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Mechanisms of Synthesis of Waxy Esters in Broccoli (*Brassica oleracea*)*

P. E. Kolattukudy

ABSTRACT: Broccoli (*Brassica oleracea*) leaf is shown to contain enzymes capable of synthesizing waxy esters from fatty alcohols by direct esterification with fatty acids and by an acyl transfer from phospholipids and acyl coenzyme A (acyl-CoA). Young broccoli leaves readily incorporated [U-^{14}C]stearyl alcohol mostly into waxy esters. The mechanism of esterification was studied with acetone powder prepared from the leaves. This material readily incorporated labeled stearyl alcohol (C_{18}) and cetyl alcohol (C_{16}) (but not cholesterol) into esters by utilizing endogenous acyl compounds. Radio gas–liquid partition chromatography of the esters showed that the major endogenous acyl moiety was a palmityl group (C_{16}). The pH optimum was 5.0, and half the maximal rate was obtained with 2.5×10^{-5} M stearyl alcohol with the acetone powder suspension. Bovine serum albumin inhibited the reaction, and this inhibition could be partially reversed by free fatty acids which when added alone showed some inhibition. Neither adenosine triphosphate (ATP) and CoA supplied with palmitic acid nor palmityl-CoA stimulated esterification of [^{14}C]stearyl alcohol. Significant incorporation of [U-^{14}C]palmitic acid into the ester suggested the occurrence of direct esterification of free fatty acids with stearyl alcohol. The [^{14}C]-palmityl moiety of exogenous phospholipids (and triglycerides) was much more efficiently incorporated into waxy ester than would be expected from the quantity of free ^{14}C fatty acids produced from them, suggesting the occurrence of an acyl transfer from a phospholipid (and triglyceride) to the fatty alcohol. The relative participation of direct esterification in wax synthesis increased when the concentration of fatty alcohol was increased. About one-third of the esterifying activity of the acetone powder was soluble in buffer, and most of this activity was precipitated at 25–60% saturation with $(NH_4)_2SO_4$. Palmityl-CoA served as a substrate for esterification with this fraction. The ammonium sulfate precipitated enzyme was further purified by Sephadex G-100 gel filtration by which fatty acid binding proteins could be separated from the enzyme. The gel filtration increased the stimulation of esterification caused by palmityl-CoA, and the enhanced esterification produced mainly stearyl palmitate. The esterification reaction that required acyl-CoA had a broad pH optimum above 6.0, and the reaction was inhibited by bovine serum albumin and to a lesser extent by free fatty acids. The major part of the esterifying activity of broccoli leaf homogenate was located in the soluble proteins, but this system was not stimulated by addition of ATP, CoA, and palmitic acid, or of palmityl-CoA.

Waxy esters constitute one of the most common class of compounds found in the surface lipids of plants (Douglas and Eglinton, 1965; Martin, 1964; Silva Fernandes *et al.*, 1964), animals (Nicolaides, 1965), and insects (Baker *et al.*, 1960; Gilby and Cox, 1963). Certain unusual oils such as oil from the head of sperm whale (spermaceti), castor oil fish (*Ruvettus pretiosus*) (Cox and Reid, 1932), "Mutton bird oil" (Carter, 1921), and Mullet (*Mugil cephalus*) roe oil (Iyengar and Schlenk, 1967) contain waxy esters as the major component. The alcohol and acid moieties usually range in chain length from C_{10} to C_{30}, and carbon chains with an even number of carbon atoms predomi-

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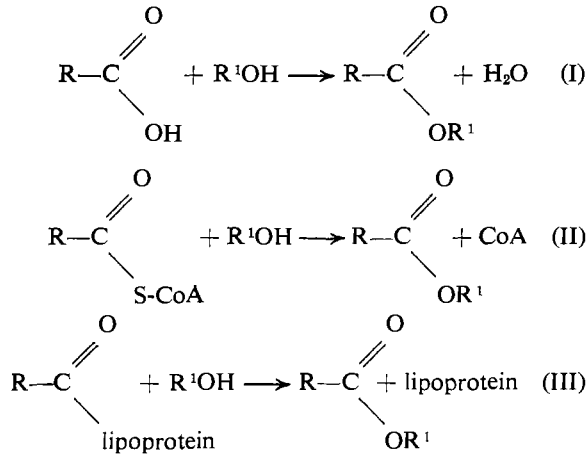
nate. Usually saturated straight carbon chains are found in plants although unsaturation and branching have been found in the acid and alcohol moieties of waxy esters of other organisms. Certain microorganisms accumulate waxy esters inside the cells (Raymond and Davis, 1960; Davis, 1964) while others excrete waxy esters into the medium (Stewart *et al.*, 1959) when they are grown on paraffins. Thus a variety of organisms synthesize waxy esters, but the mechanism of their synthesis remains largely unknown save for a recent report describing a liver enzyme which catalyzed waxy ester synthesis by the reversal of a hydrolytic reaction (Friedberg and Greene, 1967).

Experiments with labeled precursors have demonstrated that acetate is incorporated into waxy esters of plants (Matsuda, 1962; Kolattukudy, 1965; Mazliak, 1963), animals (Nicolaides *et al.*, 1955), and insects (Piek, 1964). Longer fatty acids were more readily incorporated into waxy esters of broccoli (*Brassica oleracea* var. italica (Plenck)) (Kolattukudy, 1966). Although the mechanism of the waxy ester synthesis is not known, certain possible pathways can be suggested in analogy to known esterification reactions, especially the esterification of cholesterol in animals. A variety of esterases is known (Schwartz *et al.*, 1964; Myers, 1960; Hofstee, 1960) and these enzymes can catalyze a direct esterification (Scheme I) (reaction I)

SCHEME I



of free fatty acids with alcohols (Hansen and Mead, 1965). Fatty acyl-CoA[1] is known to be involved in cholesterol esterification in certain animal tissues (Mukherjee *et al.*, 1958; Goodman *et al.*, 1964; Shyamala *et al.*, 1966) and an analogous reaction (reaction II) may be responsible for waxy ester synthesis. Acyl-CoA has been suggested to be involved in the synthesis of short-chain esters that are produced during alcoholic fermentation by yeast (Nordstrom, 1966). In animal serum, acyl moieties have been shown to be transferred from lipoprotein to cholesterol (Glomset, 1962; Glomset *et al.*, 1962, 1966), and a similar mechanism (reaction III) could also operate in waxy ester synthesis. In this paper experimental results are described which demonstrate the occurrence of the acyl-CoA mechanism and strongly suggest the presence of the other two mechanisms for waxy ester synthesis in *B. oleracea*.

Experimental Section

Acetone Powder. Midribs were removed from young broccoli leaves (second and third from the apex) collected from plants grown in the greenhouse as described earlier (Kolattukudy, 1965). The leaf blade tissue (25 g) was washed briefly with cold acetone and then homogenized in an explosion-proof Waring laboratory Blendor for 1 min (55% of the line voltage) with acetone (250 ml) previously cooled to −20°. The powder was collected by filtration and rehomogenized twice (0.5 min each time) with 150-ml portions of cold (−20°) acetone. The final powder was rinsed in the Buchner funnel with cold acetone (200 ml) and then transferred to sheets of 3MM filter paper. The powder was manipulated with a spatula until dry and then stored at −20° in sealed jars until required. The yield of acetone powder was usually almost 10% of the fresh weight. The acetone powder could be stored at −20° for several weeks without loss of activity.

For purification of the soluble enzyme activity, 15 g of acetone powder was stirred with 400 ml of 0.1 M sodium acetate buffer at pH 5.0 for 1 hr and the residue was removed by centrifugation. To the supernatant fluid (340 ml) crystalline ammonium sulfate was slowly added with stirring to 25% of saturation, and after stirring the suspension for an additional 30 min the precipitate was collected by centrifugation. Addition of ammonium sulfate was continued and the residues representing 25–60% saturation and 60–85% of saturation were collected. All three protein fractions contained esterifying activity, but the 25–60% fraction had most of it and accordingly this fraction was further fractionated by Sephadex G-100 gel filtration.

