Using either culture chamber, the plant material was prepared for culturing by sectioning the 3 to 5-mm diameter candelilla stems into 3 cm segments. For uniformity, the segments were obtained from an area 20 to 30 cm below the stem apex. The segments were then sliced in half longitudinally and quickly placed cut side down into a large petri dish containing a small amount of water to prevent drying.

Eleven (or nine when cultures were made in Mason jars) half-cylindrical stem segments were selected at random and transferred cut side down into the culture solutions. The chambers containing the cultures were next placed in controlled light and temperature boxes maintained at 27.5 ± 1.5 C and 700 ft-c light. Illumination was supplied using three 15-watt daylight and three 15-watt cool white fluorescent lamps and the light intensity was measured using a Weston Model 603 light meter held at culture level. Dark cultures were placed in coffee cans alongside the light treatments. The total time which elapsed from first cutting of stems to initiation of culture was no longer than one-half hour.

In most studies with candelilla, the stems were obtained from selected plants grown in different areas of the greenhouse and were mixed thoroughly before as well as after sectioning. When direct comparisons were made between two treatments, however, all culture material was obtained from a single plant; in addition, the adjacent halves of each stem segment were placed in the opposing treatments.

Cultures of jojoba were run using the same chambers and in about the same manner as that used for candelilla. Instead of stem sections, however, nearly mature embryos were sliced lengthwise into 2 mm thick sections which were

placed one layer deep in the culture dishes. The number in each dish was the maximum possible without overlapping.

III. ANALYTICAL PROCEDURES

A. Carbon dioxide collection and analysis:

Except in studies which required the addition of gases to cultures, the respired CO_2 was collected during the culture period using 2 ml of 1 N NaOH. After collection, the carbonate was precipitated as $BaCO_3$ by the addition of 2 ml of 4 M NH_4Cl and 2 ml 1 M $BaCl_2$, and centrifuged. The precipitate was next washed twice with 4 ml water, taken up in alcohol, and plated onto a tared, flat, 22 mm copper planchet. The planchet was dried in a pan over a steam bath, reweighed, and the radioactivity of the sample was determined using a Tracerlab SC-16 windowless flow counter. To prevent self absorption errors, the radioactivity of each planchet was determined using a 4 to 6 mg sample of $BaCO_3$; total weight was determined later after adding the remainder of the precipitate.

Square Mason jars were used exclusively as culture chambers in studies aimed at determining the effect of added CO_2 on acetate incorporation into waxes. Each rubber stopper which sealed the jar was drilled and fitted with an air inlet tube which extended into the chamber, an air outlet tube that projected only lightly into the container, and a bent eye dropper and bulb filled with 10 percent

perchloric acid. The eye dropper was shaped so the acid could be dropped into a small beaker inside the chamber which contained $BaCO_3$. After the culture, excess CO_2 was removed from the chamber and collected in the hood by bubbling the gas through a series of tubes that each contained 5 ml of 1 N NaOH. The collection of gas was effected by repeatedly evacuating the chamber by bubbling the CO_2-air mixture into the NaOH solutions, then filling with CO_2-free air for a total of 10 minutes. The CO_2-free air was obtained by filtering air through a 1 x 12 in. soda lime column (Baker's 8-16 mesh). The recovered CO_2 was then weighed and counted for radioactivity as described previously.

B. Separation and analysis of waxes:

The results from jojoba were obtained mostly from studies of acetate incorporation into the wax fraction as a whole. After culture, the soluble radioacetate was washed from the 2 mm thick embryo slices and the slices were transferred to a beaker containing approximately 50 ml of ligroine (0.67-0.69 density). The ligroine was then heated to boiling on a hot water bath; after 1 min the supernatant solution of ligroine and wax was transferred to a second tared beaker. The procedure was repeated twice using 30 ml ligroine each time and the supernatant solutions were all combined. The combined solution in each beaker was then cooled to room temperature, washed once

with 20 ml of 1 percent K_2CO_3, then 3 times with 20 ml each of distilled water to remove soluble acetate. After all solvent and water were removed, each beaker was reweighed, made up to 15 ml with ligroine and an aliquot was removed for radioassay.

The limited supply of young jojoba fruits prevented an extensive study of the biosynthesis of waxes by this plant. On the other hand, the greenhouse supply of candelilla was considerable. As a result, this research has been directed primarily toward the biosynthesis of waxes in candelilla and the analysis of the waxes of candelilla was considerably more thorough and detailed. The steps required for candelilla wax separation and analysis are presented below.

1. Removal of resin from candelilla wax.

The process for removal of resin from candelilla wax is a slight modification of that suggested by Wilder (Wilder, E. A., personal communication, S. C. Johnson and Son, Racine, Wisconsin): it depends on the solubility of terpene-type resins and insolubility of candelilla wax in alcohol at 25 C.

At the termination of each culture, the plant material was washed free of adhering acetate solution with water and transferred to a 150 ml beaker containing hot 95 percent ethyl alcohol, and killed immediately. The alcohol

was then boiled for 10 minutes and the hot alcohol solution of waxes and resins was filtered through Whatman #1 filter paper into a second 150 ml beaker. The boiling and filtration process was repeated twice with about 20 ml amounts of alcohol, and the combined wax and resin solution was evaporated to 50 ml. On standing overnight at 25 C, the waxes precipitated and the resins remained in solution. The beaker containing the total mixture was then placed in the hood. The resin-containing solution was next filtered through Whatman #1 filter paper and the filtrate containing resins was discarded. The residue of wax was transferred quantitatively to a tared 150 ml beaker with hot toluene, and the final solution of about 70 ml was cooled, washed twice with 30 ml of 1 percent K_2CO_3 and 3 times with 30 ml of distilled water to remove free acetate and other short chain compounds. In this manner, nearly 30 percent of a sample of crude commercial candelilla wax was found to be of resinous material.

Since a large number of toluene dissolved wax samples was washed at one time with aqueous solutions, the use of separatory funnels was found to be quite cumbersome. Consequently, all washings were done directly in the tared beakers. After each washing, the lower aqueous phase was removed by using a clean, thin, U-shaped glass tube that was connected to a portable vacuum trap. The vacuum trap

consisted simply of a 250 ml Erlenmeyer flask which was attached to a 3 hole rubber stopper that contained the U-shaped extraction tube, the vacuum line tube, and a thumb-controlled vacuum regulation hole. Careful use of the apparatus showed less than 1 percent loss of wax.

2. Displacement chromatography of candelilla wax into paraffin, ester, alcohol and acid fractions.

General discussion - Since suitable methods for the separation of 50 mg samples of waxes were not available, the techniques used by Cole (16), Broadhead et al. (9) and Wilder (Wilder, E. A., personal communication, S. C. Johnson and Son, Racine, Wisconsin) were modified so that smaller samples could be used. The materials used for the standardization of the method and the subsequent separation of waxes into different chemical groups are listed below:

Crystalline paraffin
: Commercial crystalline paraffin was washed in successive batches of fresh concentrated sulfuric acid until no charring occurred when the acid was heated to 130 C (63). After the final heating and cooling, the paraffin plug was removed from the acid, washed with water, and recrystallized from acetone.

Stearic acid
: Matheson, Coleman and Bell (69-70 C) stearic acid was recrystallized twice

from ligroine (0.64 density) at 25 C. Observed MP = 70.3-71.2 C, reported MP (28) = 69.6 C.

Octadecanol

Matheson, Coleman and Bell (56-58 C) octadecanol was recrystallized 3 times from ligroine (0.64 density). Observed MP = 58.8-59.3 C, reported MP (74) = 58.5 C.

Octadecyl octadecanoate

The stearic acid prepared above was converted to the acid chloride (97) and the latter was reacted with octadecanol (96) to form the ester. The esterification was conducted under vacuum in a rotating flask at 2-4 mm Hg pressure and 97 0 (boiling water bath). The reaction time was 1 hour. The product was recrystallized 3 times from acetone at 15 C, MP = 59.0-59.5 C. Observed ester value = 100.4, Theoretical ester value = 104.5. Purity = 98.5%.

n-heptane

Phillips Petroleum, Bartlesville, Oklahoma (99 mol%) pure grade n-heptane was dried with Drierite and redistilled before use.

Silica gel

Davidson Chemical Company grade 12, 28-200 mesh silica gel was used without further treatment.

Alumina

Fisher Scientific Company, alumina, absorption Cat. #A-540, Lot #283097 was used without further treatment.

n-Propanol

Baker's analyzed, reagent grade.

Ethylene Dichloride

J. T. Baker, purified grade.

Acetic acid

Baker's Analyzed, reagent grade.

Various synthetic waxes were prepared in order to standardize the separation procedure. The waxes were prepared using crystalline paraffin, octadecanol, stearic acid, and octadecyl octadecanoate either singly or in different combinations. The specific mixtures used are listed in the appendix.

The separation method which was adopted showed 94 to 102% recovery of each of the materials used. In addition, there was no evidence that hydrolysis of esters occurred. Analysis of the resin-free candelilla wax from both commercial sources and plants used in research almost always showed 85% or better recovery of the total wax. While the recovery was not complete, it was considered satisfactory in view of the fact that weighing errors would contribute significantly to the total errors when only 20 to 100 mg resin-free wax was available for study.

Separation procedures- The columns used for separating waxes into chemical families by absorption chromatography were made by fusing a short length of 5 mm glass tubing to the base of a 6 in. culture tube that had been blown out previously. The extension of the culture tube was then tapered until a flow rate of about 30 ml water/min was obtained when the culture tube was 3/4 filled. Regulation of flow rate using tygon or neoprene tubing was not satisfactory since solubilization of the

tubing walls occurred.

In preparing for chromatography, the columns were plugged first with a piece of glass wool to prevent leakage of adsorbent. Six grams of silica gel were next placed into the columns and tamped into place. A layer of glass wool was inserted into the top of the adsorbent to prevent overflow of the column material, and the column was warmed with hot (nearly boiling) n-heptane.

The total wax fraction obtained after resin removal was taken up as soon as possible in hot n-heptane and quantitatively transferred to the silica gel column. Additional amounts of hot n-heptane were then passed through each column until about 150 ml of paraffin-containing eluent was collected in a tared 150 ml beaker. Next, a hot 10 percent by volume ethylene dichloride/heptane solution was passed through the column into another tared beaker until 150 ml ester-containing eluent was collected. In the same way, the alcohols and acids were removed as a mixture from the column by using hot 10 percent by volume n-propanol/heptane solution.

Although most of the studies in this research used silica gel extracts, a further separation of the alcohols from the acids was performed in some instances. In making the separations, a column containing 6 g of alumina was prepared in the same fashion as that used for silica

gel. The alcohols were removed first from the alumina column using 150 ml hot 2 percent by volume n-propanol/ heptane. The acids were removed later using 150 ml of hot 4 percent by volume acetic acid in heptane.

3. Determination of the radioactivity of the various wax fractions of candelilla.

After the wax samples were separated into the different components and the total weights determined, each wax was transferred quantitatively into a small, calibrated vial and made up to exactly 15 ml with a 10 percent n-propanol/heptane solution. Two 1/2 ml portions were then pipetted out and plated onto separate planchets and counted in a Tracerlab SC-16 windowless flow counter; the results were averaged. No corrections for self absorption were necessary since a plot of counts/planchet as a function of weight of added wax showed a linear relationship from 1 to 6 mg. For routine studies on radioactivity, none of the samples contained more than 4 mg wax.

Specific activities were determined as cpm/mg (counts per minute per milligram of sample). Total radioactivities were determined by multiplying the planchet cpm by 30, since each planchet contained 1/30 of the total wax fraction.

4. Vapor phase separation of the paraffin fraction of candelilla wax.

Preparation of standards- The n-paraffins used as standards for the gas chromatographic separation and analysis of the hydrocarbons in candelilla were 7, 14, 21, 27, 28, 29, 31, 33 and 35 carbon atoms in length. Heptane was obtained from Phillips Petroleum Company and was used as the solvent. The 14, 21 and 28 chain length paraffins were obtained from Eastman Kodak. The 27, 29, 31, 33 and 35 carbon length paraffins were prepared by using essentially the method of Piper et al. (63) who prepared C_{27}, C_{31} and C_{35} paraffins by a modified Clemmensen reduction of ketones formed by iron catalyzed reactions involving myristic, palmitic and stearic acids, respectively. The methods used in the study reported here differed from those used by Piper et al. (63) only in that the original Clemmensen method (15) was used to reduce the ketones since their modifications as well as the Wolff-Kishner method of Huang-Minlon (33) yielded products that dissolved completely in concentrated sulfuric acid. The reactions involved were:

Ketone formation (63):

$$2\ CH_3\text{-}(CH_2)_{16}\text{-}COOH \xrightarrow[Fe\ catalyst]{300^{\circ}C} CH_3\text{-}(CH_2)_{16}\text{-}\overset{\overset{O}{\|}}{C}\text{-}(CH_2)_{16}\text{-}CH_3$$

2 molecules stearic acid → Stearone

Clemmensen reduction of ketone (15):

$$CH_3(CH_2)_{16}\overset{O}{\overset{\|}{C}}(CH_2)_{16}CH_3 \xrightarrow[HCl]{Zn(Hg)} CH_3(CH_2)_{16}\overset{H}{\overset{|}{\underset{H}{|}}}C(CH_2)_{16}CH_3$$

Stearone — Pentatriacontane

In addition to the C_{27}, C_{31} and C_{35} paraffins, the C_{29} and C_{33} paraffins were prepared using the Piper et al. (63) method. The C_{29} and C_{33} symmetrical ketones were prepared from the C_{15} and C_{17} acids, respectively. The C_{17} acid was obtained from Eastman Kodak and was crystallized 3 times from ligroine (0.64 density). The C_{15} acid was synthesized from the C_{16} acid. The C_{16} acid was first brominated at the α-position using the Hell and Sadomsky (31) procedure and the α-bromo acid was converted to the α-hydroxy acid using the Le Sueur (45) technique. Finally, the α-hydroxy acid was oxidized with permanganate to the C_{15} acid according to Levene and West (44).

