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# THE BIOSYNTHESIS OF LONG-CHAIN FATTY ACIDS BY LETTUCE CHLOROPLAST PREPARATIONS

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SUMMARY

1. It is demonstrated that the major site of biosynthesis of saturated fatty acids from $C_{10}$ to $C_{18}$ and of oleic acid in leaves is the chloroplast.

2. The co-factors required in the presence of light are ATP, CoA, $Mg^{2+}$, $CO_2$ and inorganic phosphate.

3. In the dark greatly diminished synthesis occurs despite the presence of a full complement of cofactors.

4. As in photosynthetic phosphorylation the synthesis of fatty acids is inhibited by $NH_3$ and 3(*p*-chlorophenyl)1,1-dimethyl urea but not by dinitrophenol.

5. Under anaerobic conditions the biosynthesis of oleic acid drops markedly but that of the saturated acids is relatively unaffected.

6. Isolated chloroplasts are less effective in utilising the $C_8$, $C_{10}$, $C_{12}$ and $C_{14}$ saturated fatty acids for the biosynthesis of oleic acid than is the intact leaf. Both malonic and acetic acids are, however, readily utilised.

## INTRODUCTION

It has been shown that isolated leaves effciently synthesize long-chain fatty acids from acetate, octanoate, decanoate, dodecanoate and tetradecanoate by JAMES[1]. The localisation of enzymes and the nature of the co-factor requirements were, however, not known.

In 1956 SISAKYAN AND SMIRNOV[2] observed that isolated chloroplasts from sunflower leaves incorporated labelled acetate into long-chain fatty acids both in the light and the dark. These data were extended in 1960 by SMIRNOV[3] who demonstrated that the conversion of acetate into higher fatty acids by spinach chloroplasts required ADP, CoA, $Mg^{2+}$ and light. He concluded that photophosphorylation was not involved and suggested a unique photoacetylation reaction.

The chloroplasts of higher plants contain a considerable amount of lipid; on a dry weight basis about 20–35 % of the chloroplast is lipid. The structural role of

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lipids in the photosynthetic apparatus has been recognised by many workers[4]. The composition of spinach-chloroplast lipid has recently been analysed by ZILL AND HARMON[5] who identified as the major components, glycolipids, phospholipids and sulpholipids. WOLF *et al.*[6] showed that at least eleven different fatty acids occurred in isolated spinach chloroplasts, linolenic acid being the major component. DEBUCH[7] has obtained similar results with other plant material.

We show in this paper that a chloroplast system from lettuce leaves efficiently and rapidly incorporates [$^{14}$C]acetate into long-chain saturated acids and oleic acid when light, ATP or ADP, $P_i$, CoA, $CO_2$, $Mg^{2+}$ and $NADP^+$ are supplied. Photophosphorylation appears to be tightly coupled to lipid synthesis. A preliminary account of this work has already appeared[8].

## METHODS AND MATERIALS

### *Preparation of chloroplasts*

Approx. 200 g of thoroughly washed lettuce leaves, purchased from a local source (*Lactuca sativa capitata*, variety Blackpool, employed during the months of November and December, and variety Number 2 employed thereafter) are homogenized for 30 sec at top speed in a chilled Waring Blender to which had been added 250 ml of cold suspending medium, the composition of which will be described below. The suspension is pressed through five layers of cheese-cloth and the resulting green filtrate is passed through ten layers of cheese-cloth without pressing, to remove any leaf fragments which may have passed through the first filtration process. The filtrate is then centrifuged in a refrigerated MSE (London) centrifuge for 10 min at 1000 $\times$ *g*. The supernatant is carefully decanted and discarded; the green pellet is suspended in 20 ml of fresh suspending medium and recentrifuged for 10 min at 1000 $\times$ *g*. The supernatant is again discarded and the sediment is collected with a minimum amount of fresh suspending medium and homogenized for about 10 sec with a Teflon pestle in a Potter homogenizer to give a smooth suspension which is then analyzed for chlorophyll content by BRUINSMA's modification[9] of ARNON's method[10]. Fresh suspending medium is now added to yield a final chlorophyll concentration of 0.5 mg per 0.5 ml. This suspension is then stored at 1° until ready for use. Disrupted chloroplasts are prepared by substituting distilled water for the final suspending medium.