A column (2 × 45 cm) of Sephadex G-100 was washed with 0.1 M sodium acetate buffer (pH 5.0) overnight, and then 5 ml of the enzyme solution (about 100 mg of protein) in the same buffer was placed on it. The buffer was also used for elution and 4-ml fractions were collected. The proteins that bind fatty acids were located by mixing the enzyme solution with 27 mμmoles of [U-^{14}C]palmitic acid before adding it on the column. The protein was assayed by the biuret method (Gornall *et al.*, 1949), the fatty acid binding protein was located by assaying for ^{14}C, and enzyme activity was measured as described below. After locating the enzyme activity, the contents of tubes that contained most of the enzyme were pooled, and this solution was used as the source of enzyme for further experiments. The enzyme activity deteriorated when stored

[1] Abbreviations used: CoA, coenzyme A; ATP, adenosine triphosphate; BSA, bovine serum albumin.

for a few days either as an ammonium sulfate precipitate or as eluted from the Sephadex column. All operations were conducted at 5° or lower.

Intracellular Fractions. Young broccoli leaves (midribs removed) were homogenized for 15 sec at full speed in a Waring Blendor with four to five volumes of a homogenizing medium which consisted of 0.1 M buffer (sodium acetate (pH 5.0) or potassium phosphate (pH 7.0)), 0.4 M sucrose, and 0.005 M mercaptoethanol. After being filtered through four layers of cheese cloth, the suspension was centrifuged at 1000*g* for 5 min. The residue was discarded, and the supernatant liquid was centrifuged at 13,000*g* for 15 min. The pellet, which included broken chloroplasts and mitochondria, is referred to as the "particulate fraction." The supernatant fluid was centrifuged at 110,000*g* for 90 min and the pellet produced is called the "microsomal fraction." The final supernatant was saturated with ammonium sulfate, and the precipitate was collected by centrifugation. This protein fraction is referred to as "soluble."

Assay for Enzyme Activity. Acetone powder (usually 10–100 mg) or an aliquot of soluble enzyme (1–2 mg of protein) was mixed with about 200 mμmoles of [1-^{14}C]stearyl alcohol (with or without added acyl moiety) in a total volume of 3 ml of buffer, and the reaction mixture was incubated with shaking at 30°. At the end of the desired experimental time the reaction mixture was shaken with 60–100 ml of a 2:1 mixture of chloroform and methanol. After about 1 hr the chloroform layer was separated as described by Folch *et al.* (1957). The chloroform solution of lipids was evaporated to dryness under reduced pressure or nitrogen and then chromatographed on silica gel G thin layer plates. To help in the location of the alcohol and the esters, the sample was mixed with nonradioactive standards before chromatography. After locating the alcohol and the esters with 2′,7′-dichlorofluorescein the silica gel from the corresponding areas was scraped into counting vials, shaken with scintillation mixture, and assayed for radioactivity as described before (Kolattukudy, 1965). Every experiment included controls with boiled enzyme and no enzyme; no evidence of any effect of bacterial contamination on the substrates could be obtained and the control values were always insignificant.

Administration of Substrates. The substrates that are almost insoluble in water (stearyl alcohol, palmitic acid, etc.) were dispersed in water with the aid of a detergent in the following manner. The lipid substrate of the required specific activity (stearyl alcohol usually 5 μc/μmole) was dissolved in a few milliliters of ethyl ether in a volumetric flask and a few small drops of Tween 20 or Triton X-100 were added. After evaporating the ether with a stream of nitrogen, hot distilled water was added with shaking. After a thorough shaking the flask was cooled to room temperature to give an almost clear to slightly turbid solution. Portions of this stock solution were pipetted into the reaction mixtures which usually contained a final concentration of the detergent 130–250 μg/ml.

Chromatography. Column chromatography with Silica AR cc-4 100–200 mesh (Mallinckrodt Chemical Works, St. Louis, Mo.) was used for purifying the enzymatically or chemically synthesized waxy esters. Elution with 5% ethyl ether in hexane gave a waxy ester fraction which was further purified by preparative thin layer chromatography. For analytical thin layer chromatography, 0.25-mm thick silica gel G plates were used with hexane–ethyl ether–formic acid (40:10:1, v/v) as developing solvent as described before (Kolattukudy, 1967a).

Gas–liquid partition chromatography was carried out on a Perkin-Elmer gas chromatograph equipped with a flame ionization detector, an effluent splitter, and a Barber Colman radioactivity monitor. The waxy esters were separated on 5% silicone gum rubber (SE 30); the details of the experimental conditions are given in the legends.

Determination of Radioactivity. Radioactivity in the thin layer chromatographic fractions and lipid solutions was determined as described before (Kolattukudy, 1965). An internal standard of [^{14}C]toluene was used to determine the efficiency of counting; in general, 60% efficiency was obtained and counting was done with a standard deviation of less than 3%. Radioactivity in the effluent from the gas chromatograph was continuously monitored. Radioactivity on the thin layer plates was located by scanning them with a Packard radiochromatogram scanner.

Materials

Standards for waxy esters were prepared by refluxing stearyl alcohol with the acid chlorides in the presence of pyridine. The waxy ester fraction was isolated and purified by column chromatography on basic alumina (or Silica AR cc-4 as described); benzene eluted the waxy esters which were crystallized from hot ethanol and dried. Infrared spectra and thin layer chromatography showed the product to be pure waxy ester, and gas–liquid partition chromatography revealed only minor impurities.

Radioactive Phospholipids. Young broccoli leaves (3–4 g) were incubated with 300 μc of sodium [1-^{14}C]-acetate for 5–6 hr under 2000 ft-candles of incandescent light. The total lipids were isolated as described before (Kolattukudy, 1966) and the polar lipid fraction was separated from it by preparative thin layer chromatography (Kolattukudy, 1967a). This fraction, which contained large amounts (about half) of acyl moieties other than palmitate, was used as such for preliminary experiments. In order to make a more valid comparison between incorporation of ^{14}C from free [^{14}C]palmitic acid and ^{14}C phospholipids into wax, phospholipids with all of their radioactivity in the palmityl moiety had to be prepared. For this purpose [1-^{14}C]palmitate was administered to chopped young broccoli leaves and, after 4–6 hr of metabolism, the phospholipids were isolated by preparative thin layer chromatography. Essentially all of the radioactivity of this lipid was in the palmityl moiety with minor amounts of ^{14}C in longer saturated fatty acids.

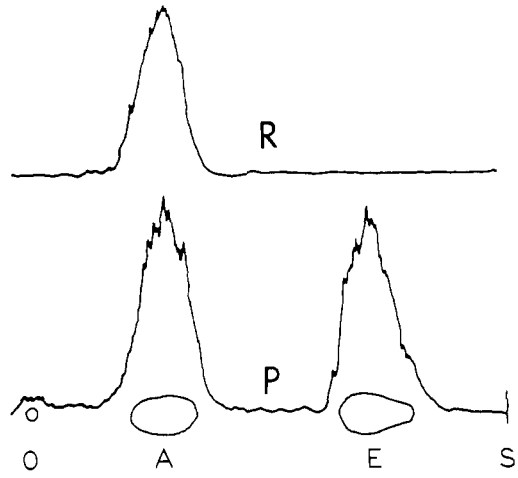


FIGURE 1: Radio thin layer chromatogram of lipids isolated from the reaction mixture. (R) Before incubation; (P) after incubation; (O) origin; (A) stearyl alcohol; (E) waxy ester; and (S) solvent front. Acetone powder (100 mg) was incubated with 200 mμmoles of [1-^{14}C]stearyl alcohol for 15 hr at 30° in 0.1 M acetate buffer at pH 5.0. Thin layer chromatography was carried out with 0.3-mm thick silica gel G plates and hexane–ethyl ether–formic acid (40:10:1, v/v) as the developing solvent. Prior to chromatography, nonradioactive fatty (stearyl) alcohol and waxy ester (stearyl myristate) were added, the lipid components were located by spraying the plates with 2′,7′-dichlorofluorescein, and fluorescing spots are outlined in the figure. This reaction mixture contained no Tween 20 and the radioactivity in the ester spot was found to be mostly in stearyl palmitate by subjecting the eluted waxy ester to radio gas–liquid partition chromatography. Boiled enzyme control gave the same pattern as R and no effect of bacterial contamination could be found on the substrate. With lesser amounts of stearyl alcohol similar patterns could be obtained with much shorter incubation periods.