Some of the properties of the paraffins are given in Table I.

Conditions for gas chromatography of paraffins- Gas chromatography of paraffins was conducted using a modification of the method used by the Wilkins Instrument and Research Corporation (2). All studies were made using the Aerograph A-100 instrument at a filament current of 235 ma. Retention curves were plotted using Varian G-10 recorder. The column used was a 5 foot silicone column

(5 mm diameter) containing GE SF-96 high methyl silicone supported on C-22 firebrick in a ratio of 75 (firebrick)/ 25 (silicone) by weight. Helium was used as the inert gas phase and was passed through the column at the rate of 360 ml/min. The temperature of the column was maintained at 300 C. Paraffins were added as a n-heptane solution in amounts from 2 to 12 mg total paraffin.

Gas chromatography of standard paraffin samples- Prior to the analysis of the natural paraffins present in candelilla wax, suitable vapor phase procedures were developed using the standard paraffin samples. The studies with the prepared paraffins shed light on both the limitations and the sensitivity of the method and the quality of the synthesized product.

The retention times for the standards, when gas chromatographed, are shown in Table II; the graph of the log of the retention times as function of the number of carbon atoms in the paraffin molecules shows a linear relationship between C_{17} and C_{31} hydrocarbons (Figure 1). A composite diagram of the actual curves obtained (Figure 2) gives an indication of the purity of the standards used.

As shown in Figure 2, the diagram of the retention curves for C_{21}, C_{27}, C_{31} and C_{35} paraffins were quite sharp, indicating a high purity of material. On the other

TABLE I. Melting points and yields of the solid paraffins used as standards for gas chromatographic analysis; melting points and yields of ketones used in the preparation of the paraffins.

No. Carbon Atoms in Paraffin	Melting Point of Paraffin		Yield of Paraffin*	Melting Point of Ketone		Yield of Ketone**
	Observed (C)	Reported (C)	(%)	Observed (C)	Reported (C)	(%)
21	37.5-37.9	...	...	...	...	...
27	60.0-60.2	59.0-59.2 (63)	35	74.0-74.1	76.5 (27)	25
28	63.4-63.6	...	...	...	...	...
29	67.3-67.6	62.7-63.0 (63)	47	77.4-77.5	...	24
31	68.1-68.3	67.6-67.8 (63)	46	83.9-84.6	83.0 (27)	18
33	73.0-73.2	...	50	88.2-88.4	...	23
35	74.8-75.0	74.4-74.6 (63)	67	88.0-89.0	88.5 (27)	14

* Starting weights of ketones used in Clemmensen (15) reduction to paraffins were: C_{27}, 2.0 g; C_{29}, 1.9 g; C_{31}, 3.0 g; C_{33}, 1.4 g; C_{35}, 2.1 g.

** Starting weights of acids used for the formation of ketones (63) were: C_{14}, 15 g (formed C_{27} ketone); C_{15}, 25 g (C_{29} ketone); C_{16}, 25 g (C_{31} ketone); C_{17}, 15 g (C_{33} ketone) C_{18}, 15 g (C_{35} ketone).

TABLE II. Retention of paraffins of different chain lengths in a silicone column used in gas chromatography*.

No. Carbon Atoms in -Paraffin	Average Retention Time (min)	Log Retention Time
7	0.3	9.48 - 10
14	0.45	9.65 - 10
21	1.5	0.18
27	4.2	0.62
28	5.7	0.76
29	7.5	0.88
31	9.5	0.98
33	14.7	1.17
34 **	18.2	1.26
35	22.2	1.37

*A 5 mm diam x 5 ft long silicone (GE SF-96, high methyl) column was used in an Aerograph Model A-100 gas chromatograph (Wilkins Instrument and Research, Inc.). The silicone was supported on C-22 fire brick in a packing ratio of 75 (fire brick)/25 (silicone) by weight. Operating temperature was 300 C; helium was used at the rate of 360 ml/min; filament current was 235 ma; retention curves were recorded on a Varian G-10 recorder.

**This material appeared as an impurity in the C_{33} preparation.

hand, the retention curve for the C_{29} paraffin showed two peaks, one of which coincided with the C_{31} standard. Also, the retention curve for the C_{33} paraffin was quite spread out and three rather indistinct peaks were evident. The peaks coincided with the calculated value for the C_{33} and C_{34} paraffins and the observed value for the C_{35} paraffin. The presence of "accessory" peaks suggests, after consideration of the procedure for synthesis of the paraffins, that impurities were present in the odd-chain length acids before the ketones were formed. For example, if C_{17} and C_{18} acids were present together during the formation of the C_{33} symmetrical ketone, three different products could have resulted. Two C_{17} acids, two C_{18} acids and a C_{17} and C_{18} acid could react to give the C_{33}, C_{35} and C_{34} ketones, respectively. The results indicate that the higher homologs were present in both cases. Although the objective of the study was not to show the presence of impurities in the standards, the results do show that gas chromatographic methods provide excellent sensitivity for detecting the presence of paraffins separated by two carbon atoms. They also show that homologs below C_{33} which are separated by one carbon atom are not easily separable using the particular method. Undoubtedly, some of the difficulties were the result of injections of large amounts of paraffins into the column. The large quantity permitted the detection of

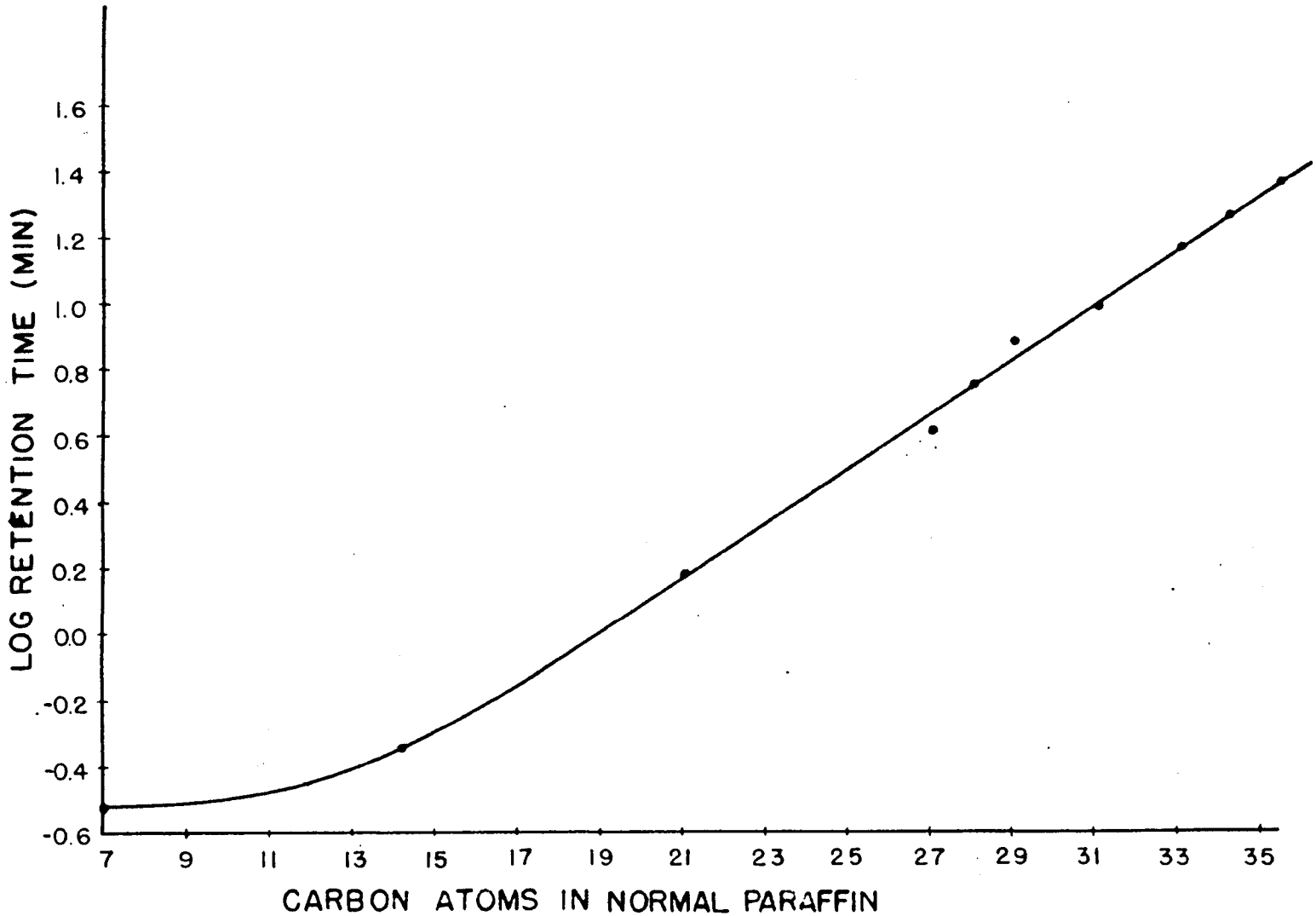


FIGURE 1. Retention times of paraffins of different chain length. A 5 mm diam x 5 ft long silicone (GE SF-96, high methyl) column was used in an Aerograph Model A-100 gas chromatograph (Wilkins Instrument and Research, Inc.). The silicone was supported on C-22 fire brick in a packing ratio of 75 (fire brick)/25 (silicone) by weight. Operating temperature was 300 C; helium was used at the rate of 360 ml/min; filament current was 235 ma; retention curves were recorded on a Varian G-10 recorder.

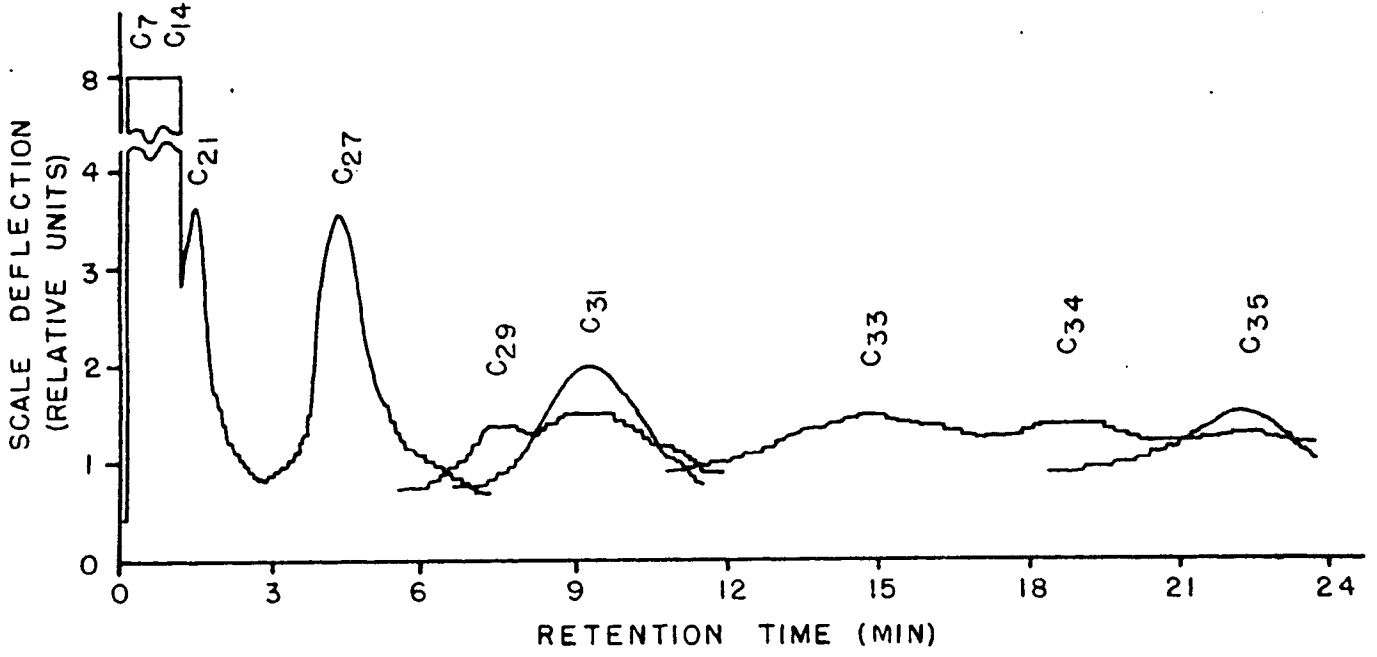


FIGURE 2. Gas-chromatographic retention curves for standard samples of n-paraffins. A 5 mm diam x 5 ft long silicone (GE SF-96, high methyl) column was used in an Aerograph Model A-100 gas chromatograph (Wilkins Instrument and Research, Inc.). The silicone was supported on C-22 fire brick in a packing ratio of 75 (fire brick)/25 (silicone) by weight. Operating temperature was 300 C; helium was used at the rate of 360 ml/min; filament current was 235 ma; retention curves were recorded on a Varian G-10 recorder. Sample weights were 4 mg; n-heptane was used as the solvent.

paraffins but also resulted in bleeding of one chain length fraction into the subsequent one.