### *Procedure*

Reactions are conducted in 50-ml Erlenmeyer flasks placed in a water bath which is cooled by a copper coil through which cold tap water is rapidly circulated. The water bath is placed above a battery of Philips Photolita bulbs (240 V, 500 W). Under these conditions for a period of 1 h the temperature range is 20–23°. Approx. 2000 ft candles of light strike the flasks. The reaction mixtures which are employed are described in detail in the appropriate table or figure. After 1 h, 0.1 ml of 1 N KOH is added to stop the reaction and hydrolyze thioesters; after 5 min 0.2 ml of 5 N $H_2SO_4$ and 50 ml of chloroform – methanol (2:1, v/v) are added and the flasks are shaken to form a one-phase system. 15 min later, the contents of each flask are poured into a 100-ml separatory funnel, about 15 ml of water are added and the funnel vigorously shaken. The suspension rapidly separates into two phases with the lipid-containing chloroform layer at the bottom. This layer is drawn off into a 125-ml Erlenmeyer

flask containing anhydrous $Na_2SO_4$ to remove traces of water. Vigorous shaking for a few seconds clarifies the chloroform solution which is then filtered into a large test tube (25 × 180 mm). The solvent is evaporated at 90° with the aid of a jet of nitrogen. The lipid residue is taken up in a minimum of diethyl ether and aliquots taken for radioactive counting on aluminium planchets with a thin-window Geiger–Müller counting system of 5 % efficiency.

Lipids are saponified by refluxing for at least 2 h at 90° in the presence of 10 % KOH in methanol. The hydrolyzed mixture is then acidified with 5 N $H_2SO_4$ and the free fatty acids extracted several times with diethyl ether. The ether solution is dried over $Na_2SO_4$ and the free fatty acids esterified with a slight excess of freshly prepared and distilled diazomethane obtained by the usual procedures from nitrosomethyl urea. Excess solvent is removed and the methyl esters, dissolved in a minimum of petroleum ether (40–60°) are analysed for composition and radioactivity by the method of JAMES AND PIPER[11].

Lipids were fractionated into hydrocarbons and carotenes, glycerides (mono-, di-, and triglycerides) and free fatty acids, and phospho- and glyco-lipids by the procedure of FREEMEN *et al.*[12]. Free fatty acids were separated from neutral lipids by the method of BORGSTRÖM[13].

*Degradation procedures*

1–2-mg quantities of palmitic, stearic, oleic, linoleic and linolenic acids as well as unknown components were separated as methyl esters by gas–liquid chromatography on a polyethyleneglycol adipate column at 180°. Fractions were condensed in cotton-packed traps at room temperature and eluted with diethyl ether.

*Saturated acids*: Since by use of gas radiochromatography (JAMES AND PIPER[11]) it is easy to measure the radioactivity of a series of related compounds, it is an advantage to use a degradation reaction capable of removing more than one carbon atom at a time. The isolated methyl esters of fatty acids were hydrolysed to the free acids with alkali, extracted and diluted 3-fold with unlabelled material. The acid was dissolved in ether and carefully evaporated in a 3-in length of 4-mm internal diameter glass tube. Two drops of thionyl chloride and one drop of bromine were added, the tube was cooled, sealed, and heated in an oven at 120° for 17 h. The tube was cooled, opened and the excess thionyl chloride and bromine removed *in vacuo*. The residual α-bromo-acid bromide was removed by dissolving in ether, evaporated in a 2-ml round-bottomed flask and hydrolysed with 1 N aq. KOH for 20 h. The alkaline solution was acidified with 5 N sulphuric acid and the α-hydroxy acid extracted with ether. The ether solution was evaporated in a small conical centrifuge tube and oxidised with 10 mg of $KMnO_4$ in two drops of glacial acetic acid for 1 h at 90°. Water was then added, a few milligrams of potassium bisulphite were added to remove $MnO_2$ and $KMnO_4$ and the solution was extracted with ether and dried. The resultant mixture of shorter-chain saturated acids was methylated with diazomethane and analysed by gas–liquid radiochromatography in an Apiezon-L column at 200°. A typical result obtained with palmitic acid is shown in Fig. 1. The major product is pentadecanoic acid, with appreciable amounts of tetradecanoic, tridecanoic, dodecanoic and undecanoic acids resulting from overoxidation.