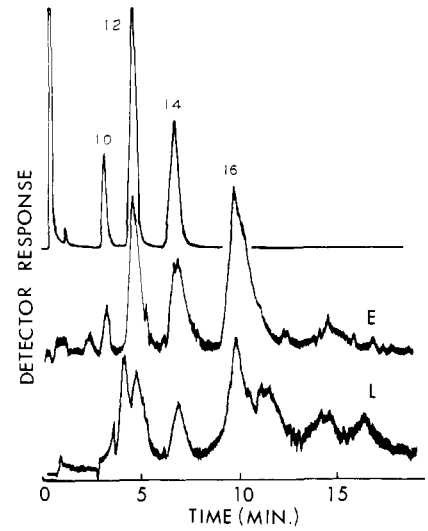


FIGURE 2: Radio gas–liquid partition chromatogram of the waxy ester synthesized from [1-^{14}C]stearyl alcohol by acetone powder suspension (E) and from [U-^{14}C]-stearyl alcohol by chopped broccoli leaves (L). The top tracing represents the flame ionization detector response of chemically synthesized waxy esters from stearyl alcohol; the chain length of the acyl moiety of each ester is shown on the peaks. Experimental conditions: 4-ft (0.25 o.d.) coiled copper column, 5% silicone gum rubber (SE 30) on 90–100 mesh Anakrom SD, and temperatures of column and injector 308 and 380°, respectively. Carrier gas, argon at 75 cc/min. The only major peak was stearyl palmitate when the reaction mixture did not contain Tween 20. The recorder tracing beyond 20 min is omitted from this figure because no more radioactive esters could be detected even when the run was continued for several hours. If much longer esters were present in the mixture they represent less than 20% of the radioactivity.

[1-^{14}C]Tripalmitin, [1-^{14}C]palmitic acid, [1-^{14}C]acetate, [1-^{14}C]stearyl alcohol, [1-^{14}C]palmityl alcohol, and [4-^{14}C]cholesterol were purchased from Nuclear-Chicago. Bovine serum albumin and palmityl-CoA were purchased from Sigma Chemical Co. The reagents for gas chromatography were from Analabs, Hamden, Conn. Stearyl alcohol and cetyl alcohol were gifts from Dr. B. J. Humphrey of Humphrey Chemical Co., North Haven, Conn.

Results and Discussion

Stearic acid was found to be an excellent precursor of cuticular wax components in *B. oleracea* (Kolattukudy, 1966). The C_{18} acid was readily incorporated into the fatty alcohol and waxy ester fractions of the leaf. Since only the acid has been tested the fate of externally administered fatty alcohol was studied. When chopped broccoli leaves were incubated with [U-^{14}C]stearyl alcohol for 2 hr, two-thirds of the recovered radioactivity (80–90% of the added ^{14}C was recovered) was found to be in the waxy ester fraction, almost all of the remaining radioactivity being in the alcohol fraction. Thus the externally administered fatty alcohol is primarily converted into waxy esters; any other metabolic reactions the alcohol may have undergone in the leaf were quantitatively small when compared to the esterification pathway.

An acetone powder suspension prepared from young broccoli leaves was also capable of converting [1-^{14}C]stearyl alcohol into waxy esters. The preliminary identification of the product was based on its thin layer chromatographic behavior (Figure 1). The radioactive components isolated from the reaction mixture had R_F values corresponding to stearyl alcohol and waxy esters (cabbage wax ester and chemically synthesized ester). The product that migrated like waxy ester was saponified by refluxing it with 10% alcoholic KOH under nitrogen, and the products were isolated after acidification. When chromatographed on the

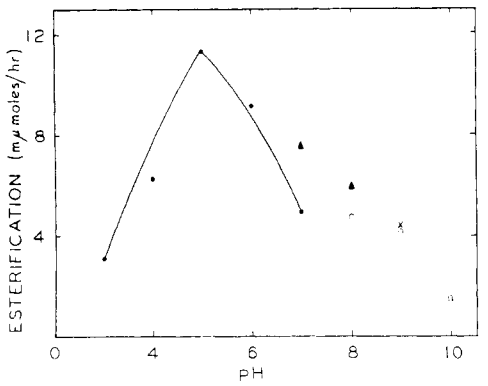


FIGURE 3: Effect of pH on [1-^{14}C]stearyl alcohol esterification by acetone powder suspension, (–●–) 0.1 M citrate–phosphate buffer; (▲) 0.1 M phosphate buffer; (○) 0.1 M Tris buffer; (×) 0.1 M borate buffer; and (□) 0.1 M glycylglycine buffer. Each reaction mixture contained 100 mg of acetone powder and 163 mμmoles of [1-^{14}C]stearyl alcohol in a total volume of 3.2 ml; incubation at 30° for 2 hr.

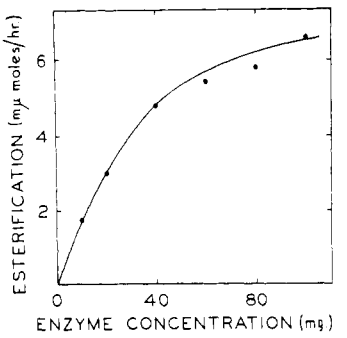


FIGURE 4: Effect of increasing amounts of acetone powder on [1-^{14}C]stearyl alcohol esterification. Each reaction mixture contained 200 mμmoles of [1-^{14}C]stearyl alcohol and the acetone powder in 4 ml of 0.1 M acetate buffer (pH 5.0); incubation at 30° for 1 hr.

same thin layer system, essentially all of the radioactivity was located in the fatty alcohol fraction.

Further evidence for the identification of the waxy ester comes from gas–liquid partition chromatography. In order to ascertain which acyl moieties were participating in the esterification process, the waxy ester fraction synthesized from [1-^{14}C]stearyl alcohol by the acetone powder was purified by column chromatography and thin layer chromatography. This purified fraction was analyzed directly by radio gas–liquid partition chromatography and the results are shown in Figure 2. Since stearyl alcohol was the only source of radioactivity, and the radioactivity of the ester was located only in the alcohol moiety, all of the esters represented by the radioactivity peaks contained C_{18} alcohol. The retention times of the esters show that the acyl moieties of these esters must be members of a homologous series. As seen in Figure 2, two peaks coincided with chemically synthesized stearyl laurate and stearyl myristate. Since all peaks contain the acyl moieties of a homologous series the other peaks are easily identifiable. Thus the major acyl moieties were C_{16}, C_{12}, and C_{14} in decreasing amounts with still smaller amounts of C_{10} and C_{18}. A similar pattern of radioactivity was obtained when the waxy esters produced by chopped broccoli leaves were examined in the same way. However, as shown in Figure 2, there are a few extra peaks which were not further examined.