RESULTS AND DISCUSSION

I. THE COMPOSITION OF WAX FROM YOUNG AND OLD CANDELILLA STEMS

Although the present understanding of the composition of candelilla wax is far from complete, studies by a number of workers have given some idea of its qualitative and quantitative make-up. Buchner (10) suggested that candelilla wax is about 20% resin, 50-52% C_{31} paraffin, 15% "mellisic" (C_{31}) acid which is present in both the free and esterified forms, and 5% "myricyl" (C_{31}) alcohol. Later studies by Chibnall et al. (14) and Shuette and Baldinus (70, 71) have shown that paraffin, acid and alcohol fractions are all mixtures of homologs rather than pure compounds. Chibnall et al. (14) indicated that the paraffin fraction consists of a 95:5 molar mixture of C_{31}:C_{33} homologs; later, Shuette and Baldinus (71) found the C_{29} homolog and suggested a C_{29}:C_{31}:C_{33} proportion of 5:90:5. Chibnall et al. (14) felt that both the acid and alcohol fractions are mixtures of 28, 30, 32 and 34 carbon-length homologs. In either fraction, the C_{30} and C_{32} homologs were thought to be present in about equimolar amounts and together represent about 80% of the total; the C_{28} and C_{34} were felt to constitute the remainder.

In an attempt to understand the biosynthesis of

waxes in candelilla, use was made of this information and waxes were separated into fractions containing only paraffins, esters or a mixture of acids and alcohols. In some cases, also, the acids and alcohols were separated further.

Before attempting to study wax metabolism using radio-carbon containing compounds, comparisons were made first of the wax content and composition of young and old stem sections. It was hoped that if changes of composition with age do occur, the differences would give some idea of the pathway of synthesis of the wax.

In studies with candelilla, "young" stems were considered to be those which were distinctly green and which were obtained in the region from 0 to 10 cm from the apex of shoots possessing ephemeral leaves. "Old" stems were distinctly grayish in appearance and were obtained from 20 to 30 cm away from the apex. Also, young stems appeared to possess (and actually did possess) less wax than the older stems. On a dry weight basis, older stems contained about 2.5 percent wax whereas younger stems contained less than 1 percent wax. The values in both cases were lower than the 3.5 percent reported by Shuette and Baldinus (70), which indicated wax synthesis was not terminated in the old stems.

The resin-free waxes obtained from young and old stems were then separated into paraffins, esters, and a

mixture of acids and alcohols. The percentages of the 3 fractions in young stems were then compared with those present in older stems, (Table III). In Table III and in all other studies of the same type, paraffin percentages were higher and mixed alcohol and acid percentages were lower in waxes from older stems. Ester percentages were not different. The differences which were noted were due to actual changes in composition rather than analytical errors resulting from solubility differences since nearly identical results were obtained using different sample weights of commercial candelilla wax.

From the standpoint of wax metabolism, the increase in paraffin and decrease in alcohol and acid percentages in older stems could have occurred as the result of formation of paraffins from acids or through changes in the relative rates of formation of paraffins, alcohols and acids. Thus, although alternate possibilities exist, the data are consistent with the hypothesis (12) that paraffins are formed by decarboxylation of the next higher acid.

Following the column chromatographic separation of waxes into different chemical groups, the paraffins alone were analyzed using gas chromatography (Figure 3) (Table IV). Figure 3 shows curves that are typical of all curves obtained in analyzing the paraffins from candelilla.

TABLE III. Paraffin, ester, and combined alcohol and acid percentages of waxes from young and old sections of candelilla stems.

Source of Wax	Total Wax* (g)	Paraffins (g)	%	Esters (g)	%	Alcohols & Acids (g)	%	Total Recovery %
ANALYSIS BY SEMI-MICRO METHOD**								
Seven 4-cm stem sections, 0-4 cm from apex	0.0277	0.0082	28.6	0.0045	16.2	0.0099	35.9	81.6
Seven 4-cm stem sections, 20-24 cm from apex	0.0944	0.0454	45.7	0.0145	14.6	0.0279	28.1	93.0
Commercial unrefined candelilla wax	0.0328	0.0183	55.8	0.0045	13.7	0.0068	20.8	90.2
Commercial unrefined candelilla wax	0.1000	0.0589	58.9	0.0112	11.2	0.0193	19.3	89.4
ANALYSIS BY S. C. JOHNSON METHOD***								
Six 10-cm stem sections, 0-10 from apex	0.0888	0.0280	31.5	0.0224	25.2	0.0393	44.2	101
Six 10-cm stem sections, 10-20 cm from apex	0.1650	0.0825	50.0	0.0351	21.3	0.0526	31.9	104

*Denotes weight of resin-free wax.

**Method is outlined in text. Each treatment represents the mean of 3 replications. Statistical comparisons of stem waxes were made separately from commercial wax. In stems, the 0-4 cm apical section had a significantly lower paraffin (5% level) and higher alcohol & acid percentage (1% level) than the 20-24 cm section. Ester values were not different (5% level). Sample weight had no effect on analysis of commercial wax (5% level).

***Each value represents a single analysis.

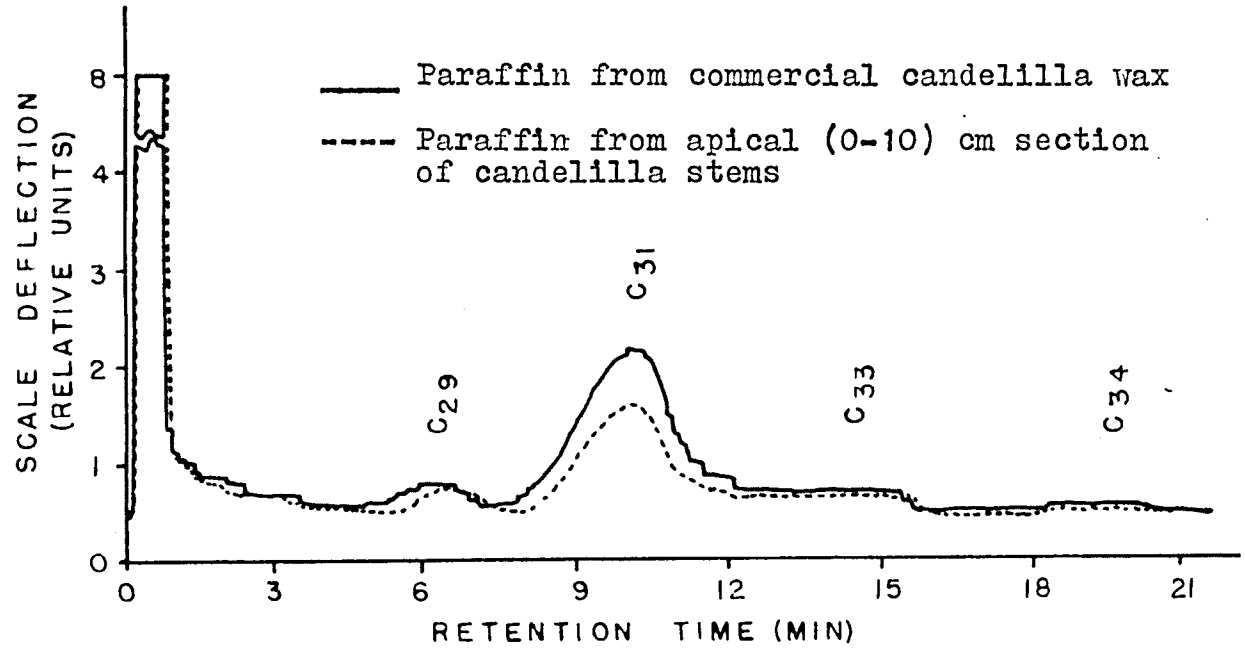


FIGURE 3. Gas chromatographic retention curves for paraffins from two sources of candelilla wax. A 5 mm diam x 5 ft long silicone (GE SF-96, high methyl) column was used in an Aerograph Model A-100 gas chromatograph (Wilkins Instrument and Research, Inc.). The silicone was supported on C-22 fire brick in a packing ratio of 75 (fire brick)/25 (silicone) by weight. Operating temperature was 300 C; helium was used at the rate of 360 ml/min; filament current was 235 ma; retention curves were recorded on a Varian G-10 recorder. Sample weights were: paraffin from commercial wax, 5 mg; paraffin from apical section, 4 mg; n-heptane was used as solvent.

TABLE IV. The chain lengths of paraffins present in young and old candelilla stem sections and commercial candelilla wax*.

Section of Plant Analyzed	Trial No.	Percent of Total Paraffin			
		C-29	C-31	C-33	C-34
Ten cm section, 0-10 cm from apex	1	6	75	19	Trace
	2	8	76	16	Trace
Ten cm section, 10-20 cm from apex	1	5	71	24	Trace
	2	4	79	14	Trace
Ten cm section, 20-30 cm from apex	1	3	82	15	...
	2	3	82	15	Trace
	3	3	80	17	Trace
	4	2	77	22	...
Commercial candelilla wax	1	5	80	15	Trace
	2	6	76	18	Trace

*The plants used were from a random selection of clones. The apical 10 cm sections were noticeably younger than the rest. The percentages of paraffins were estimated from comparisons of areas below curves of the type shown in Figure 3.

The percentages of different paraffin homologs were determined by analyzing the areas beneath the curves. As shown in Table IV, all samples, whether from young or old stems or commercial wax, contain about 5% C_{29}, 80% C_{31}, 15% C_{33} and trace amounts of C_{34} paraffins.

The existence of a constant proportion of paraffins in all samples presented suggestions on the nature of the enzymes controlling the metabolism of this group of compounds. Probably, degradation of paraffins in candelilla does not occur to a great extent or if it does occur, there is no noticeable preferential attack on any particular homolog. In the same light, it appeared that if paraffins are formed by decarboxylation of the next higher acids and if the decarboxylation is mediated by a fairly non-specific enzyme, the proportion of 29, 31 and 33-carbon paraffins should have been duplicated by the 30, 32 and 34 carbon acids. Unfortunately, the attempts to study the homolog ratios of the acids of candelilla were not completed and comparisons of acid to paraffin homologs were not made.

In addition to suggesting possibilities for further work, the comparison studies served as a "proving ground" for analytical methods as applied to natural samples. As a whole, the gas chromatographic separations of paraffins were not completely satisfactory since large amounts of odd-chain length homologs could have masked the detection

of small amounts of the next even-chained compound, particularly in the region from C_{27} to C_{33}. However, the ready detection of the C_{34} paraffin (which has not been reported previously) coupled with the ease of operation, suggests that gas chromatography could be developed into an ideal method of wax analysis. Undoubtedly, the use of smaller sample sizes along with a more sensitive recorder (2) should yield considerable improvement in the procedure.

II. STUDIES ON THE DETERMINATION OF THE SEQUENCE OF WAX FORMATION.

In addition to using changes in wax composition as indicators of the possible sequence of wax formation, more direct attempts were made using C^{14} tracer compounds. Initially, a kinetic study was made using sodium acetate-1-C^{14} as the substrate (Table V). Cultures were run for durations of 1, 2, 3, 8 and 20 hours in the light; in addition, a comparison was made using an 8-hour dark period. Following the culture period, the waxes were extracted, fractionated, and their radioactivities were determined. Another kinetic study using a series of both light and dark treatments was run and analyzed in the same manner (Table VI).

In Table V, the radioactivities of the wax fractions are expressed in terms of both total and specific activities. In Table VI and in most other cases, on the other hand, primary emphasis was placed on determining only total radioactivities since specific activities are affected by the large amounts of waxes present in the stems prior to culture. In the latter instances, however, specific activities can be estimated since cultures with 11 half-sections of candelilla stems yielded approximately 22 g

TABLE V. Kinetic study showing the distribution of radiocarbon from sodium acetate-1-C^{14} in CO_2 and wax fractions of candelilla*.

Culture Duration (hrs)	Carbon Dioxide			Paraffins		Esters		Alcohols & Acids	
	Wt (mg)	Specific Activity (cpm/mg)	Total Activity (cpm)	Specific Activity (cpm/mg)	Total Activity (cpm)	Specific Activity (cpm/mg)	Total Activity (cpm)	Specific Activity (cpm/mg)	Total Activity (cpm)
Light									
1	1.0	16,000	16,000	50	1,200	1,100	5,300	2,000	20,000
2	1.7	43,000	73,000	160	4,100	2,800	15,000	4,200	38,000
4	1.1	130,000	145,000	490	11,000	7,100	39,000	14,000	145,000
8	1.8	230,000	410,000	850	19,000	14,500	61,000	38,000	250,000
20	4.8	260,000	1,200,000	1,300	29,000	19,000	115,000	46,000	610,000
Dark									
8	6.2	530,000	3,300,000	890	17,000	20,000	110,000	23,000	240,000

* Eleven half-sections of candelilla stems (3 cm long) were placed in 3 ml of 0.01 M KH_2PO_4-K_2HPO_4 buffer solution (pH 6.5) which contained 0.36 mg sodium acetate-1-C_{14}. Total solution radioactivity was 0.051 mc (1.1×10^8 dpm); light intensity was 700 ft-c; temperature was 27.5 ± 1.5 C; cultures were run in duplicate under conditions of continuous CO_2 removal and results were averaged.

TABLE VI. Kinetic study made in the light and dark showing the distribution of radiocarbon from sodium acetate-1-C^{14} in CO_2 and different wax fractions of candelilla stems*.