*Unsaturated acids*: The acids were oxidised in the same way as the α-hydroxy acids described above (JAMES AND WEBB[14]). After methylation the mixture of mono-

and dicarboxylic acids was separated on a gas-radiochromatogram using an Apiezon-L column at 200°. Unlabelled mono- and dicarboxylic acids were often added as markers.

*Gas–liquid chromatography*

Gas-radiochromatograms obtained on polyethyleneglycol adipate and Apiezon-L columns were used to determine radioactivity of the biosynthesized fatty acids. Analytical separations and fraction collections were carried out on instruments having both argon ionisation and gas density meter detectors.

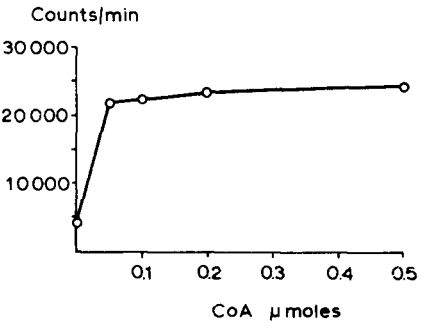



Fig. 1. Relationship between coenzyme A concentration and incorporation of [$^{14}$C]acetate into long-chain fatty acids. The conditions of the experiment are described in Table I.

*Chemicals*

ATP (dipotassium salt, ADP and AMP were obtained from Nutritional Biochemical Corporation; CoA (75% purity) was purchased from Sigma Company, St. Louis; $TPN^+$ and $DPN^+$ were supplied by C. F. Boehringer and Sons, Mannheim, West Germany. Sodium [2-$^{14}$C]acetate (5 mC/mmole) was purchased from the Radiochemical Centre, Amersham, England.

## RESULTS

*General properties*

Two kinds of chloroplast preparations were employed in the work to be described. Preparation I was used in the early work and Preparation II for the later and major number of experiments. Both preparations employ the same general procedures for the isolation of chloroplasts as described in METHODS and differ only in the composition of the suspending medium. Preparation I employs a suspending medium composed of 0.35 M NaCl, 0.01 N sodium ascorbate, 0.01 M phosphate buffer (pH 7.4), while preparation II uses a suspending medium composed of 0.50 M sucrose, 0.01 M sodium ascorbate, 0.01 M NaCl, all adjusted to pH 7.4. Both media contain 0.001 M Versene. The major difference in activity between Preparations I and II is a consistent and far more pronounced requirement for adenosine nucleotides by Preparation II in contrast to Preparation I. These differences are illustrated in Table I. Presumably a critical amount of nucleotide leaks out of chloroplasts obtained by Preparation II which necessitates a further addition to complete the nucleotide needs. Both preparations are reasonably stable when stored in an ice bath for at least 3 h. However, if stored overnight at −10° or frozen and then promptly thawed, complete loss of acetate incorporation activity is observed. Preparations in which Tris buffer at pH 7.4 to 8.2 has been substituted for phosphate buffer showed low activity even when phosphate has been added to reaction mixtures. Since Tris does not affect photophosphorylation, it must be assumed that Tris inhibits the system involved in fatty acid

synthesis. Indeed, BARRON, SQUIRES AND STUMPF[15] have observed a Tris inhibitory effect with water extracts of acetone powder of avocado mesocarp particles. However, veronal buffer systems have been used in place of phosphate buffers whenever phosphate-free reaction mixtures were desired. No inhibitory effects were noticed.