The lauryl residue of the waxy ester was suspected to be derived from the Tween 20 (laurate ester) used to dissolve the stearyl alcohol. Therefore waxy esters synthesized by acetone powder suspension in the absence of Tween 20 (Triton X-100 was used instead) were examined, and most of the radioactivity of the ester fraction was then found to be in stearyl palmitate. Thus the major endogenous acyl moiety is palmitate.

Specificity. The broccoli acetone powder was capable of esterifying stearyl alcohol (C_{18}) and cetyl alcohol (C_{16}); other fatty alcohols were not studied. Cholesterol was not esterified by this acetone powder. A liver enzyme system which catalyzes esterification of cetyl alcohol, but not cholesterol, has been recently reported (Friedberg and Greene, 1967). The epidermis of *Senecio odoris*, a species in which the epidermis is readily stripped from the mesophyl cells, and acetone powder prepared from potato tuber could also be shown to catalyze esterification of fatty alcohol. Since waxy esters are found naturally in most plants this esterification reaction would be expected to be of widespread occurrence.

Enzymatic Hydrolysis of Waxy Ester. Waxy ester synthesized by chopped broccoli leaves was purified by column and thin layer chromatography, and this ester fraction was emulsified in 0.1 M acetate buffer at pH 5.0 with the aid of 2 drops of Tween 20. When the ester emulsion was incubated at 30° with 100 mg of acetone powder and 200 mμmoles of stearyl alcohol, a slow exchange of radioactivity occurred between the ester and the alcohol; after 16 hr only about 10% of the radioactivity could be detected in the alcohol fraction. Thus hydrolysis (or exchange) of waxy esters that occurs during the experimental periods used in this study is small if it occurs at all.

Effect of pH on Stearyl Alcohol Esterification. In Figure 3 the effect of pH on stearyl alcohol esterification is shown. At pH 5.0, the esterification catalyzed by the acetone powder suspension was at a maximum, and lower pH values resulted in a rather sharp decline. The pH profile is reminiscent of the cholesterol esterifying activity (Shyamala *et al.*, 1966). In adrenal homogenates they observed two kinds of cholesterol esterifying activity, one with a pH optimum of 5.0 and the other at pH 6.6. The peak at pH 6.6 was small when compared to the activity at pH.5 and it also required cofactors. The pH profile shown in Figure 3 was obtained without added cofactors. Probably because of this a clear biphasic pH profile was not observed with

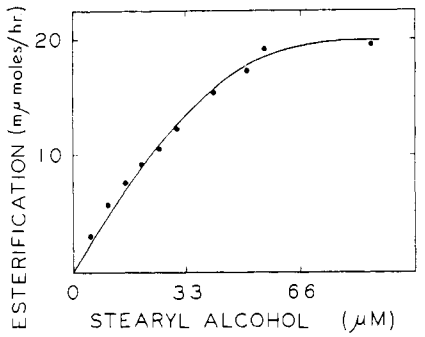


FIGURE 5: Effect of increasing concentrations of stearyl alcohol on waxy ester synthesis. Each reaction mixture contained 100 mg of acetone powder and appropriate amounts of [1-^{14}C]stearyl alcohol in a total volume of 3 ml of 0.067 M acetate buffer at pH 5.0; incubation 30° for 1 hr.

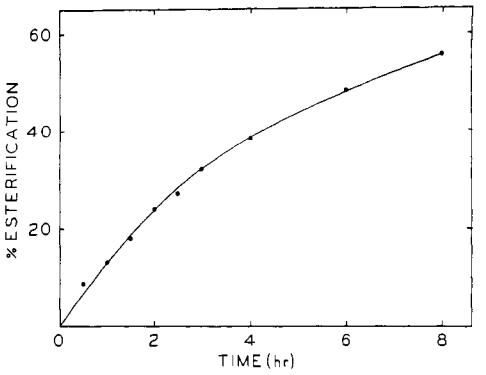


FIGURE 6: Effect of time on incorporation of [1-^{14}C]-stearyl alcohol into waxy ester by acetone powder. Identical reaction mixtures were incubated at 30° for the time indicated. Each reaction mixture contained 163 mμmoles of [1-^{14}C]stearyl alcohol and 100 mg of acetone powder in a total volume of 3.1 ml of 0.1 M acetate buffer (pH 5.0).

the broccoli acetone powder although the preparation did contain a component which requires acyl-CoA and has a pH optimum near neutrality (shown later in this paper).

Effect of Time and Concentrations of the Enzyme and Substrate. In Figure 4 the increase in conversion of stearyl alcohol into waxy esters with increasing concentration of the enzyme is shown. A linear increase in conversion with increasing amounts of acetone powder is found when small amounts of acetone powder are used under the present experimental conditions. Figure 5 shows the effect of increasing concentrations of stearyl alcohol on the extent of its conversion into waxy esters. About 25 μM stearyl alcohol gave half the maximal rate of esterification under the experimental conditions. Figure 6 shows the time course of esterification of stearyl alcohol. The reaction rate was approximately linear for several hours without addition of any acyl compound. After about 3 hr the rate began to decrease slowly, but this decrease appears to be primarily due to a decrease in the concentration of stearyl alcohol. Thus the endogenous pool of the acyl compounds does not seem to be exhausted even after such long periods of time.

Participation of Free and Esterified Palmitic Acid in Waxy Ester Synthesis. Results thus far discussed suggested that the acyl moiety is present in the acetone powder in sufficient quantities to allow esterification for extended periods. Attempts were made to detect possible involvement of acyl groups of phospholipids (or glycerides) in the waxy ester synthesis by using radioactive acetone powder prepared from leaves that had metabolized [1-^{14}C]palmitate. When this acetone powder, that contained about 80% of its ^{14}C in the phospholipid fraction, was incubated at 30° in 0.1 M sodium acetate buffer (pH 5.0), radioactivity in the phospholipid fraction decreased and there was a corresponding increase in the free fatty acid and waxy ester fractions. Thus it became evident that phospholipids did participate in the waxy ester synthesis either directly or *via* conversion to free fatty acids. In the preliminary experiments (Kolattukudy, 1967b) the exogenous ^{14}C phospholipid used was obtained from leaves that had metabolized [1-^{14}C]acetate, and this phospholipid fraction contained significant proportions (about 50%) of [^{14}C]acyl groups other than the palmityl moiety and the incubation time was relatively short (2 hr). Under these conditions incorporation of ^{14}C from [U-^{14}C]palmitic acid, tri[1-^{14}C]palmitin, and ^{14}C phospholipids into waxy esters could not be demonstrated. Longer incubation periods (8–16 hr) in the presence of acetone powder washed with 2:1 mixture of chloroform and methanol indicated that exogenous esterified fatty acids could participate in waxy ester synthesis. A series of experiments with the washed acetone powder suspensions were therefore done with [U-^{14}C]palmitic acid, tri[1-^{14}C]palmitin, and ^{14}C phospholipids isolated from broccoli leaves that metabolized [1-^{14}C]palmitate (essentially all the radioactivity in this phospholipid is known to be in the palmityl moiety with small amounts in longer saturated acids). Typical experimental results are summarized in Table I. Exogenous [^{14}C]tripalmitin was hardly hydrolyzed and the incorporation of the acyl moiety from it into waxy esters was not readily measurable although it was detectable. On the other hand, ^{14}C phospholipids underwent hydrolysis and incorporation of their acyl moiety into waxy esters more readily. This incorporation could result either from a transfer of acyl moiety to the alcohol (transesterification) or esterification of the free fatty acid produced from the phospholipids. Significant incorporation of exogenous free ^{14}C fatty acids into waxy esters shows that this system contains an enzyme that can catalyze a direct esterification of fatty acids with alcohols. In spite of the initial high specific activity of the fatty acid pool in the case of exogenous [U-^{14}C]palmitic acid (in the initial periods of incubation free fatty acids are virtually absent and