Culture Duration (hrs)	Wt (mg)	Carbon Dioxide Specific Activity (cpm/mg)	Carbon Dioxide Total Activity (cpm)	Total Radioactivity of Wax Fractions Paraffins (cpm)	Esters (cpm)	Alcohols & Acids (cpm)
			Light			
0.5	2.9	3,800	11,000	1,400	300	2,300
1	1.7	15,000	25,000	5,800	800	6,400
2	1.7	65,000	110,000	10,000	1,600	14,800
4	2.0	140,000	273,000	25,000	6,300	59,300
8	3.3	170,000	560,000	32,000	13,400	146,000
20	2.3	420,000	973,000	65,000	23,300	252,000
			Dark			
1	2.8	24,000	68,000	6,300	900	12,000
2	2.1	155,000	330,000	2,600	1,400	14,400
4	3.7	200,000	740,000	8,900	2,500	27,400
8	4.1	450,000	1,800,000	13,000	3,700	57,300
20	12.6	1,840,000	23,000,000	21,000	14,100	246,000

*Eleven half-sections of candelilla stems (3 cm long) were placed in 3 ml of 0.01 M KH_2PO_4-K_2HPO_4 buffer (pH 6.5) which contained 0.36 mg sodium acetate-1-C^{14}. Total solution radioactivity was 0.051 mc (1.1×10^8 dpm); light intensity was 700 ft-c; temperature was 27.5 ± 1.5 C; cultures were run in duplicate with continuous CO_2 removal.

paraffins, 4 g esters, and 7 and 4 g of alcohols and acids, respectively. Cultures which contained nine half-sections yielded a proportionately smaller amount of wax.

The data from both Table V and VI show that the total radiocarbon content of the fractions containing mixtures of acids and alcohols are higher although their total weights are less than corresponding paraffin fractions. However, the results are not identical. In Table V, the alcohols and acids are 10 to 20 times more radioactive than the paraffins whereas in Table VI, only a 2-fold difference exists. Since the culture methods were identical, the results suggest that the metabolic state of the plant was altered somehow during the two week interval which separated the two cultures.

Although differences do exist between the two cultures, the results from both studies gave further support to Sutton's (81) suggestion that acetate or some substance formed easily from acetate is the natural substrate in wax formation by candelilla. Table VI shows that acetate is easily converted into wax since some radiocarbon from acetate-1-C^{14} is found in all wax fractions even after only 1/2 hour of culture. Also, Table V shows that in a 20 hour culture, a considerable (0.7%) transfer of radiocarbon from acetate to wax does occur. The presence of radioactivity in the wax fractions was not a result of

contamination of the fractions by acetate. This was shown by a subsequent determination of the radioactivity of the paraffins following their gas-chromatographic separation into component homologs. Although the collection technique was not refined sufficiently to permit the designation of specific values of radioactivity to specific homologs, the results did show that the incorporated radiocarbon was associated with C_{29}, C_{31} and C_{33} paraffins and not with impurities which may have been eluted along with the major fractions in the column chromatographic process.

The kinetic studies with acetate-1-C^{14} were undertaken with the hope that comparisons of changes in radioactivity would give some indication of the pathway of wax synthesis. Since many biological reactions are "steady state" reactions which yield an apparent constant concentration of intermediates, it was felt that if wax acids are intermediates in the formation of other fractions, radioactivities of the acid fractions should reach a maximum and level off. However, all fractions, including the acids, continued to increase in radioactivity with duration of culture (Table V). Although other factors (e.g., slow diffusion of acetate into plant material) may have been responsible for the lack of "leveling off", the results indicate that acids, as such, are not precursors to other fractions. More likely, it appears that all

fractions including the acids are end-products of metabolism.

In another type of kinetic study, candelilla stems were first cultured in sodium acetate-1-C^{14}, then washed and transferred to solutions which contained non-radioactive acetate of the same concentration (Table VII). Under these experimental conditions, it was felt that if acids are intermediates in the biosynthesis of other compounds, their radioactivities should decrease following exposure to non-labeled acetate solutions. The results show no changes in radioactivity in the acids or any other fraction, except in the 0 and 1-hr values for the alcohol fraction. This again indicates that all fractions are end-products of metabolism.

TABLE VII. Kinetic study showing changes in radiocarbon content of wax fractions of candelilla stem sections cultured *in vitro* after removal from a pre-culture solution containing sodium acetate-1-C^{14}*.

Culture Duration After Removal From Acetate-1-C^{14} Solution (hrs)	Paraffins		Esters		Alcohols		Acids	
	Specific Activity (cpm/mg)	Total Activity (cpm)	Specific Activity (cpm/mg)	Total Activity (cpm)	Specific Activity (cpm/mg)	Total Activity (cpm)	Specific Activity (cpm/mg)	Total Activity (cpm)
0	3,800	70,000	7,300	60,000	4,100	22,000	5,500	18,000
1	6,000	130,000	12,500	110,000	17,000	120,000	9,300	41,000
4.5	5,000	100,000	15,000	130,000	8,500	60,000	6,100	24,000
7	5,900	110,000	17,000	140,000	9,700	65,000	14,000	35,000

* Eleven half-sections of candelilla stems (3 cm long) were placed initially in 3 ml solution containing a total of 0.051 mc (1.1×10^8 dpm) sodium acetate-1-C^{14}. After 3.5 hrs, the stems were removed, washed, and transferred immediately to solutions containing an equivalent weight of non-radioactive sodium acetate (0.36 mg). Light intensity throughout was 700 ft-c; pH was maintained at 7.0 with 0.01 M KH_2PO_4-K_2HPO_4 buffer; temperature was 27.5 ± 1.5 C; cultures were run in duplicate and results averaged.

In a further attempt to elucidate the sequence of wax formation, C^{14} -labeled long-chain fatty acids were used as the sole source of carbon in candelilla stem cultures. The acids, which were biosynthesized previously by candelilla stems cultured in sodium acetate-1-C^{14}, were converted to water dispersible sodium soaps prior to use. The results (Table VIII) show that the radiocarbon of wax fatty acids can be transferred to other wax fractions. On the surface, this appears to conflict with the interpretations of results given in Tables V and VI. However, other studies with added CoA gave a tentative suggestion on how indirect rather than direct transformations of acids to paraffins and alcohols could occur in the event the latter compounds are actually formed from the acids. In the 27-hour cultures with added CoA (Table VIII), 43% of the radiocarbon of the fatty acid soaps was degraded into CO_2 whereas only 7.6% was transformed in the culture containing no added CoA. Similar percentage differences were obtained using 4-hour cultures but total radioactivities were proportionately less. The response to CoA suggests that degradation of wax acids, like degradation of glyceride fatty acids (38, 51, 53), requires the formation of an acyl CoA intermediate. By extrapolating further, since acyl CoA compounds are intermediates in the synthesis of glyceride fatty acids (7, 87), it seems likely that CoA derivatives of wax acids rather

TABLE VIII. Incorporation of long-chain fatty acids into wax fractions of candelilla*.

Cofactors Added**	Total CO_2 Radioactivity (cpm)	Recovery of Added Radiocarbon as CO_2 (%)	Total Radioactivity of Wax Fractions		
			Paraffins (cpm)	Esters (cpm)	Alcohols & Acids (cpm)
27 hr culture duration					
None	2,100	7.2	30	41	690
CoA	12,500	43.0	17	103	780
Thiamine	300	1.3	17	49	840
CoA, Thiamine	900	3.1	8	24	900
4 hr culture duration					
None	190	1.3	...	...	...
CoA	1,400	9.9	...	...	...

*Radioactive fatty acids were biosynthesized by candelilla stems from sodium acetate-1-C^{14}. The acids, believed to be 28, 30, 32 and 34 carbon atoms in length, were made into sodium soaps (pH 7.0) and used at the rate of 2 mg/2 ml culture solution; total radioactivity was 29,000 cpm/culture; temperature was 27.5± 1.5 C; light intensity was 700 ft-c. Results represent single cultures.

**Concentration of CoA (Pabst Laboratories, Lot 411) and thiamine HCl (California Foundation for Biochemical Research) was 10^{-3} M.

than the acids proper would be the intermediates in the formation of the wax components.

The study with labeled fatty acid soaps was a preliminary one and a number of unknown factors were present in the experiment. Although the wax acids were believed to be mostly 30, 32 and 34 carbon atoms in length, the actual chain length or position of labeling was not known. Also sodium soaps are probably not the natural substrates present in the plants, but solubility problems required their preparation and use. Nevertheless, candelilla stems are able to both degrade wax acids into CO_2 and transform them into other wax fractions. This, in turn, suggests future studies with specific long-chain compounds labeled in known positions should yield considerable information on both degradative and synthetic pathways involved in wax metabolism.

A preliminary study was also made to determine if soaps of labeled glyceride fatty acids could be transferred to the paraffin and ester fractions of the wax. There was no evidence of incorporation of radiocarbon from stearate and palmitate into the two fractions. However, there is, as yet, no reason to doubt that modifications in culture methods will give some transformation of these acids to waxes. By thorough use of labeled wax acids and labeled glyceride fatty acids and the techniques of Gastambide-Odier

and Lederer (20), it may be possible to show definitely that paraffins are formed by decarboxylation of wax fatty acids or by reactions of two glyceride fatty acids.

III. THE METABOLISM OF ACETATE IN CANDELILLA.

A. General considerations.

Although both kinetic studies with acetate-1-C^{14} (Tables V, VI) show that wax fractions generally increase in radiocarbon content with duration of culture, they show distinct differences in other aspects of acetate metabolism. The effect of light and dark were particularly evident. The following results were considered to be significant:

1) Radioactivities of wax fractions were usually higher in cultures exposed to 700 ft-c light than in comparable cultures in the dark.

2) In illuminated cultures, the total weight of evolved CO_2 was fairly constant whether the culture period was 1/2 hour or 20 hours. This indicates that most of the CO_2 was evolved at the start of culture and only a small amount was released later. On the other hand, dark cultures continued to evolve CO_2 throughout the culture period. After 20 hours, the total weight of evolved CO_2 in the dark was about 5 times that of the light cultures (Table VI).

3) The radioactivities of CO_2 evolved from cultures in the dark were from 3 to 20 times higher than in

corresponding illuminated cultures.

4) After 20 hours of culture in the dark (Table VI), about 20 percent of the added radiocarbon was converted into CO_2. In the light cultures, however, only about 1 percent of the added radiocarbon was released as gaseous CO_2.

5) In either the light or dark cultures, the total weight of evolved CO_2 was far more than that which could have been obtained from the added acetate. Thus, most of the evolved CO_2 was produced from endogenous sources.

The combined results of the kinetic studies suggest that light stimulates wax synthesis and inhibits respiration in candelilla. The evidence, however, is not conclusive since radioactivities of wax fractions were not always higher in the light than in the dark. Furthermore, although the cultures were run under conditions of continuous CO_2 removal, the extent of direct transfer of respired CO_2 to the CO_2-absorbing photosynthetic processes was not determined. Therefore, further studies were made to try to understand some of the factors involved in acetate metabolism and wax synthesis. Initially, attempts were made to eliminate or understand the factors responsible for the lack of consistency which occurred in the studies.

Table IX shows that waxes from short-term kinetic studies run at 17.5 C are about 1/2 or 1/4 as radioactive

TABLE IX. Kinetic study showing the effect of two temperatures on the distribution of radiocarbon from added sodium acetate-1-C^{14} in CO_2 and wax fractions of candelilla*.

Culture Period (hrs)	Carbon Dioxide Wt (mg)	Specific Activity (cpm/mg)	Total Activity (cpm)	Total Radioactivity of Wax Fractions Paraffins (cpm)	Esters (cpm)	Alcohols & Acids (cpm)
			17.5 C			
0.25	2.2	35,000	77,000	150	280	890
0.50	1.3	69,000	90,000	560	460	2,000
2.50	0.8	100,000	80,000	1,800	2,500	8,800
			27.5 C			
0.25	0.3	130,000	39,000	680	360	980
0.50	0.2	210,000	42,000	850	1,100	2,800
2.50	0.7	64,000	45,000	8,400	12,000	28,000

* Nine half-sections of candelilla stems (3 cm long) were placed in 2 ml of 0.01 M KH_2PO_4-K_2HPO_4 buffer (pH 6.5) which contained 0.24 mg sodium acetate -1-C-^{14}. Total solution radioactivity was 0.034 mc (7.3×10^7 dpm); light intensity was 700 ft-c; cultures were run in duplicate under conditions of continuous CO_2 removal and the results were averaged.

as waxes from cultures made at 27.5 C. The total radioactivities of the evolved CO_2 tended to be slightly higher in the 17.5 C cultures, however. These results show the importance of temperature control in acetate metabolism studies. Conceivably, some of the lack of consistency obtained in the determinations of wax radioactivity is due to unavoidable variations in temperature of the culture solution.

Studies were also made to determine the effect of pH on acetate transformation into waxes and CO_2 (Table X). The total radioactivity in either the wax fractions or evolved CO_2 was not affected by altering buffer solutions to give pH values ranging from 5 to 8. Furthermore, in studies using malonic acid (Table XIII) no significant difference existed between treatments which contained no malonic acid and those which contained 10^{-2}M malonic acid. Since the pH value of the external solution which contained the malonic acid was 2.6, the lack of variation due to pH differences indicates that the plant possesses a high internal buffer capacity. In addition, because bacteria are quite sensitive to pH changes, the absence of a "pH effect" represents evidence that bacteria were not primarily responsible for the metabolism of acetate in the cultures.