TABLE I

NUCLEOTIDE REQUIREMENT BY PREPARATIONS I AND II

Each reaction mixture contained 0.5 ml of freshly prepared chloroplast preparation (0.5 mg of chlorophyll), 100 μmoles of phosphate buffer (pH 8.2), 0.5 μmole of [$^{14}$C]acetate (0.5 μC, 55500 counts/min), 0.2 μmole of CoA, 1.0 μmole of $Mg^{2+}$, 30 μmoles of sodium bicarbonate, 0.2 μmole of $NADP^+$ and varying amounts of nucleotide as indicated. Time of reaction incubation is 60 min; temperature is 20°, and 2000 ft candles of light strike the flasks. Total volume is 1.2 ml.

| *Preparation* | *Nucleotide concentration (μmoles)* | *[$^{14}$C] Acetate incorporation* | |
|---|---|---|---|
| | | *ATP* | *ADP* |
| I | — | 9 800 | 9 800 |
| | 1 | 11 600 | 11 000 |
| | 10 | 6 000 | 9 600 |
| II | — | 12 800 | 12 800 |
| | 1 | 23 300 | 26 000 |
| | 10 | 6 900 | 11 000 |

The pH optimum for the lipid synthesis system is between pH 8.0 and 8.2. Below pH 7.0 and above pH 9.0 little, if any, activity is observed. It is of interest that the pH optimum for photophosphorylation is approx. 8.0–8.2. Because of the complexity of both the photophosphorylation and the lipid synthesis system, that is, the number of enzymes involved in catalyzing the reactions, little however, can be gained by speculating on the significance of these pH optima.

*Cofactor requirements*

Detailed cofactor studies were carried out with both preparations and typical results are found in Tables II and III. In Table II in the absence of all cofactors, incorporation of radioactive acetate drops to a low level under both light and dark

TABLE II

COFACTOR REQUIREMENTS FOR ACETATE INCORPORATION INTO LONG-CHAIN FATTY ACIDS UNDER LIGHT AND DARK CONDITIONS WITH PREPARATION I

Conditions the same as in Table I. Cofactor concentrations as indicated.

| *Cofactors (μmoles)* | *Total lipid (counts/min)* | | |
|---|---|---|---|
| | *Light* | *Dark* | *Light/dark* |
| Complete | 15 600 | 1900 | 8.2 |
| −all cofactors | 1 600 | 580 | 2.7 |
| −ATP (10) | 18 360 | 624 | 29.4 |
| −CoA (0.5) | 6 000 | 680 | 8.8 |
| $-Mn^{2+}$ (0.25) | 13 260 | 1400 | 9.6 |
| $-Mg^{2+}$ (0.25) | 16 240 | 1660 | 9.9 |
| $-NADP^+$ (0.2) | 22 500 | 1900 | 11.5 |
| $-CO_2$ (30) | 7 440 | 1100 | 6.8 |

conditions. When ATP is omitted from the complete system (Preparation I) a surprising stimulation under light conditions is noted, suggesting a definite inhibitory effect by ATP. Under dark conditions there is a drop in incorporation suggesting that ATP is not readily regenerated in the dark. CoA is a limiting factor in incorporation both under light and dark conditions whereas metal and $NADP^+$ requirements do not show under both dark and light conditions. Presumably in the intact chloroplast a sufficient concentration of these compounds may be present to fulfil their requirements. $CO_2$ is required under both experimental conditions which is in line with the modern concept of fatty acid synthesis[16] whereby $CO_2$ carboxylates acetyl CoA to yield the actual condensing unit, malonyl CoA. Table III illustrates typical results

TABLE III

COFACTOR REQUIREMENTS FOR ACETATE INCORPORATION IN PREPARATION II

See Table I for details.