TABLE I: Comparison of ^{14}C Incorporations from [U-^{14}C]Palmitic Acid and ^{14}C Phospholipids into Waxy Esters by Broccoli Leaf Acetone Powder.[a]

Substrate:	% Distribution of Total Radioactivity					Esterification as % of ^{14}C in Free Fatty Acid Fraction[b]		
	[U-^{14}C]-Palmitic Acid	^{14}C Phospholipids				FA[c]	P[c]	P/FA
Tlc[c] Fraction: Stearyl alcohol (mμmoles)	Waxy Ester	Waxy Ester	Triglyceride	FA	P			
Control (boiled enzyme)		0.14	0.89	0.52	98.5			
0	0.0	1.3	3.7	61.8	33.5	0	1.7	
50	0.3	2.0	0.80	65.6	31.7	0.3	2.9	9.6
100	0.5	2.8	1.0	65.3	31.0	0.51	4.1	8.0
200	1.0	4.7	0.84	63.1	31.0	1.04	7.0	6.8
400	2.6	10.7	1.4	59.0	29.4	2.75	16.3	5.9
1200	10.5	13.2	0.9	50.0	36.0	11.2	20.1	1.8

[a] Each reaction mixture consisted of 100 mg of acetone powder (washed three times by agitating for 30 sec in a Waring Blendor with a cold 2:1 mixture of chloroform and methanol), stearyl alcohol, and the radioactive substrate in a total volume of 4 ml of 0.05 M sodium acetate buffer (pH 5.0). The incubation period at 30° was 10 hr. The total lipids were isolated and analyzed by thin layer chromatography. To aid the detection of the lipids on the thin layer plate nonradioactive palmitic acid, tripalmitin and stearyl myristate were added before chromatography. Palmitic acid (27 mμmoles) (sp act. 93 mc/mmole) was used and approximately the same amount of radioactivity was contained in the ^{14}C phospholipid added, but the specific activity of the palmityl moiety of this phospholipid was lower than that of the free [U-^{14}C]palmitic acid because it was isolated from leaves that metabolized [^{14}C]palmitic acid of sp act. 31 mc/mmole. A control reaction mixture with boiled enzyme was included in all series of experiments. The standard deviation in thin layer chromatographic analysis was less than 8% in all cases and it ranged from less than 1% in the case of fatty acid fraction to 7.8% in the case of waxy ester fraction. [b] In the case of [U-^{14}C]palmitic acid per cent esterification was recalculated based on the actual ^{14}C found in free fatty acid fraction (a value of 94% was used in all cases). In the case of phospholipids per cent esterification was recalculated on the basis of the ^{14}C in free fatty acids generated during the experimental period (65% hydrolysis was assumed in all cases). [c] FA, free acid; P, phospholipids; tlc, thin layer chromatography.

they are generated during incubation), the extent of incorporation of its radioactivity into waxy ester was always smaller than the incorporation of ^{14}C from phospholipids. If phospholipid contributed its radioactivity to waxy esters *via* free fatty acids, the extent of incorporation of radioactive fatty acids produced from ^{14}C phospholipids into waxy esters would be expected to be similar to or less than that of exogenous [U-^{14}C]-palmitic acid. However, as shown in Table I, based on the radioactive fatty acids produced from phospholipids, the incorporation of ^{14}C from it into waxy ester is very much higher than that of [U-^{14}C]palmitic acid. This evidence suggests that the acyl moiety from phospholipid is incorporated into waxy ester not only *via* free fatty acid but also by another route presumably a transesterification.

Without added stearyl alcohol, incorporation of radioactivity from [U-^{14}C]palmitic acid could not be demonstrated under the experimental conditions, but incorporation of ^{14}C from phospholipid into waxy esters could readily be observed (Table I). Apparently the low concentration of endogenous fatty alcohol present in the acetone powder is sufficient to permit a transesterification reaction but not sufficient for the direct esterification of free fatty acids. Since the latter represents reversal of esterase activity, it would be expected to require higher concentrations of the substrates. Therefore the relative participation of the direct esterification pathway in waxy ester synthesis would be expected to increase as the concentration of alcohol is increased. The ratio of incorporation into wax of the acyl moiety of ^{14}C phospholipid (based on the radioactive free fatty acids produced from it) to [U-^{14}C]palmitic acid incorporation decreased from a large value (with only endogenous stearyl alcohol there was almost no [U-^{14}C]palmitic acid incorporation and therefore the ratio became very large) to less than 2 as the amount of exogenous stearyl alcohol increased from 0 to 1.2 μmoles. Thus, as the concentration of stearyl alcohol increased, the participation of the direct esterification pathway increased. Direct esterification of free fatty acids with alcohol appears to be of widespread occurrence. In rat adrenal homogenates (Shyamala *et al.*, 1966) and in rat liver (Goodman *et al.*, 1964) free fatty acids have been shown to be directly esterified with cholesterol. In the adrenal

homogenate, triglycerides were rapidly hydrolyzed (90% during the experiment) and the free fatty acids thus produced were directly esterified with cholesterol (Shyamala *et al.*, 1966). In the recently described liver system (Friedberg and Greene, 1967), free fatty acids were found to be directly esterified with fatty alcohol. Triglycerides were readily hydrolyzed in this system also, and the resulting fatty acids in turn were esterified with the fatty alcohol.

The transesterification in broccoli is reminiscent of serum cholesterol esterification where acyl groups from phospholipids (lecithin) of lipoproteins are transferred to cholesterol (Glomset, 1962; Glomset *et al.*, 1966). Incorporation of radioactivity from phospholipids into triglycerides in serum indicated that the hydroxyl groups of glycerol are capable of participating in the transesterification. Similarly glyceride participation in transesterification in the broccoli system is indicated by the small but significant amount of ^{14}C from ^{14}C phospholipids incorporated into triglycerides. This incorporation occurred much less (if at all) in the presence of exogenous stearyl alcohol (Table I) the hydroxyl group of which must have successfully competed with the hydroxyl groups of glycerol for the acyl moiety of the phospholipids. Furthermore, based on the ^{14}C free fatty acids produced from tri[1-^{14}C]palmitin, the acyl moiety from the glyceride was more efficiently incorporated into waxy ester than from free ^{14}C fatty acids.

Hydrolytic enzymes such as proteolytic enzymes are known to be capable of catalyzing transesterification reactions in which amino acid residues are transferred to alcohols rather than to water (Glazer, 1966a,b; Bernhard *et al.*, 1965). Various lipases can transfer the particular enzyme-bound moiety involved to suitable acceptors. For example, the reaction catalyzed by phospholipase D involves an enzyme-bound phosphatidyl moiety (Long *et al.*, 1967) and this group can be transferred to the hydroxyl groups of suitable acceptors such as glycerol (Dawson, 1967). Lipases that apparently involve enzyme-bound acyl groups have been shown to be capable of catalyzing acyl transferase type reactions; the conversion of lysolecithin to lecithin in a rat liver system (Marinetti *et al.*, 1958), for example, was suggested to occur by the transacylase activity of the hydrolytic enzyme (Lands, 1960), and the protein-bound acyl moiety was indicated to be involved in the transesterification between lecithin and the mono- or diglycerides of serum catalyzed by Pancreatin (Marinetti, 1961). A similar transfer of an enzyme-bound acyl moiety of a lipase to the fatty alcohols could be responsible for the acyl-transfer mechanism of wax synthesis reported here. In the case of phospholipase D, however, the transferase activity was not thought to be important in the living plant cell because of the high concentration of the acceptors required to compete with water (Dawson, 1967). The acyl transfer to fatty alcohols reported here, on the other hand, did not require such high concentrations of the alcohol, even in the reaction mixtures that overwhelmingly favor water. For example at a concentration of water about 2×10^6 times that of stearyl alcohol, the hydrolysis was not even 30 times as fast as the acyl transfer to the alcohol. In the leaf the environment of the enzyme is probably much less favorable to water, and therefore the acyl transfer of alcohol probably takes place much more efficiently. In any case, with the crude enzyme source used in this study it is not possible to establish whether the hydrolysis and transfer functions can be attributed to a single enzyme.