In conducting the studies using acetate-1-C^{14}, candelilla stems had been selected at random from a number

TABLE X. Effect of pH of culture solutions on incorporation of radiocarbon from added sodium acetate-1-C^{14} into CO_2 and wax fractions of candelilla*.

	Carbon Dioxide			Total Radioactivity of Wax Fractions		
pH	Wt (mg)	Specific Activity (cpm/mg)	Total Activity (cpm)	Paraffins (cpm)	Esters (cpm)	Alcohols & Acids (cpm)
5	1.0	120,000	120,000	130,000	89,000	64,000
6	0.8	135,000	110,000	110,000	51,000	57,000
6.5	1.1	200,000	220,000	79,000	63,000	46,000
6.5 Fe**	1.1	130,000	140,000	88,000	62,000	50,000
7	1.0	180,000	180,000	63,000	52,000	64,000
8	1.4	81,000	110,000	67,000	41,000	49,000

* Eleven half-sections of candelilla stems (3 cm long) were placed in 3 ml 0.01 M KH_2PO_4-K_2HPO_4 buffer solution which contained 0.36 mg sodium acetate-1-C^{14}. Total solution radioactivity was 0.051 mc (1.1×10^8 dpm); light intensity was 700 ft-c; culture duration was 4 hrs at 17.5± 1.5 C; cultures were run in duplicate under conditions of continuous CO_2 removal and results were averaged and analyzed statistically. Statistical analysis (5% level) showed that pH value of culture solutions had no effect on radioactivities of any fraction.

** Iron was added at the rate of 20 ppm Fe+++ as ferric citrate.

of clones which were grown in the greenhouse. A later observation of the plant supply showed that even genetically identical plants were physically different, depending on the location of the clone in the greenhouse. In all cases, the plants were grown for at least one year in the same locations before culture studies were initiated. Plants which received a high amount of sunlight (10,000-15,000 ft-c) during daylight hours were about 4 mm in diameter whereas those grown in the low light intensity area (2,000-4,000 ft-c) were about 2 to 3 mm in diameter and appeared to have a lower wax content. Since plants from the two areas were different physically, comparisons of the two types of plants were made to determine if they would give different results when cultured in acetate -1-C^{14}.

Table XI shows the results from cultures run on stems from two genetically identical plants which were grown in different areas of the greenhouse. Plant E_1 received light of low intensity whereas plant E_2 received light of high intensity during growth. Stems from both plants were cultured in acetate-1-C^{14} under 0 and 700 ft-c of light. After each stem section was sliced in half, one of the halves was placed in a culture solution which received light and the other half was placed in a comparable culture solution in the dark. The results from Experiment 1,

TABLE XI. Incorporation of radiocarbon from sodium acetate-1-C^{14} into CO_2 and wax of genetically identical plants grown in the greenhouse under different conditions*.

Plant Used for Culture**	Culture Light Intensity (ft-c)	Carbon Dioxide Wt	Specific Activity	Total Activity	Total Radioactivity of Waxes Paraffins	Esters	Alcohols & Acids
			Experiment 1 ***				
E_2	0	8.1	30,000	$240,000_a$	$18,000_a$	$34,000_a$	$83,000_a$
E_2	700	3.1	15,500	$48,000_b$	$22,000_a$	$31,000_a$	$120,000_a$
E_1	0	2.9	48,000	$140,000_a$	$7,700_a$	$14,300_a$	$56,000_a$
E_1	700	2.0	17,000	$36,000_b$	$20,500_a$	$25,000_a$	$110,000_a$
			Experiment 2 ***				
E_1	0	...	...	...	$4,300_a$	$22,000_a$	$23,000_a$
E_1	700	...	...	...	$7,100_b$	$15,000_a$	$39,000_b$

* Nine half-sections of candelilla stems (3 cm long) were placed in 2 ml 0.01 M KH_2PO_4-K_2HPO_4 buffer solution (pH 6.5) which contained 0.034 mc (0.24 mg) sodium acetate-1-C^{14}. CO_2 was removed continuously during culture; temperature of culture was 27.5 ± 1.5 C; culture duration was 5 hrs.

** Plants E_1 and E_2 were from the same rootstock. Plant E_1 was grown with approximately 2000-4000 ft-c maximum light intensity; plant E_2 received 15,000 ft-c during daylight hours.

*** In Experiment 1, 3 replicate cultures/light treatment were made for E_2 and 2 were made for E_1. Experiment 2 was run with 4 replications/treatment. Statistical comparisons were made of total radioactivities within fractions. Values found different at the 5% level have different subscript letters. Separate comparisons are indicated by horizontal lines.

Table XI, indicate that 700 ft-c illumination during culture has no effect on the radioactivities of the wax fractions of cultures which contained E_2 stems. Results of cultures of E_1 stems made at the same time suggest but do not show statistically that illumination increases the radioactivity of waxes. A later study (Experiment 2, Table XI) shows statistically that incorporation of radiocarbon into the paraffins and also the combined alcohols and acids of stems from plant E_1 is increased while incorporation into esters is not affected when cultures are illuminated.

Immediately after the cultures shown in Table XI were begun, plant E_2 (high light) was placed in complete darkness and preliminary acetate-1-C^{14} culture studies were made 6 and 14 days afterwards (Table XII). Studies made both 6 and 14 days after the plant was placed in the dark showed that cultures that received 700 ft-c light had twice the radioactivity in the alcohol and acid fraction as comparable dark treatments. The radioactivity values of the esters were not affected but the results for the paraffin fractions were surprising. When the plant was grown in the dark for 6 days before the stems were cultured, no difference existed in the radioactivity of paraffins of dark or illuminated cultures. After the plant was grown in the dark for 14 days, however, illumination of cultures resulted in an 8-fold increase in the radiocarbon content

TABLE XII. Effect of duration of exposure of candelilla plants to total darkness on the subsequent incorporation of radiocarbon into waxes of stem sections cultured in sodium acetate-1-C^{14}*.

Experiment No.	Days After Plant Placed in Total Darkness	Duration of Culture (hrs)	Culture Light Intensity (ft-c)	Total Radioactivity of Wax Fractions Paraffins (cpm)	Esters (cpm)	Alcohols & Acids (cpm)
1	0	5	0	18,000	34,000	83,000
	0	5	700	22,000	31,000	120,000
2	6	5	0	23,000	40,000	100,000
	6	5	700	19,000	36,000	210,000
3	14	8	0	1,700	16,000	81,000
	14	8	700	14,000	25,000	190,000

* Nine half-sections of candelilla stems (3 cm long) were placed in 2 ml of 0.01 M KH_2PO_4-K_2HPO_4 buffer (pH 6.5) which contained 0.24 mg sodium acetate-1-C^{14}. Total solution radioactivity was 0.034 mc (7.3×10^7 dpm). CO_2 was removed continuously during culture. Plant used for culture (E_2) was given up to 15,000 ft-c light during daylight hours for over 1 yr before being placed in the dark. Three replications/treatment were made in Experiment 1; 2 replications/treatment were made for Experiment 2; a single run was made for each of the treatments of Experiment 3.

of the paraffins.

The specific causes for the results obtained in both Tables XI and XII are still not known. However, the results of the two studies show that light definitely can increase the radioactivity of waxes when stems are cultured in sodium acetate-1-C^{14}. The results also show that conditions of growth prior to culture affect the response of cultures to light. Conceivably, the earlier kinetic studies (Tables V, VI) were influenced by differences in growth conditions of plants before culture as well as by temperature fluctuations during culture.

Interestingly, results of the light-dark studies correlate well with a recent observation made by Juniper (34). From electron microscopic data, he found that light definitely increased exudation of wax by Alaska peas which were grown previously in the dark. Quite possibly, light may be a direct stimulant for wax synthesis in a great many plants.

B. The effects of various additives on wax synthesis and other aspects of acetate metabolism.

Since light is a form of energy and since it appears to affect wax synthesis, further attempts were made to understand the energy relationships involved in wax synthesis and the possible role of light in the formation process. Initially, various inhibitors were added to

acetate-1-C^{14} cultures of candelilla stems to determine the energy relationships involved in wax synthesis and the possible pathways of wax synthesis. Table XIII shows results obtained from stem cultures in the presence of 10^{-2} M malonic acid, 5 x 10^{-1} M hydroxylamine hydrochloride, and 10^{-3} M 2,4-dinitrophenol (DNP). The inhibitor concentration used in each case was selected after preliminary studies were made with serial dilutions of each inhibitor.

Malonic acid, a specific inhibitor of succinic dehydrogenase in the citric acid cycle, was added with the objective of stopping the formation of ATP through aerobic respiration. Preliminary studies with 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} M malonic acid showed that the acid had no effect on the radioactivity of CO_2 or the wax fractions. Since 10^{-2} M malonate is a relatively high concentration of inhibitor, a second study was made using 3 replications/treatment of the same concentration to recheck the results (Table XIII). The data from the latter study also show that 10^{-2} M malonate has no effect on the radioactivity of CO_2 or the wax fractions. The use of 10^{-1} M malonate, on the other hand, results in a drastic reduction in CO_2 production and radioactivity of CO_2 and wax fractions (Table XIV). The results are somewhat confusing since 10^{-2} M malonate usually results in a distinct inhibition of the citric acid cycle in most other organisms. The inhibition by 10^{-1} M malonate cannot

TABLE XIII. Effect of malonic acid, hydroxylamine and DNP on the incorporation of radiocarbon from sodium acetate-1-C^{14} into respired CO_2 and wax fractions of candelilla*.

Inhibitor Added**	Carbon Dioxide Wt (mg)	Carbon Dioxide Specific Activity (cpm/mg)	Carbon Dioxide Total Activity (cpm)	Paraffins Specific Activity (cpm/mg)	Paraffins Total Activity (cpm)	Esters Specific Activity (cpm/mg)	Esters Total Activity (cpm)	Alcohols & Acids Specific Activity (cpm/mg)	Alcohols & Acids Total Activity (cpm)
None	1.4	45,500	$63,000_a$	2,200	$62,000_a$	5,100	$37,000_a$	10,000	$135,000_a$
Malonic	1.1	88,000	$96,000_a$	3,600	$75,000_a$	6,700	$37,000_a$	11,000	$120,000_a$
$NH_2OH \cdot HCl$	2.5	60,000	$150,000_a$	80	$1,300_b$	980	$4,800_b$	1,600	$19,000_b$
DNP	2.6	160,000	$420,000_b$	240	$4,200_b$	810	$4,900_b$	800	$10,000_b$

* Eleven half-sections of candelilla stems (3 cm long) were cultured for 4 hrs in 3 ml 0.01 M KH_2PO_4-K_2HPO_4 buffer solution containing 0.36 mg sodium acetate-1-C^{14}. Total radioactivity was 0.051 mc (1.1×10^8 dpm). Original pH of 6.5 was maintained only for control treatment and DNP treatment; pH of malonic acid was 2.6; pH of hydroxylamine treatment was 4.7. Temperature of culture was 27.5± 1.5 C; light intensity was 700 ft-c; results are means of 3 replications. Total radioactivities were analyzed statistically. Values within any column having the same subscript letters are not significantly different at the 5% level. Values with different subscript letters are different at the 1% level.

** Concentrations of inhibitors were: malonic acid, 10^{-2} M; DNP, 10^{-3} M; $NH_2OH \cdot HCl$, 5×10^{-1} M.

TABLE XIV. Effect of malonic acid and DPNH on the incorporation of radiocarbon from sodium acetate-1-C^{14} into evolved CO_2 and wax fractions of candelilla*.

Molar Conc of Additives	Carbon Dioxide**		Total Radioactivity of Wax Fractions**		
	Wt (mg)	Total Activity (cpm)	Paraffins (cpm)	Esters (cpm)	Alcohols & Acids (cpm)
None added	1.0	$71,000_a$	$41,000_a$	$29,000_a$	$98,000_a$
10^{-6} DPNH	0.9	$110,000_a$	$39,000_a$	$31,000_a$	$81,000_a$
10^{-1} Malonate	Trace***	$5,000_b$	600_b	800_b	$2,800_b$

* Nine half-sections of candelilla stems (3 cm long) were placed in 2 ml 0.01 M KH_2PO_4-K_2HPO_4 buffer solution (pH 6.5) that contained 0.24 mg sodium acetate-1-C^{14}. Malonate conc, however, was so high that pH was reduced to 1.6. Total radioactivity of culture was 0.034 mc (7.3×10^7 dpm); culture time was 6 hrs; temperature was 27.5 $\pm$ 1.5 C; CO_2 was removed continuously from culture. Values are means from 3 replicate cultures.

** Statistical analysis was run on values within columns. Values having different subscript letters are significantly different at the 1% level; values having the same subscript letters are not different at the 5% level.

*** CO_2 determination for the treatments containing malonic acid required the addition of a small amount of potassium carbonate as co-precipitant.

be considered proof that wax synthesis depends on aerobic respiration because the particular concentration of acid reduced the pH to 1.6 and other effects (e.g., protein denaturation) could have been responsible for the observed results. It is possible that candelilla has a mechanism for utilizing or degrading any malonic acid which is present in the stems.