| *Cofactors (μmoles)* | *Total lipid (counts/min)* |
|---|---|
| Complete | 32 000 |
| −all cofactors | 600 |
| −CoA (0.25) | 5 300 |
| −ATP (1.0) | 16 000 |
| $-CO_2$ (30) | 8 200 |
| $-Mn^{2+}$ (0.25) | 31 600 |
| $-Mg^{2+}$ (0.25) | 18 600 |
| $-NADP^+$ (0.2) | 34 000 |

of a similar study with Preparation II. Under these conditions a slightly different picture emerges. Thus, the system shows a definite requirement for low concentrations of ATP; omission of cofactors gives a much lower blank and a $Mg^{2+}$ requirement is now revealed. It is possible that the chloroplast preparation has had its permeability somewhat altered and therefore these effects become more noticeable. Figs. 1–6 show the relation of activity to increasing concentrations of various cofactors. (Note in Figs. 2 and 3 the marked inhibitory effects of high concentrations of ATP and ADP on the lipid-synthesizing system.) Preparation II was employed in all these concentration studies. A similar series was not carried out with Preparation I.

The role of inorganic phosphate was examined in detail with Preparation II. In Fig. 7 the incorporation of [$^{14}$C]acetate by chloroplasts with increasing amounts

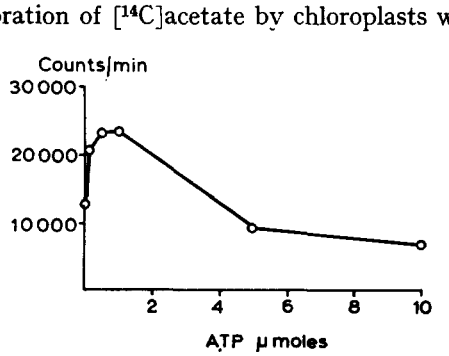



Fig. 2. Relationship between concentration of ATP and [$^{14}$C]acetate incorporation. Conditions are described in Table I.

of $P_i$ is depicted with and without ATP. Preparation 1 also shows a definite response to phosphate ions*.

These cofactor studies suggest that ATP, CoA, and $CO_2$ are involved in the reaction responsible for fatty acid synthesis. Light and inorganic phosphate are most likely involved in the process of photophosphorylation, which makes possible the rapid and efficient synthesis of ATP required for activation and carboxylation of acetate units. Since photophosphorylation is not inhibited by large amounts of ATP, the ATP inhibitory effects must be on some phase of fatty acid synthesis. Moreover, since photophosphorylation also requires $Mg^{2+}$ for activation but still functions effectively in the presence of large amounts of ATP, the ATP effect cannot be readily attributed to possible chelation reactions of ATP with divalent cations.

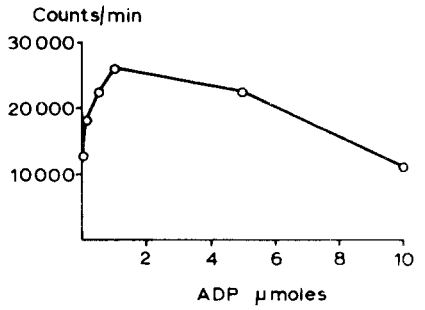



Fig. 3. Relationship between concentration of ADP and [$^{14}$C]acetate incorporation. Conditions are described in Table I.

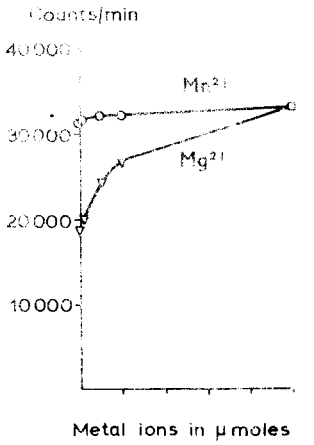



Fig. 4. Relationship between concentration of $Mg^{2+}$ and $Mn^{2+}$ ions and [$^{14}$C]acetate incorporation. Conditions are described in Table I.

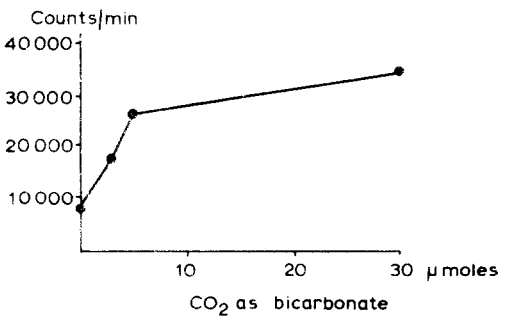



Fig. 5. Relationship between concentration of sodium bicarbonate and [$^{14}$C]acetate incorporation. Conditions are described in Table I.