Effect of Cofactors and Bovine Serum Albumin on Stearyl Alcohol Esterification. With the whole acetone powder as the enzyme source, addition of free palmitic acid, ATP, CoA, and magnesium ions in any combina-

TABLE II: Effect of Cofactors on Esterification of Stearyl Alcohol by Broccoli Acetone Powder.[a]

pH	Enzyme	Addition	% Esterification
		Expt 1	
5.0	Total	None	18.0
5.0	Total	ATP + CoA	17.5
5.0	Total	Palmityl-CoA	15.5
5.0	Total	Palmitic acid	15.5
5.0	Total	Palmitic acid + ATP + CoA	15.0
7.0	Total	None	15.0
7.0	Total	ATP + CoA	15.0
7.0	Total	Palmityl-CoA	13.0
7.0	Total	Palmitic acid	14.0
7.0	Total	Palmitic acid + ATP + CoA	14.0
		Expt 2[b]	
5.0	Insoluble	None	20.0
	Insoluble	Palmitic acid	13.0
	Insoluble	Palmitic acid + ATP + CoA + Mg	13.3
	Soluble	None	9.0
	Soluble	Palmitic acid	4.9
	Soluble	Palmitic acid + ATP + CoA + Mg	6.7

[a] In expt 1, 100 mg of acetone powder was incubated with 163 mμmoles of [1-^{14}C]stearyl alcohol with the additions shown in the table in a total volume of 3 ml. ATP (6 μmoles), CoA (0.5 μmole), palmitic acid (0.5 μmole), and palmityl-CoA (0.5 μmole) were used where indicated. [b] In expt 2, the enzyme was incubated with 63 mμmoles of [1-^{14}C]stearyl alcohol in a total volume of 3.7 ml with the additions shown in the table. ATP (10 μmoles), CoA (1 μmole), $MgCl_2$ (20 μmoles), and palmitic acid (3 μmoles) were used where indicated. A buffer extract of the acetone powder is referred to as soluble enzyme and the residue is called the insoluble enzyme. In both experiments, incubation was carried out for 90 min at 30°; 0.1 M acetate buffer (pH 5.0) or 0.1 M phosphate buffer (pH 7.0) was used.

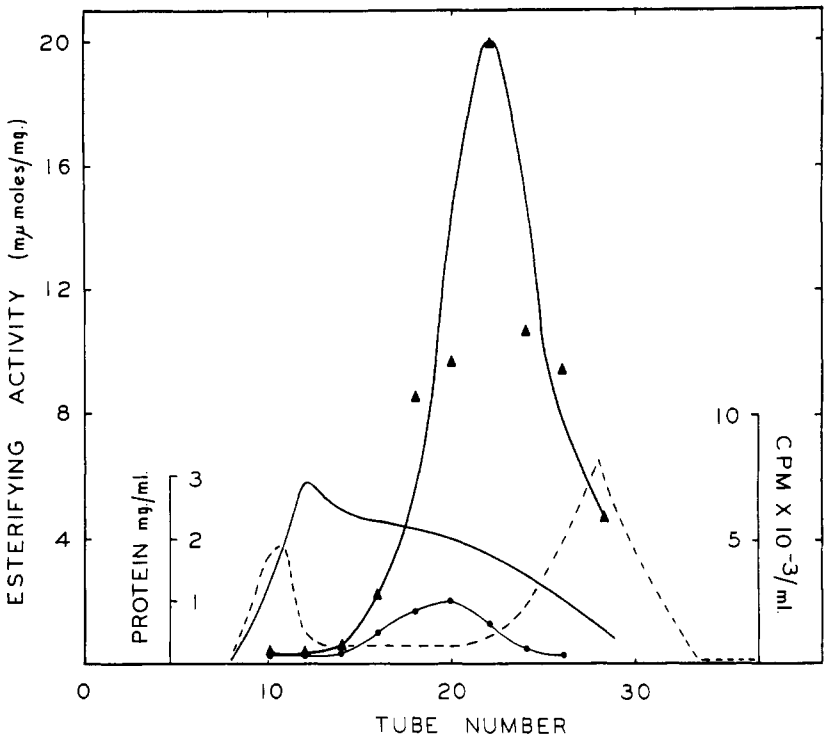


FIGURE 7: Sephadex G-100 gel filtration of the soluble enzyme. Protein (5 ml, about 100 mg) solution was administered on 2 × 45 cm Sephadex G-100 column and elution was carried out with 0.1 M acetate buffer (pH 5.0), 4-ml fractions being collected in each tube. The solid line represents protein concentration (milligrams per milliliter); (●—●) represents esterifying activity without added acyl moiety; and (▲—▲) esterifying activity with 500 mμmoles of palmityl-CoA. For each assay 163 mμmoles of [1-^{14}C]stearyl alcohol was incubated for 2 hr at 30° with 1.1 mg of protein with or without palmityl-CoA in a total volume of 3 ml 0.1 M acetate buffer (pH 5.0). The broken line represents elution of radioactivity when 27 mμmoles of [U-^{14}C]palmitic acid was mixed with the protein solution before adding it on the column. The data for ^{14}C were taken from a separate run and therefore represent the approximate positions of peaks. These radioactivity peaks were always separated from the peak of esterifying activity. Free [U-^{14}C]palmitic acid administered without protein on the Sephadex column was not eluted even with 400 ml of the buffer, but when 100 mg of bovine serum albumin was added to this column the palmitic acid bound to the Sephadex was eluted with the albumin.

tion at pH 5 and 7 failed to stimulate esterification. Palmityl-CoA was also not effective in enhancing the esterification reaction (Table II). It is possible that the acetone powder contains enough hydrolytic activity so that acyl-CoA does not survive long enough to react with the alcohol.

Addition of bovine serum albumin resulted in inhibition of the esterification process (Table III). This inhibition could be partially reversed, especially in the case of the soluble enzyme, by the addition of free fatty acids which when added alone showed some inhibition. Cholesterol esterification catalyzed by rat liver enzymes is known to be inhibited by free fatty acids (Swell *et al.*, 1964). However bovine serum albumin could completely overcome the inhibition and albumin itself did not significantly inhibit cholesterol esterification. Bovine serum albumin usually contains tightly bound fatty acids that are not readily removed by crystallization, and these acids inhibit certain reactions (Mudd and Stumpf, 1961). The inhibition of waxy ester synthesis is not caused by such fatty acids because the inhibition by albumin could be at least partially reversed by supplying free fatty acids. Albumin caused inhibition probably by binding the alcohol (Ray *et al.*, 1966) and these binding sites would be made unavailable by adding free acids.

When the acetone powder suspension was separated into soluble (extracted by 0.1 M acetate buffer at pH 5.0) and insoluble (residue remaining after the extraction) parts, addition of ATP and CoA showed some stimulation of esterification catalyzed by the soluble part but not that catalyzed by the insoluble part (Table II). Washing the acetone powder with cold 2:1 mixture of chloroform and methanol prior to the extraction by the buffer did not change the results. Thus the only indication about the nature of the acyl compound that participates in the esterification process is that the soluble protein may contain an esterifying activity that requires an activated acid.

Acyl-CoA-Linked Esterification of Stearyl Alcohol. The inability of added acyl-CoA to stimulate esterification of [1-^{14}C]stearyl alcohol by acetone powder

TABLE III: Inhibition of Stearyl Alcohol Esterification by BSA and Reversal of Inhibition by Fatty Acid.