Although the role of hydroxylamine as an inhibitor is not understood well, in candelilla studies, the material was added with the intention of converting carbonyl-containing compounds (particularly any possible keto acid intermediates of wax formation) into non-metabolizable oximes. It is recognized that the action of hydroxylamine is not as specific as malonic acid and keto compounds other than possible wax intermediates (e.g., α-keto glutaric acid) could have been affected. In addition, since hydroxylamine is considered to be an intermediate in amino acid metabolism (84), it could even enhance some metabolic processes. In this respect, the use of 10^{-4} M hydroxylamine hydrochloride actually appeared to stimulate paraffin synthesis in a preliminary study although the synthesis of other fractions did not appear to be affected. On the other hand, the use of 5×10^{-1} M hydroxylamine hydrochloride in acetate-1-C^{14} cultures reduced the radioactivities of all wax fractions significantly (Table XIII). The radioactivity of CO_2 was

not affected. The reduced radioactivity of wax fractions probably was not due to pH depression since solutions containing 10^{-2} malonate had lower pH values and did not affect the radioactivity of either CO_2 or wax fractions. It appears, then, that hydroxylamine or the Cl^- ion (added as hydroxylamine hydrochloride) reacts with intermediates involved in the formation of all wax fractions and thereby reduces total wax synthesis. The citric acid cycle is probably not affected.

Table XIII also shows results obtained with DNP. DNP was added to cultures to determine if ATP was the particular high energy compound involved in wax synthesis. Initially, DNP was recognized as an inhibitor which specifically uncoupled high energy phosphates from ATP produced in oxidative phosphorylation (48) but recent studies have shown that the compound also uncouples phosphates from ATP produced in photophosphorylation (4, 93). The results of Table XIII show that DNP reduces wax synthesis and at the same time increases the radioactivity of the evolved CO_2. This indicates that ATP is the high energy compound required for the endergonic process of wax synthesis.

Since DNP results in a reduction of wax synthesis, studies were made to determine if the addition of ATP to cultures would, on the other hand, stimulate wax synthesis

(Table XV). A preliminary culture in the light suggests that 10^{-2} M ATP can increase the synthesis of all wax fractions. A later study in the light supported the results but differences between treatments receiving 0 or 10^{-2} M ATP in the dark were not so definite. Also, even in studies conducted in the light, the differences in the second study were not as conspicuous as in the first experiment.

The erratic behavior in the presence of ATP suggests other factors are also involved. Conceivably, the ATP was decomposed in the three week interval which separated the two experiments, or other compounds, perhaps a pyridine nucloetide, may also participate in wax synthesis. It seemslikely that pyridine nucleotides would be involved in wax synthesis because both TPNH (7, 76, 87) and DPNH (30, 42) have been shown to be required for the synthesis of glyceride fatty acids. However, a study with 10^{-6} M DPNH without ATP (Table XIV) shows that the particular concentration of material has no effect on the radioactivity of the wax fractions. Since concentration levels may be a critical factor, further studies using both DPNH and TPNH along with ATP will be required before it can be determined if these compounds also are involved in wax metabolism.

TABLE XV. Effect of ATP on the incorporation of radiocarbon from sodium acetate-1-C^{14} into the wax fractions of candelilla*.

Experiment No.	Treatment		Total Radioactivity of Wax Fractions			
	Light Intensity (ft-c)	ATP Conc (M)	Paraffins (cpm)	Esters (cpm)	Alcohols (cpm)	Acids (cpm)
1	700	0	43,000	66,000	55,000	30,000
	700	10^{-2}	250,000	170,000	235,000	97,000
2	0	0	23,000	70,000	36,000	15,000
	700	0	25,000	52,000	41,000	23,000
	0	10^{-2}	23,000	130,000	48,000	61,000
	700	10^{-2}	40,000	93,000	77,000	56,000

* Nine half-sections of candelilla stems were placed in 2 ml of 0.01 M KH_2PO_4-K_2HPO_4 buffer solution (pH 6.5) that contained 0.24 mg sodium acetate-1-C^{14}. Total radioactivity of culture solution was 0.034 mc (7.3×10^7 dpm); culture duration was 5 hrs; temperature was 27.5 $\pm$ 1.5 C; CO_2 was removed from cultures continuously. Values from Experiment 1 are from single cultures; values from Experiment 2 represent the mean from 2 replications.

C. Some effects of light on acetate metabolism and wax synthesis in candelilla.

In comparing results from light and dark cultures of candelilla stems in acetate-1-C^{14} (Tables V, VI, XI), all data show that total production of CO_2 and radioactivities of CO_2 are considerably lower in cultures run in the light. It is noteworthy that the depression of CO_2 production by light is not a characteristic which is unique to candelilla. Many workers using other plants (1, 40, 62, 82, 92) have noted the same effect and future studies will show, undoubtedly, the phenomenon to be a fairly general one.

In a system which bears many similarities to candelilla, Abdul Wahab (1) noted that light reduced CO_2 evolution in flax embryos and also increased the synthesis of total lipides believed to be mostly glyceride fatty acids. Recently, Smith (75) confirmed the observations and showed that visible light of all wave lengths stimulated the synthesis of stearic and oleic acids. As an explanation for the effect, Abdul Wahab suggested photophosphorylation in the light created at least part of the ATP required for fat synthesis; in the dark, fat synthesis was believed to be dependent totally on aerobic respiration and the rate of CO_2 evolution was consequently increased. It is possible that the same type of phenomenon is involved in candelilla and other green, wax synthesizing plants.

In green plants, considerable difficulty exists in the measurement of respiration rates in the light since CO_2 produced in respiration can be recycled back into the plant through photosynthetic fixation of CO_2. However, in the studies shown in Tables V, VI and IX, the CO_2 evolved from the plants was trapped continuously in both light and dark cultures and little recycling probably occurred. Under the circumstances, since illumination of cultures reduced the total amount of evolved CO_2 as well as CO_2 radioactivity, it was felt that light reduced the rate of respiration in candelilla.

The results of Table XVI suggest that even if CO_2 was not removed continuously during culture, the recycling of CO_2 in candelilla cultures would not be extensive. The treatment containing added radioactive CO_2 and non-radioactive acetate showed that only a small amount of radiocarbon from CO_2 was incorporated into the waxes. A comparison of the acetate-1-C^{14} illuminated culture in which CO_2 was removed continuously with the illuminated culture which contained added non-radioactive CO_2 showed little, if any, difference in wax fraction radioactivity. The lack of a dilution effect suggests that CO_2 contributes only a small part of the total carbon in waxes in cultures containing substantial amounts of acetate.

TABLE XVI. Effect of added CO_2 on the incorporation of radiocarbon from sodium acetate-1-C^{14} into respired CO_2 and the wax fractions of candelilla; incorporation of $C^{14}O_2$ into the waxes of candelilla*.

CO_2 Added + (mg)	Light Intensity (ft-c)	CO_2 Recovered		Total Radioactivity of Wax Fractions		
		Wt (mg)	Total Activity (cpm)	Paraffins (cpm)	Esters (cpm)	Alcohols & Acids (cpm)
0**	0	...	...	4,200	15,000	25,000
0**	700	1.2	93,000	30,000	53,000	69,000
4.4	0	6.6	310,000	7,300	25,000	14,000
4.4	700	5.0	68,000	18,000	33,000	55,000
4.4 $C^{14}O_2$	700	4.0	250,000	12	60	1,050

* All cultures except that containing 4.4 mg $C^{14}O_2$ were conducted using a 0.01 M KH_2PO_4-K_2HPO_4 buffer solution (pH 6.5) that contained 0.24 mg sodium acetate-1-C^{14} (0.034 mc). The treatment with $C^{14}O_2$ contained an equivalent weight of non-radioactive acetate. Nine half-sections of candelilla stems were used in each culture; culture duration was 4 hours. Temperature was 27.5 ± 1.5 C. Total radioactivity of CO_2 in the treatment with 4.4 mg $C^{14}O_2$ was 320,000 cpm. Results are values from single runs made in 500 ml culture chambers.

** Cultures containing 0 mg CO_2 also contained a vial containing 2 ml of 1 N NaOH which removed evolved CO_2 continuously during culture.

Although there is good evidence which indicates that light reduces respiration in candelilla stem cultures, attempts to link the lowered respiration to a production of high amounts of ATP by photophosphorylation were inconclusive. The studies with DPN (Table XIII) showed that ATP is required for wax synthesis but the source of ATP generation was not found. The concentration of malonic acid required for inhibition in candelilla stems was so high that unqualified statements could not be made regarding the nature of this inhibition (Table XIV). In a further attempt to clarify the role of light in respiration and wax synthesis, a preliminary study was made using continuous N_2 flow to create anaerobic conditions (Table XVII). In a N_2 atmosphere, it was felt that ATP formation from oxidative phosphorylation would be reduced under circumstances which were considerably milder than in the malonic acid study. At the same time, it was felt that photophosphorylation would not be inhibited since it is known to occur in a N_2 atmosphere (3). In the N_2 atmosphere studies, little or no difference in the radioactivities of CO_2 from light and dark treatments was found, but the radioactivities of the wax fractions were higher in the light. The paraffins, particularly, showed a 25-fold increase in radioactivity. Although further studies must be made before definite conclusions can be drawn, it appears that the increased radiocarbon content of waxes in the

TABLE XVII. Effect of N_2 gas on the incorporation of radiocarbon from sodium acetate-1-C^{14} into CO_2 and the wax fractions of candelilla*.

N_2 Flow Rate (ml/min)	Light Intensity (ft-c)	CO_2 Recovered		Total Radioactivity of Wax Fractions		
		Wt (mg)	Total Activity (cpm)	Paraffins (cpm)	Esters (cpm)	Alcohols & Acids (cpm)
0	0	2.7	650,000	14,000	22,000	19,000
0	700	2.2	91,000	24,000	18,000	23,000
50	0	4.4	160,000	900	9,800	11,000
50	700	2.8	120,000	23,000	14,000	36,000

* Nine half-sections of candelilla stems (3 cm long) were cultured for 5.5 hrs in 2 ml 0.01 M KH_2PO_4-K_2HPO_4 buffer solution (pH 6.5) that contained 0.24 mg sodium acetate-1-C^{14}. Total radioactivity was 0.034 mc (7.3×10^7 dpm); CO_2 was trapped continuously during culture. Temperature of culture was 27.5 $\pm$ 1.5 C. Results are from single runs using 500 ml culture chambers.

light may very well be the result of a direct transfer of ATP from photophosphorylation to steps in wax synthesis.

The effect of light on acetate metabolism in candelilla is not confined to respiration and wax synthesis, however. In studies on radioactivities of candelilla stems in acetate-1-C^{14} cultures, only about 20 percent of the total added radiocarbon was recovered in the two fractions even in the maximum culture duration (Table VI). Since 80 percent of the added radiocarbon was not accounted for, an attempt was made to determine the amount incorporated into the plant and the amount which was left in solution (Table XVIII). It is interesting to note that although illuminated cultures converted a far smaller amount of acetate-1-C^{14} to CO_2 (1 percent versus 15.7 percent), they absorbed a much higher amount of acetate into the plant tissues (86 percent versus 49.2 percent). The particular compounds formed following uptake into the plant tissue are not known but the results show the significance of light in still other aspects of acetate metabolism.

IV. SUGGESTIONS ON THE PATHWAY AND ENERGY SOURCES INVOLVED IN THE FORMATION OF WAXES IN CANDELILLA.

The combined data from all candelilla studies have not been sufficiently conclusive to yield a clear-cut understanding of the pathway of wax formation in the plant.

TABLE XVIII. The distribution of radiocarbon from acetate-1-C^{14} into CO_2, waxes, and other fractions of candelilla stems after 22.5 hours of culture*.

Light Intensity (ft-c)	Radioactivity Recovered as CO_2 (% of total activity added)	Radioactivity Recovered as Waxes (% of total activity added)	Radioactivity Left in Culture Solution (% of total activity added)	Radioactivity Incorporated into Non-wax Fractions** (% of total activity added)
0	15.7	0.8	34.3	49.2
700	1.2	1.0	11.6	86.2

* Nine half-sections of candelilla stems (3 cm long) were placed in 2 ml 0.01 M KH_2PO_4-K_2HPO_4 buffer solution (pH 6.5) which contained 0.24 mg sodium acetate-1-C^{14}. Actual radioactivity was 13.4 x 10^6 cpm. Culture temperature was 27.5 ± 1.5 C; CO_2 was removed continuously during culture - 1.6 mg was trapped in light cultures; 5.5 mg was trapped in dark treatments. All values are means of 4 replications.

** Radioactivities of non-wax fractions were determined by difference.

However, they do permit a tentative scheme of biosynthesis which can be used as a guide for future studies. Also, they have yielded suggestions on the energy sources and relationships involved in wax formation and other aspects of metabolism in candelilla. The accumulated information from the different studies has been summarized in Figure 4.

In the candelilla studies reported here, primary emphasis was placed on events which occurred after acetate was formed. Consequently, the natural formation of acetate in candelilla was accepted to be the conventional one involving photosynthetic formation of sugars followed by their breakdown through glycolysis or the pentose shunt pathway. After acetate is formed, it is then utilized partly in aerobic respiration, partly as a substrate for wax formation, and partly as a substrate for the formation of other compounds which have not been studied yet. Evidence for the first two uses is given by the fact that stems cultured in acetate-1-C^{14} invariably produced radioactivity in both evolved CO_2 and wax. However, Table XVIII shows that most of the acetate is not used in respiration or wax formation but is absorbed or incorporated into some other materials in the plant.