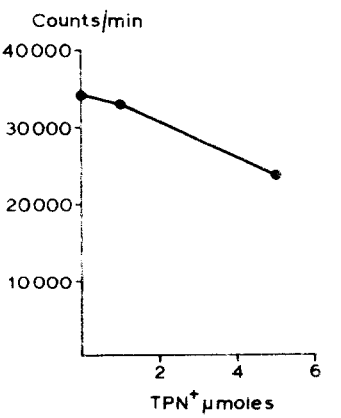



Fig. 6. Relationship between concentration of $NADP^+$ and acetate incorporation. Conditions are described in Table I.

* In unpublished experiments by STUMPF, BOVE AND GOFFEAU an active non-cyclic photophosphorylation system which readily synthesizes NADPH, ATP and $O_2$ can be demonstrated in isolated lettuce chloroplast.

*Inhibition studies*

Three compounds tested for inhibitory effects on lipid synthesis were ammonium ion, 3(*p*-chlorophenyl)1,1-dimethyl urea, and dinitrophenol. Ammonium ion has been shown by KROGMAN *et al.*[17] and TREBST *et al.*[18] to inhibit phosphorylation in photophosphorylation; 3(*p*-chlorophenyl)1,1-dimethyl urea participates at the site of the photo-oxidation of water[19] and dinitrophenol, while it does not inhibit photophosphorylation and indeed has been shown to be a redox dye in the Hill reaction[20], uncouples oxidative phosphorylation. In Table IV, experiments are presented to show that both ammonium ions and 3(*p*-chlorophenyl)1,1-dimethyl urea, effective inhibitors of photophosphorylation, also inhibit lipid synthesis while dinitrophenol, an effective uncoupler of oxidative phosphorylation, has in fact a slight stimulatory effect.

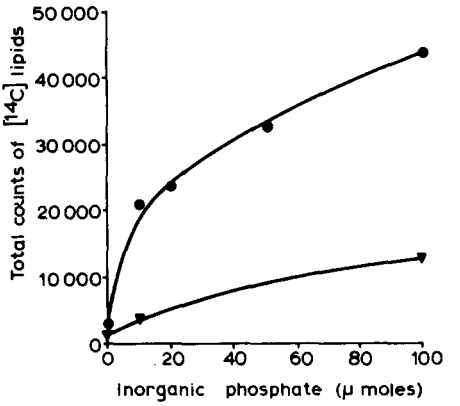



Fig. 7. Incorporation of [2-$^{14}$C]acetate into lipids as a function of inorganic phosphate and ATP in the presence of light. ●—●, in the presence of ATP; ▼—▼, in the absence of ATP; both in the presence of light. Each reaction mixture contained 0.5 ml of fresh chloroplast preparation (Preparation II) which contains 0.7 mg of chlorophyl, 0.1 μmole of [2-$^{14}$C]acetate (55 500 counts/min), 0.2 μmole CoA, 1 μmole of ATP where indicated, 2.5 μmoles of $MnSO_4$, 2.5 μmoles of $MgSO_4$, 30 μmoles of $NaHCO_3$, 0.1 μmole of $TPN^+$, and varying amounts of phosphate buffer (pH 8.2). pH of reaction mixtures without phosphate was 8.2 and no changes were observed.