Experimental Conditions	Esterification (mμmoles)
Expt 1[a]	
Insoluble enzyme + substrate	22.4
Insoluble enzyme + substrate + 20 mg of BSA	10.9
Insoluble enzyme + substrate + 20 mg of BSA + 0.5 μmole of palmitic acid	10.4
Soluble enzyme + substrate	5.9
Soluble enzyme + substrate + 20 mg of BSA	0.7
Soluble enzyme + substrate + 20 mg of BSA + 0.5 μmole of palmitic acid	2.7
Expt 2	
Soluble enzyme + substrate + 200 mg of BSA	0.2
Soluble enzyme + substrate + 200 mg of BSA + 10 μmoles of palmitic acid	3.5

[a] The enzyme was incubated at 30° for 90 min with 130 mμmoles of [1-^{14}C]stearyl alcohol in a total volume of 3 ml of 0.1 M acetate buffer (pH 5.0); 100 mg of acetone powder was stirred for 45 min with 3 ml of buffer and then centrifuged at 10,000*g* for 10 min, the supernatant and residue being called soluble and insoluble enzymes, respectively.

TABLE IV: Effect of Palmityl-CoA and BSA on the Stearyl Alcohol Esterification by Purified Enzyme.[a]

Additions	Esterification (mμmoles)
Expt 1	
None	3.4
Palmityl-CoA (0.5 μmole)	16.7
Palmitic acid (0.5 μmole)	2.9
BSA (0.5 mg)	0.9
BSA (2 mg)	0.3
Expt 2	
None	1.8
Palmityl-CoA (0.05 μmole)	2.1
Palmityl-CoA (0.10 μmole)	3.4
Palmityl-CoA (0.4 μmole)	6.1
Boiled enzyme, 0.5 μmole of palmityl-CoA	0.02

[a] The enzyme was incubated with 260 mμmoles of [1-^{14}C]stearyl alcohol with the addition indicated, for 2 hr at 30° in a total volume of 3 ml of 0.1 M acetate buffer (pH 5.0). In expt 1, 1 mg of protein/assay and in expt 2, 1.8 mg of protein/assay was used. The enzyme preparation was eluted from two Sephadex G-100 columns and the contents of the tubes containing the peak of the enzyme activity were pooled.

suspensions could have resulted from the rapid hydrolysis of the added acyl-CoA. Attempts were therefore made to purify the enzyme. As shown in Table II less than one-third of the total esterifying activity of the acetone powder was extracted by the buffer. An ammonium sulfate fraction, at 25–60% of saturation, contained most of the enzyme activity, and this fraction was then further purified by Sephadex G-100 gel filtration (Figure 7). When radioactive palmitic acid was added to the enzyme solution prior to gel filtration, radioactivity could be detected in two fractions of the eluent. Part of the radioactivity was associated with the heaviest protein fraction and another part was eluted with the lightest. When the radioactive fatty acid solution was placed on the Sephadex G-100 column without any protein, no radioactivity could be eluted with the buffer, but a solution of bovine serum albumin which is known to bind fatty acids readily eluted the radioactive palmitic acid from the column. Thus there seems to be two fatty acid binding activities in the enzyme solution, one associated with the heaviest proteins and the other with the lightest.

The esterifying activity was clearly separated from both these proteins (see Figure 7). At this stage of purification palmityl-CoA was required for maximal activity; addition of 1.7×10^{-4} M palmityl-CoA showed about 20-fold stimulation at the peak of activity. Esterification was dependent on palmityl-CoA concentration (see Table IV). Free palmitic acid was not a substrate, but it inhibited, and so did bovine serum albumin.

The esterification stimulated by palmityl-CoA would be expected to produce primarily stearyl palmitate if palmityl-CoA served as a substrate. Therefore a purified fraction of the enzyme was used to prepare waxy esters from [1-^{14}C]stearyl alcohol, and palmityl-CoA and the esters were analyzed by radio gas–liquid partition chromatography. The results in Table V show that the production of stearyl palmitate was preferentially stimulated by the addition of palmityl-CoA. However the product formed was not exclusively the palmitate, laurate making up a significant part of it. The substrate solutions used in this experiment were prepared with the aid of Tween 20, which is a laurate ester, and this acyl moiety apparently participated in the esterification process presumably by transesterification. The fact that only C_{12} was a significant part of the acyl moiety (other than C_{16}) of esters from palmityl-CoA-stimulated system supports this explanation. Furthermore, when Tween 20 was avoided, significant amounts of stearyl laurate were not found in the esters produced by the palmityl-CoA-stimulated system.

It was later observed that the enzyme precipitated at 25–60% saturation of ammonium sulfate also required acyl-CoA for maximal activity, and esterification

TABLE V: Effect of Palmityl-CoA on the Composition of the Waxy Esters Synthesized by Broccoli Enzyme.[a]

Chain Length of the Acyl Moiety of Esters	% Composition of Ester Produced by	
	Whole Acetone Powder	Purified Enzyme with Palmityl-CoA
10	6.3	1.8
12	27.5	24.1
14	19.1	2.9
16	41.0	71.3
18	6.1	

[a] The purified enzyme refers to the enzyme eluted from Sephadex G-100 column (see experiments for details). The purified waxy ester was analyzed by radio gas–liquid partition chromatography and the quantities were determined from the radioactivity tracing by the triangulation method. In both cases the reaction mixture contained Tween 20, which contributed most of the C_{12} found in the waxy esters.

was dependent on the concentration of palmityl-CoA (Table VI). The stimulation due to added acyl-CoA with ammonium sulfate precipitated enzyme was always much less than that observed with eluates from Sephadex columns. With the ammonium sulfate fraction, up

TABLE VI: Palmityl-CoA Requirement for Stearyl Alcohol Esterification by Ammonium Sulfate Fractionated Enzyme.[a]

Addition	Esterification (mμmoles)
None	0.96
Palmitic acid (0.2 μmole)	0.68
Palmitic acid (0.5 μmole)	0.65
Palmitic acid (1.0 μmole)	0.68
Acetyl-CoA (0.5 μmole)	0.86
CoA (0.5 μmole)	0.90
Palmityl-CoA (0.1 μmole)	1.30
Palmityl-CoA (0.2 μmole)	2.34
Palmityl-CoA (0.3 μmole)	2.86
Palmityl-CoA (0.4 μmole)	3.38
Palmityl-CoA (0.6 μmole)	4.42
Palmityl-CoA (1.0 μmole)	9.88
Boiled enzyme, 0.6 μmole of palmityl-CoA	0.03

[a] Protein (1.6 mg, 25–60% ammonium sulfate fraction) was incubated with 260 mμmoles of [1-^{14}C]-stearyl alcohol for 2 hr at 30° in a total volume of 3 ml of 0.1 M citrate-phosphate buffer (pH 5.0).

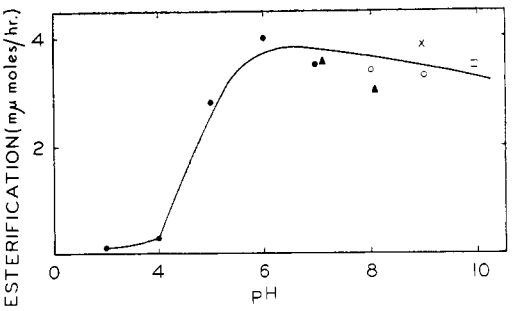


FIGURE 8: Effect of pH on palmityl-CoA-stimulated esterification of [1-^{14}C]stearyl alcohol by ammonium (25–60%)-precipitated enzyme. Each reaction mixture contained 200 mμmoles of [1-^{14}C]stearyl alcohol, 500 mμmoles of palmityl-CoA, and 1.6 mg of protein in a total volume of 3 ml of 0.067 M buffer; incubation was for 2 hr at 30°. The symbols for buffers are the same as in Figure 3.

to tenfold stimulation was obtained with palmityl-CoA whereas a 20-fold stimulation was very often found when the enzyme eluted from the Sephadex G-100 column was used. Free palmitic acid, acetyl-CoA, and free CoA did not show any stimulation of ester synthesis (Table VI).