Although a number of attempts were made to try to elucidate the steps which existed between acetate and the wax product, very little is yet known about the intermediates

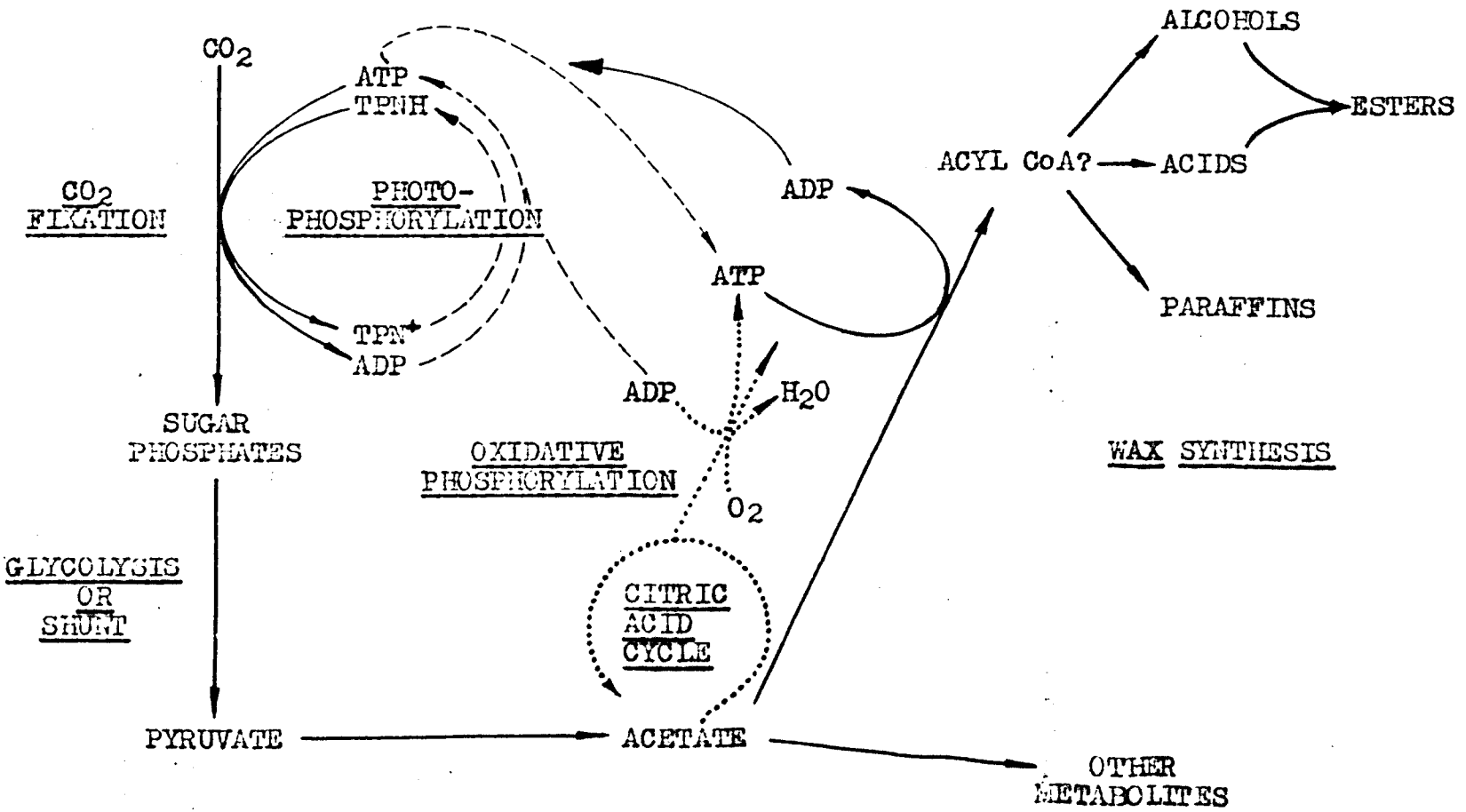


FIGURE 4. Proposed scheme of wax biosynthesis. Reactions believed to be induced by light are indicated by (---); Reactions inhibited by light are indicated by (•••); Reactions occurring in either light or dark are indicated by (——).

or reactions involved in their formation. Since acetate is incorporated into all wax fractions, it appears likely that some type of head-to-tail condensation may be involved in the formation of the components. However, it does not appear that wax acids, as such, are the precursors of the paraffins and alcohols, as was postulated by Chibnall and Piper (12). This was first indicated in a kinetic study using acetate-1-C^{14} (Table V) which showed that all fractions, including the acids, increased in radioactivity with duration of culture. The lack of "leveling off" of radioactivity suggested that each fraction was really an end product of metabolism but similarities in structure of the different chemical groups indicated the occurrence of a common precursor in their formation. When it was shown that CoA stimulated the breakdown of wax acids (Table VIII), from analogy with results from glyceride fatty acid studies, it appeared that acyl CoA compounds could very well be the postulated intermediates. A great deal more work will be required, however, before the statements are confirmed.

In retrospect, the gap which exists between labeled acetate and labeled wax components is probably so great that studies aimed at determining the sequence of formation of the different chemical groups should be made using substrates other than acetate. Since it has been demonstrated that radiocarbon from wax acids can be transferred to the

paraffin and ester fractions (Table VIII), future studies with long-chain compounds should give a considerable clarification of the sequences of formation. For example, if properly labeled compounds are used, it may be possible to show specifically that paraffins are formed by decarboxylation of wax acid derivatives or by reactions of two glyceride fatty acids.

Figure 4 also shows the energy sources believed to be involved in wax synthesis by candelilla. Wax synthesis is thought to be driven by ATP which in turn is generated either by oxidative or photophosphorylation. The reasons for this suggestion are as follows:

1) In the absence of inhibitors, cultures run in the presence of acetate-1-C^{14} invariably yielded radioactivity in all wax fractions in both light and dark. This indicates the overall metabolism of candelilla is balanced in such a fashion that wax synthesis (an energy requiring process) occurs at all times. One must assume, then, that an energy-releasing process must be occurring continuously to drive the synthetic reaction. The inhibition of acetate incorporation into wax fractions on addition of DNP (Table XIII) indicates that ATP is the specific high energy compound required for biosynthesis of waxes.

2) In the dark, photophosphorylation would not be a factor in wax synthesis and the energy for wax formation

would come necessarily from oxidative phosphorylation or some other energy releasing step. The fact that incorporation of acetate into the waxes occurs in the dark under aerobic conditions and is inhibited under dark anaerobic conditions (Table XVII) shows that ATP generation through oxidative phosphorylation can be an important factor in wax synthesis.

3) In the light, the rate of respiration is lower in all cases than in comparable dark treatments although the incorporation of radiocarbon into the waxes is usually higher (Tables V, VI, XI). In the light, then, wax synthesis is dependent to a large extent on photophosphorylation for the necessary ATP. This conclusion is supported by studies run under anaerobic conditions created by continuous N_2 flow (Table XVI). Radioactivities of wax fractions under illuminated anaerobic conditions were as high as those obtained in illuminated aerobic cultures. Wax radioactivities (particularly the paraffin fraction) of the dark anaerobic cultures were lower than in the dark aerobic cultures.

ATP, however, is not the total regulatory factor involved in wax synthesis. Radioactivities of the wax fractions were not always higher in the light than in the dark cultures (Table XI) and the addition of ATP did not always result in a stimulation of acetate transformation into

waxes (Table XV). This suggests a factor other than ATP is also involved in the regulation of wax synthesis but the factor is still unknown.

Interestingly enough, these results have given a logical basis for exploring a problem in plant physiology which is not necessarily related to wax synthesis. In the past, in studies on photosynthesis, it was generally assumed for the sake of simplicity that respiration in the light is the same as in the dark. As a result, a dogma has developed which resists any suggestions that light can alter the respiration rate in plants. In candelilla, the results very strongly suggest that respiration in the light is considerably less than in the dark and an examination of the possible causes indicates this may exist in other plants.

Conceivably, the rate of respiration in a system such as candelilla (Figure 4) can be decreased by either increasing the amount of ATP (a product of respiration) or by decreasing the amount of ADP, inorganic phosphate, or intermediates of glycolysis. On the other hand, the rate of respiration can be increased through the utilization of ATP or by increasing the amount of ADP. Indeed, Lardy and Wellman (43) have shown that additions of ADP to mitochondrial systems increases oxygen uptake 10-fold.

One might extend this knowledge by adding that which is known about higher plants. It is now known that ATP from photophosphorylation can actually drive the synthesis of starch from glucose under anaerobic conditions (52). This indicates a "leakage" of ATP from chloroplasts. In addition, the requirement of chloroplasts for ADP to carry on photophosphorylation is now known (4). In candelilla, it seems reasonable to believe that when cultures are illuminated, the ADP ordinarily used for respiration in the dark is utilized, instead, in photophosphorylation. Hence, the respiration rate decreases when cultures are illuminated. The higher rate of wax synthesis in the light is probably the result of a shunting of ATP produced in photophosphorylation toward the wax synthesis pathway.

V. THE BIOSYNTHESIS OF WAXES IN JOJOBA (*SIMMONDSIA CHINENSIS* (LINK) SCHNEIDER)

Jojoba wax, unlike candelilla and most other waxes, is an oily liquid rather than a solid. Investigations of its components have shown that the wax consists largely of esters of mono-unsaturated alcohols and acids of 20 and 22 carbon atoms in length (17, 26). Green, Hilditch and Stainsby (26) suggested that the chief acids are $\Delta^{11:12}$-eicosenoic (C_{20}) and $\Delta^{13:14}$-docosenoic (C_{22}). The alcohols are considered to be eicosenol and docosenol and

are believed to be unsaturated in the same positions as the corresponding acids. In both the acid and alcohol fractions, the C_{20} compounds are considered to be present in higher amounts. Daugherty, Sineath and Wastler (17), on the other hand, indicated that the major components are: eicosenoic acid (30.4 percent), docosenoic acid (14.2 percent), eicosenol (14.6 percent) and docosenol (33.7 percent). Thus, while the components of jojoba wax are known, some confusion still exists regarding its percentage composition.

Although it is recognized that the full understanding of the biosynthesis of the wax in jojoba will require quantitative analysis of the components, in the exploratory study presented here, no effort was made to identify the specific components present in the wax. In one case (Table XXI), however, a fractionation was conducted using the method developed for candelilla but the different fractions were not considered to be necessarily paraffins, esters and alcohols and acids. The column chromatographic method has not yet been confirmed for unsaturated compounds.

In attempting to study the biosynthesis of waxes in jojoba, some difficulty was encountered in that the date of fertilization of the flowers was not known. Consequently, the relative ages of the embryos were estimated on the basis of their relative size in relation to those of a random group of mature embryos found in the collection area.

Initial studies on wax biosynthesis were made with embryos which were about 3/4 the size of mature embryos found in the collection area (Tucson Mountains). Comparisons were made between light and dark treatments since the embryos appeared to possess chlorophyll; cultures containing biotin were also run since it has been shown to be a requirement for synthesis of glyceride fatty acids (58, 89). The results (Table XIX) show that light and biotin do not affect the radioactivity of CO_2 or lipides and that utilization of radiocarbon from acetate-1-C^{14} is considerable in all cases. The reasons for the lack of response to light or biotin are not known but the expected biotin results may not have occurred because the embryos may not have been at the proper physiological age. In flax, recent studies (58, 75) have shown that fatty acid synthesis is not altered by biotin additions if very young or very old embryos are used as the culture material.

In both the light and dark culture, 3 percent of the added acetate was converted to CO_2 and nearly 10 percent was transferred to the lipide fraction in 4-hour cultures. In candelilla, in contrast, only about 1 percent of the added radiocarbon was transferred to lipides in the light (Table XVIII). Also, about 15 percent of the radiocarbon was converted in the light in the 22.5 hour cultures.

Somewhat more mature embryos were used to determine

TABLE XIX. Effect of light and biotin concentrations on the radioactivity of CO_2 and lipides from jojoba embryos cultured in sodium acetate-1-C^{14}*.

Light Intensity (ft-c)	Biotin (ug/l)	Carbon Dioxide Wt (mg)	Carbon Dioxide Specific Activity (cpm/mg)	Carbon Dioxide Total Activity (cpm)	Lipide Total Activity (cpm)
0	0	4.8	960,000	4,600,000	11,000,000
0	1	4.6	610,000	2,800,000	12,000,000
0	10	5.0	800,000	4,000,000	8,400,000
0	100	4.9	1,000,000	4,900,000	8,400,000
700	0	4.4	780,000	3,400,000	7,600,000
700	1	4.1	860,000	3,500,000	11,000,000
700	10	3.9	990,000	3,900,000	11,000,000
700	100	3.4	980,000	3,300,000	12,000,000

* Five immature jojoba embryos were sliced in half lengthwise and placed in 3 ml 0.01 M KH_2PO_4-K_2HPO_4 buffer solution (pH 6.5) that contained 0.36 mg sodium acetate-1-C^{14}. Total solution radioactivity was 1.1 x 10^8 cpm; temperature was 27.5 $\pm$ 1.5 C; CO_2 was removed continuously during 4-hr culture. Embryos were collected on May 3, 1960. Average weight of each embryo was 260 mg. The results for lipide radioactivity are from single analysis. CO_2 results are means from 3 replicate cultures.

the effect of additives on acetate incorporation into lipides (Table XX). Characteristically, at the time of picking (June 22, 1961), all fruits were beginning to shrivel and seed coats were turning brown but were still easily removed. The results of Table XX show that jojoba embryo slices, like candelilla sections, are quite insensitive to additions of fairly high amounts of inhibitors. In the preliminary study reported in Table II, only 10^{-3} M DNP appeared to alter the radioactivity of lipides distinctly. The use of 10^{-2} M malonate, 10^{-1} M hydroxylamine hydrochloride and added CO_2 did not affect lipide synthesis from acetate-1-C^{14}. The results also indicate that CO_2 could be incorporated into the lipides.