TABLE IV

INHIBITORY STUDIES ON ACETATE INCORPORATION

Details of reaction mixture as in Table I. Final concentration of inhibitors as indicated.

| *Inhibitor* | *Final concentration (M)* | *Counts/min* | *Inhibition (%)* |
|---|---|---|---|
| None | — | 6480 | — |
| Dinitrophenol | $10^{-5}$ | 7020 | — |
| Dinitrophenol | $10^{-4}$ | 7760 | — |
| Dinitrophenol | $10^{-3}$ | 6500 | — |
| None | — | 4500 | — |
| $NH_3$ | $2 \cdot 10^{-3}$ | 2550 | 43 |
| $NH_3$ | $2 \cdot 10^{-2}$ | 1890 | 58 |
| None | — | 3860 | — |
| 3(*p*-Chlorophenyl)1,1-dimethyl urea | $10^{-4}$ | 820 | 79 |
| | $10^{-3}$ | 480 | 88 |

*The nature of the fatty acids synthesized*

In Fig. 8 is shown a typical gas-radiochromatogram of the fatty acids synthesized by the chloroplast from [2-$^{14}$C]acetate. The larger part of the label is carried by myristic, palmitic, stearic and oleic acids, both linoleic and linolenic acids were unlabelled (unlike synthesis carried out by the intact leaf). Each of the fatty acids was isolated and run on an Apiezon-L column. The labelled substances falling between myristic and palmitic (peaks a and b) and also between palmitic and stearic acids (peaks c and d) were found to have retention characteristics on both polyester and Apiezon-L columns of branched and straight-chain odd number acids of 15 and 17 carbon atoms. The specific activities of these acids were of the same order as those of stearic acid. The biosynthesis of such acids has not been noticed before in plant systems.

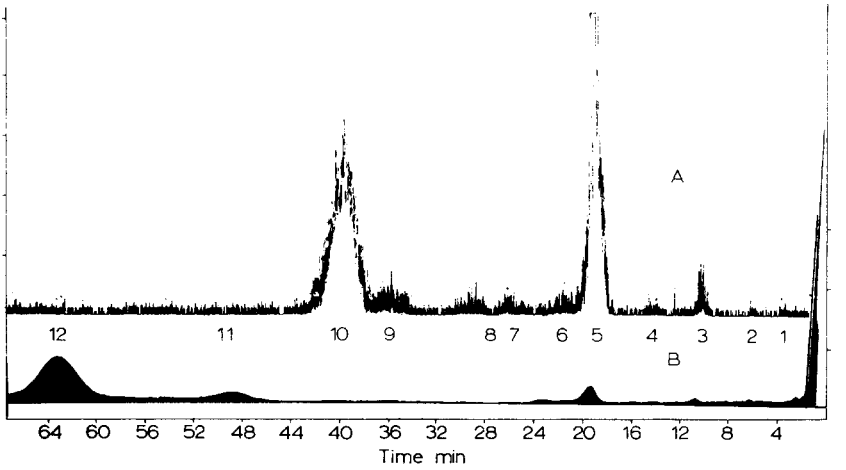
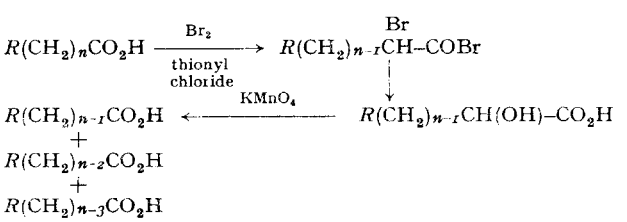



Fig. 8. Typical gas radiochromatogram of the fatty acids synthesized by chloroplasts from [2-$^{14}$C]acetate. Curve A, radioactivity; curve B, katharometer record. Peaks in order of their appearance: 1, decanoic; 2, dodecanoic; 3, tetradecanoic; 4, branched and straight-chain pentadecanoic; 5, hexadecanoic; 6, hexadecenoic; 7, branched heptadecanoic; 8, normal heptadecanoic; 9, octadecanoic; 10, $\Delta^9$-octadecenoic; 11, $\Delta^{9,12}$-octadecadienoic; 12, $\Delta^{9,12,15}$-octadecatrienoic.

*Position of label in saturated acids*

Distribution of the label in palmitic and stearic acids synthesized from [2-$^{14}$C]-acetate was studied by using the degradation method used originally by STEINBERG *et al.*[21]. This consists in brominating the free acid to the α-bromo acid bromide, hydrolysing with alkali to the α-hydroxy acid and oxidising this with permanganate in glacial acetic acid.