Effect of pH on Acyl-CoA-Stimulated Esterification. The enzyme obtained by Sephadex gel filtration showed higher activity at pH 7 than at pH 5, which was routinely used thus far. Therefore the effect of pH on the acyl-CoA-stimulated esterification was studied with the 25–60% ammonium sulfate precipitate. Unlike the acetone powder suspensions, the palmityl-CoA-stimulated activity showed a broad pH optimum from neutral to higher values (Figure 8). The effect of pH on cholesterol esterification by microsomal enzyme (acyl-CoA linked) of rat liver (Goodman *et al.*, 1964) is almost identical with the effect of pH on acyl-CoA-stimulated waxy ester synthesis shown here. The acyl-CoA-linked esterification of cholesterol by adrenal homogenate also had an optimal pH near neutrality (Shyamala, 1966). Thus it appears that the esterification which uses acyl-CoA generally has an optimum at a neutral pH whereas the esterase-type enzymes synthesize waxy esters at a lower pH.

The palmityl-CoA was suspected of undergoing hydrolysis under the conditions of the assay. Presumably because of the presence of hydrolytic activity, the esterifying activity was often not linear with enzyme concentration at high protein concentrations. Therefore whenever enzyme activity of different fractions was compared, equal amounts of protein were used in all samples. The time course of palmityl-CoA-stimulated esterification is shown in Figure 9. Although only a small fraction of the added palmityl-CoA was used for esterifying the stearyl alcohol, the rate of esterification began to decrease after about 1 hr. Endogenous alcohols could have utilized some palmityl-CoA, but hydrolysis of palmityl-CoA is probably the major cause of the

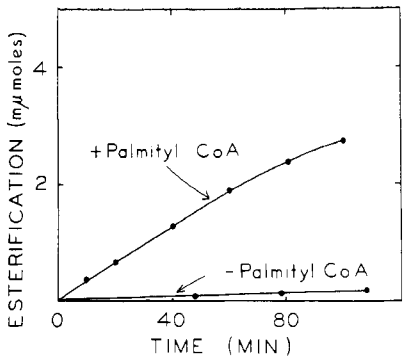


FIGURE 9: Effect of time on palmityl-CoA-stimulated esterification of [1-^{14}C]stearyl alcohol by ammonium sulfate (25–60%) precipitated enzyme. Each reaction mixture contained 200 mμmoles of [1-^{14}C]stearyl alcohol, 500 mμmoles of palmityl-CoA, and 1.6 mg of protein in a total volume of 3 ml of 0.1 phosphate buffer (pH 7.0); the incubation was carried out at 30°. Boiled enzyme control was included in the experiment but results are not shown in this figure because the ester synthesis was negligible.

TABLE VII: Esterification of Stearyl Alcohol by Subcellular Fractions.

Subcellular Fraction	% Esterification at pH 5.0 −Co-factors	pH 5.0 +Co-factors	pH 7.0 −Cofactors	pH 7.0 +Cofactors
	Expt 1[a]			
Soluble	16	17.8	13.1	12.6
Microsomal	0.6	0.6	0.96	1.1
Particulate	1.0	1.1	1.8	1.9
	Expt 2[b]			
Soluble			2.4	1.7[c]
Soluble			2.6	1.8[d]
Boiled soluble				0.05

[a] In expt 1, 2.5 mg of microsomal protein or 5 mg of soluble or particulate protein was incubated at 30° for 2 hr with 163 mμmoles of [1-^{14}C]stearyl alcohol in a total volume of 3 ml of 0.1 M citrate phosphate buffer (pH 5.0) or 0.1 M phosphate buffer (pH 7.0). Cofactors consisted of 10 μmoles of ATP, 0.5 μmole of CoA, and 20 μmoles of $MgCl_2$. [b] In expt 2, 1.6 mg of soluble protein was incubated for 2 hr at 30° with 200 mμmoles of [1-^{14}C]stearyl alcohol in a total volume of 3 ml of 0.1 M phosphate buffer (pH 7.0). [c] Cofactor is 0.5 μmole of palmityl-CoA. [d] Cofactor is 1 μmole of palmityl-CoA.

decrease in rate. This conclusion is based primarily on the two following observations. (1) Free fatty acid was detected on thin layer chromatograms of lipids isolated from the reaction mixture only when palmityl-CoA was added to the reaction mixture. (2) Increasing amounts of free fatty acid were observed after increasing periods of incubation.

Cellular Site of Esterification. All subcellular fractions contained some esterifying activity; however, the major part (about 90%) of the activity was contained in the soluble fraction (Table VII). Since different esterification mechanisms requiring different optimal pH values were indicated by the studies with acetone powder enzymes, assays were performed at pH 5.0 and 7.0 with and without exogenous ATP, CoA, and $MgCl_2$. The addition of cofactors did not substantially affect the extent of esterification in any case. In this respect the esterification resembles the recently reported liver system (Friedberg and Greene, 1967). However the leaf preparations contain a very active enzyme that hydrolyzes acyl-CoA; in addition to destroying added acyl-CoA this enzyme would produce free fatty acid which inhibits the reaction. Therefore the lack of response to ATP and CoA or to palmityl-CoA may be misleading. Fractionation of this soluble enzyme would be required before the acyl-CoA-requiring esterification can be demonstrated. Regarding intracellular location of the esterifying activity, the waxy ester synthesizing system of broccoli appears to be different from the cholesterol-esterifying systems of animals (Goodman *et al.*, 1964; Shyamala *et al.*, 1966) but similar to the waxy ester synthesizing enzyme of liver (Friedberg and Greene, 1967). However the contents of the epidermal layer of cells, which may represent large proportions of the insoluble esterifying activity of the acetone powder, were probably excluded by the short homogenization used in this study (epidermis of *Senecio* contains about 60% of the total esterifying activity of the leaf (P. E. Kolattukudy, unpublished results)).

Conclusion

The results discussed in this paper suggest the presence of three mechanisms for the synthesis of waxy esters in broccoli leaves. First, reversal of esterase reaction resulting in a direct esterification of free fatty acids with fatty alcohols. Second, an activated fatty acid is esterified with fatty alcohol by an acyl-CoA–fatty alcohol transacylase. Third, an acyl group is transferred from phospholipids (or glycerides) to fatty alcohol by an acyl transferase. The first mechanism may play only a minor role under physiological conditions, but the second mechanism is likely to be of more significance as it is thermodynamically more favorable to ester synthesis. Activated acid is apparently involved in the synthesis of waxy esters that are excreted into the medium by *Micrococcus cerificans* when it is grown with paraffins as the sole source of carbon (P. E. Kolattukudy and L. Hankin, unpublished results). The acyl transfer from phospholipids and glycerides may be of physiological significance. The waxy esters normally present in the cuticular wax are known to contain acyl

moieties much longer than C_{16} (Purdy and Truter, 1963) and presumably these longer acyl moieties are products of elongation (Kolattukudy, 1966). Very long fatty acids (C_{20}–C_{28}) produced from exogenous [U-^{14}C]-stearic acid by broccoli leaves were found to be exclusively in the esterified from (mostly in phospholipids and some in triglycerides) (Kolattukudy, 1967a). The acyl-transfer mechanism of waxy ester synthesis provides a reasonable route by which these very long fatty acids could participate in waxy ester synthesis.

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