As in the case of studies using immature embryos, the incorporation of radiocarbon into lipides is considerable. Furthermore, it seems likely that incorporation should continue for some time after seed coats initially turn brown since the measurement of the total lipides of comparable embryos showed only 8 percent lipides whereas nearly 50 percent has been reported in mature seeds (17, 26, 36, 91). In this respect, the work of Green, Hildītch and Stainsby (26) is of interest since they found 33.7 percent wax in recently matured seeds and only 6 percent in half mature seeds.

Nearly mature embryos were used also in determining

TABLE XX. Effect of various additives on the radioactivity of lipides from jojoba embryos cultured in sodium acetate*.

Additives	Acetate Used	Lipides Wt (mg)	Lipides Total Activity (cpm)
None	1-C^{14}	48	350,000
10^{-3} M DNP	1-C^{14}	36	110,000
10^{-2} M Malonate	1-C^{14}	37	240,000
10^{-1} M $NH_2OH \cdot HCl$	1-C^{14}	44	210,000
2.2 mg CO_2	1-C^{14}	43	240,000
2.2 mg CO_2 + DPNH + CoA + biotin + $MgCl_2$**	1-C^{14}	43	250,000
2.2 mg $C^{14}O_2$	Unlabeled	38	3,100

* Nearly mature jojoba embryos were sliced lengthwise into 2 mm sections and placed 1 layer deep in 2 ml 0.01 M KH_2PO_4-K_2HPO_4 buffer solution (pH 6.5) that contained 0.24 mg sodium acetate; cultures were run in 500 ml chambers for 6 hrs at 25 ± 1.5 C; CO_2 was removed continuously during culture except in instances when additional amounts were added. Total radioactivity of sodium acetate-1-C^{14} was 7.3×10^7 cpm; radioactivity of $C^{14}O_2$ was 840,000 cpm. Results are from single runs using embryos collected on June 22, 1961. Seed coats of embryos were turning brown but were easily removed.

** Materials used as cofactors were: 2×10^{-7} M ATP (Pabst); 2×10^{-7} M reduced DPN (California Foundation for Biochemical Research); 2×10^{-7} M biotin (California Foundation for Biochemical Research); 2×10^{-7} M CoA (Pabst) and 4×10^{-6} M $MgCl_2$.

the change in radioactivity of the lipides following removal from acetate-1-C^{14} solutions. The results (Table XXI) do not give a clear-cut pattern of change in radioactivity, but there is an indication that the column chromatographic method does provide some type of separation which can be developed for future studies.

The studies with jojoba, as a whole, showed that acetate-1-C^{14} and CO_2 could be incorporated into the lipide fraction and that this incorporation could occur in embryos which were of considerably different ages. They suggested, also, that studies on the metabolism of waxes could be made for some time after the seeds were quite mature. Since the composition of the wax is considerably less complex than candelilla wax and since light does not appear to affect metabolism, jojoba may be a better system than candelilla for studying such processes as alcohol formation and esterification. Future studies, however, will require a considerable development of analytical techniques since the specific compounds present in jojoba wax have not been isolated. Also, for uniformity, a more accurate method for determining embryo age will be required.

TABLE XXI. A kinetic study to determine changes in radioactivity of waxes of jojoba after removal of embryos from solutions containing sodium acetate-1-C^{14}*.

Time After Removal from Radioacetate (hrs)	Lipides Total Wt (mg)	Fraction A** Wt (mg)	%	Total Activity, (cpm)	Fraction B** Wt (mg)	%	Total Activity (cpm)	Fraction C** Wt (mg)	%	Total Activity (cpm)
0	46.2	2.6	5.6	180,000	30.0	64	97,000	7.0	15	840,000
1.3	84.9	2.8	3.3	110,000	67.0	78	270,000	6.7	8	690,000
2.3	24.5	2.2	9.0	39,000	13.1	54	57,000	3.2	13	320,000
12	42.2	2.0	4.7	54,000	38.3	91	130,000	4.4	10	290,000

* Nearly mature jojoba embryos were sliced lengthwise into 2 mm thick sections and cultured initially for 5.5 hrs in 0.01 M KH_2PO_4-K_2HPO_4 buffer solution (pH 6.5) that contained 0.24 mg sodium acetate-1-C^{14}. Radioactivity of solution was 7.3×10^7 cpm. Embryos were then washed and transferred to buffer solutions containing an equivalent weight of non-radioactive sodium acetate and allowed to remain in the solutions for the indicated periods of time before the embryos were killed in boiling alcohol. Temperature of culture was 27.5 ± 1.5 C; light intensity was 700 ft-c; cultures were run under conditions of continuous CO_2 removal.

** Fractions A, B and C represent fractions obtained using the column chromatographic methods for the separation of paraffins, esters, and alcohols and acids, respectively, of candelilla wax.

GENERAL SUMMARY

Two wax producing plants, candelilla (Euphorbia antisyphilitica Zucc.) and jojoba (Simmondsia chinensis (Link) Schneider), were used to study the pathway and factors affecting wax biosynthesis. The compositions of waxes from candelilla stems of different ages were determined and in-vitro culture studies were made using candelilla stem segments and jojoba embryo slices.

In-vitro cultures of candelilla tissues were made using 3 cm stem sections obtained from an area 20 to 30 cm below the stem apex. The sections were then sliced in half lengthwise and the half-cylindrical segments were placed cut side down in the culture solutions. Usually, sodium acetate-1-C^{14} was used as the substrate. In-vitro cultures of jojoba tissues were made using 2 mm thick embryo slices and sodium acetate-1-C^{14} as the substrate.

In studies with jojoba, the effects of various treatments on wax synthesis were measured using the total lipide fractions. In studies with candelilla, however, waxes from both in-vitro and composition studies were separated into fractions which contained only paraffins, esters, and a mixture of acids and alcohols. To achieve separation, existing column chromatographic methods (9, 16)

were modified to allow fractionation from samples containing only 20 to 100 mg total wax. Using the modified method, 94 to 102 percent recovery of synthetic waxes and 85 percent recovery of resin free candelilla wax was obtained.

Following the column chromatographic separation of the candelilla waxes, the paraffin fractions alone were analyzed in some instances using gas chromatography. Studies with standard samples of paraffins showed that gas chromatography permitted the separation and quantitative estimation of paraffins separated by two carbon atoms.

The combined results of the candelilla and jojoba studies are summarized below:

1) Comparison of the wax composition from young and old candelilla stems showed that waxes from older stems contained a higher percentage of paraffins and a lower percentage of mixed acids and alcohols than waxes from younger stems; ester percentages were not different. Analysis of the paraffins showed that all samples, whether from young stems or commercial candelilla wax, contained approximately 5 percent C_{29}, 80 percent C_{31}, 15 percent C_{33} and trace amounts of C_{34} paraffin homologs.

In terms of wax metabolism, the change toward a higher percentage of total paraffins in waxes from older stems suggested that older stems either synthesize relatively more paraffins or utilize more alcohols and acids than

younger stems. The occurrence of a constant proportion of homologs of paraffins in all samples indicated that the mechanism of paraffin formation in candelilla is the same throughout the life of the plant and biosynthesis (and possibly degradation) of paraffins is controlled by a non-specific enzyme system.

2) In addition to studying the changes in composition of the wax from candelilla, candelilla stem sections (which contained chlorophyll) and jojoba embryo slices (which contained little or no chlorophyll) were cultured in-vitro. Compounds containing C^{14} were used as substrates and transformation of radiocarbon into both waxes and CO_2 was determined. Studies using sodium acetate-1-C^{14} showed that the radiocarbon from acetate was incorporated readily into the waxes of both candelilla and jojoba. This supported and extended a previous work (81) which suggested that acetate or some substance formed easily from acetate is the natural substrate in the formation of waxes.

3) In candelilla, the incorporation of radiocarbon from acetate-1-C^{14} into waxes was not altered greatly by pH changes of the medium in the range from pH 2.6 to 8.0. However, temperature differences, light, and conditions of growth of the plant prior to culturing of stems affected the incorporation of the isotope into the wax fractions.

Waxes from stems cultured at 27.5 C were 2 to 4 times more radioactive than those from cultures at 17.5 C; waxes from stems cultured under 700 ft-c light were usually more radioactive than those from comparable cultures run in the dark. The light response depended partly on the growth of the plant prior to culture. Cultures of stems from plants grown under high light conditions in the greenhouse did not respond to illumination; cultures of stems from plants grown under low light intensity conditions in the greenhouse yielded higher radioactivity in the waxes when illuminated.

Studies with jojoba were not as extensive as those with candelilla; pH and temperature effects were not determined. However, illumination of cultures did not alter the amount of radiocarbon incorporated into the lipides of jojoba.

4) The incorporation of radiocarbon from acetate-1-C^{14} cultures into the waxes of both candelilla and jojoba was affected, in some cases, by the addition of inhibitors. Candelilla stems cultured in the presence of 5×10^{-1} M $NH_2OH \cdot HCl$ had a lower content of radiocarbon in waxes than stems cultured in the absence of the inhibitor; radioactivities of CO_2 from stems in the two treatments were not different. The reduction of radiocarbon incorporation into waxes by hydroxylamine suggested that the formation of all

wax fractions may occur through a keto-intermediate pathway.

The use of 10^{-1} M $NH_2OH \cdot HCl$ or 10^{-2} M malonic acid in cultures of both candelilla and jojoba tissues did not appear to alter radioactivity of either the wax fractions or CO_2. The reasons for the lack of response to the particular concentrations of inhibitors was not determined but it is conceivable that the materials are utilized in the metabolism of the tissues.

On the other hand, in both jojoba and candelilla studies, the waxes from cultures containing 10^{-3} M DNP had considerably less radioactivity than waxes from comparable cultures which contained no added DNP. This suggested that ATP is the high energy compound involved in wax synthesis in both organisms.

5) In candelilla, a preliminary study indicated that the energy required for wax synthesis can be produced by either photo- or oxidative phosphorylation. Under dark, anaerobic conditions, stems incorporated a lower amount of radiocarbon into waxes than stems maintained in either dark and aerobic or illuminated and anaerobic conditions.

6) Studies with candelilla also suggested that the rate of respiration of stems in the dark was higher than in the light. In most culture studies, CO_2 evolved from respiration was trapped continuously and not allowed to return to the plant as photosynthate. In all cases, the

total output of CO_2 and the specific and total radioactivities of CO_2 were higher in dark than in illuminated cultures. Although further studies will be required, this suggests that respiration was actually lower in the light cultures. The phenomenon may be the result of a photosynthetic utilization of ADP normally required for aerobic respiration.

7) Although some concept of the energy relationships involved in wax synthesis in candelilla was obtained, the understanding of the pathway of wax formation was not achieved. The ready incorporation of acetate into all wax fractions did indicate that waxes are formed by condensations of two-carbon intermediates but the successive steps were not determined. Various studies, however, have given hints on the possible pathway of formation. A preliminary study showed that labeled long-chain fatty acids can be both degraded to CO_2 and transformed into other wax fractions. Degradation was higher in the presence of CoA. From analogy with studies on the metabolism of glyceride fatty acids, it appears that acyl CoA compounds rather than wax acids are intermediates in the formation of other wax fractions. Also, other studies indicated that wax acids, alcohols, paraffins and esters were all end-products of metabolism.

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APPENDIX

I. ABBREVIATIONS:

The abbreviations used in this dissertation refer to the following compounds:

ADP & ATP, adenosine diphosphate and adenosine triphosphate; CoA, coenzyme A; DPN & DPNH, diphosphopyridine nucleotide and reduced diphosphopyridine nucleotide; DNP, 2,4-dinitrophenol; TPN & TPNH, triphosphopyridine nucleotide and reduced triphosphopyridine nucleotide.

II. SYNTHETIC WAXES USED IN THE DEVELOPMENT OF THE COLUMN CHROMATOGRAPHIC SEPARATION PROCEDURE:

Prior to the separation of candelilla wax into chemical family groups, a series of trial runs were made using various combinations of crystalline paraffin, octadecyl octadecanoate (stearyl stearate), octadecanol, and octadecanoic (stearic) acid. The materials were dissolved in n-heptane and transferred to columns containing 6 g silica gel or florisil and separations were conducted according to the methods outlined in MATERIALS AND METHODS. Using the following samples, recovery of material was 94 to 102 percent complete:

A. Synthetic waxes with one component

MATERIAL ADDED	TRIAL NO.	SAMPLE WT (MG)
Paraffin	1	10.0
	2	20.0
	3	30.0
Octadecyl octadecanoate	1	8.0
	2	12.0
Octadecanol	1	10.0
	2	15.0
Stearic acid	1	10.0
	2	15.0

B. Synthetic waxes with two components

MATERIAL ADDED	TRIAL NO.	SAMPLE WT (MG)
Paraffin	1	20.0
Octadecyl octadecanoate		10.0
Paraffin	1	20.0
Octadecanol		10.0
Paraffin	1	20.0
Stearic acid		10.0
Octadecanol	1	15.0
Stearic acid		10.0
	2	10.0
		15.0

C. Synthetic waxes with four components

MATERIALS ADDED	TRIAL NO.	SAMPLE WT (MG)
Paraffin	1	30.0
Octadecyl octadecanoate		10.0
Octadecanol		12.0
Stearic acid		10.0
	2	25.0
		8.0
		10.0
		12.0