Over-oxidation in the final stage gives rise to acids of shorter chain length and by use of the automatic gas-radiochromatogram described earlier, it is a simple matter

to measure accurately the specific activity of the homologous series of fatty acids produced. In practice it is found convenient to measure four carbon atoms along the chain at a time. Unused material can then be reacted again to move down another four carbon atoms. We were interested only in showing the expected alternation of radioactivity along the chain and in demonstrating *de novo* biosynthesis. Typical results are shown in Fig. 9 and in Tables V and VI. The label alternation indicates that some randomisation has occurred but shows clearly that the acids are largely synthesized *de novo*.

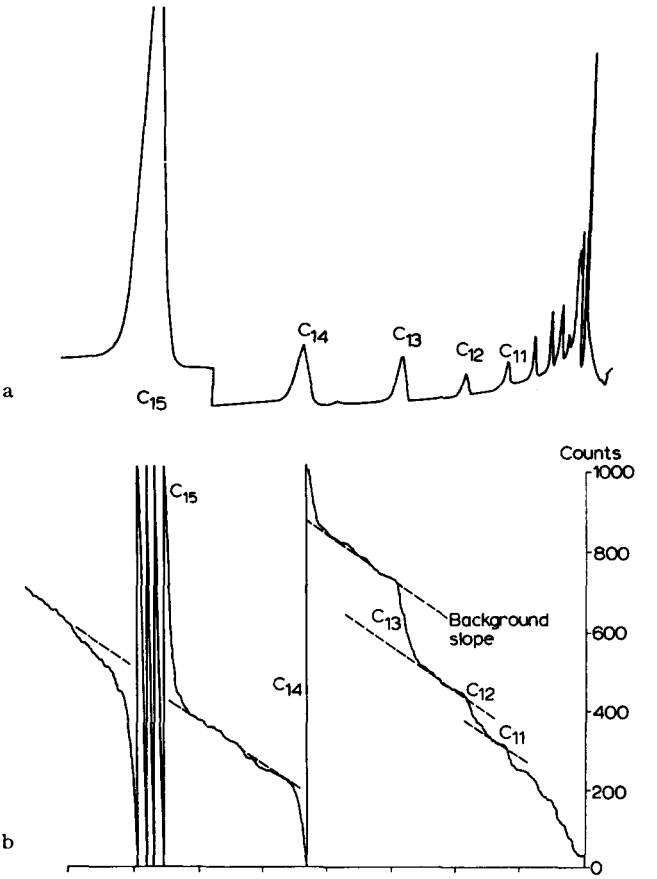



Fig. 9. Degradation of biosynthetic labelled palmitic acid by oxidation of α-hydroxy-palmitic acid. Curve a peak 1, dodecanoic acid; peak 2, tridecanoic acid; peak 3, tetradecanoic acid; peak 4, pentadecanoic acid. Curve b: integrated radioactivity of peaks.

*Unsaturated acids*

The peaks assigned to linoleic and linolenic acids had the expected retention volumes on both types of columns and gave, on oxidative degradation and gas chromatographic analysis of the products, the patterns characteristic of 9:12-octadecadienoic and 9:12:15-octadecatrienoic acids. Neither acid was radioactive.

The peak assigned to oleic acid gave the expected pattern for 9-octadecenoic acid, all the fragments produced by oxidation were radioactive, indicating general

labelling (Table VII). In earlier experiments approx. 25 % of labelled 11-octadecenoic acid was also present as shown by the presence of a labelled $C_{11}$ dicarboxylic acid after oxidative degradation. This was a minor product only in later experiments.

TABLE V

SPECIFIC RADIOACTIVITY OF THE FIRST FOUR CARBON ATOMS OF BIOSYNTHESIZED PALMITIC ACID

| *Acid* | *Counts/min/mmole* ($\times 10^{-3}$) | *Counts/min/mmole* ($\times 10^{-3}$) |
|---|---|---|
| Diluted palmitic acid | 3124 | C-1, 54 |
| Derived pentadecanoic acid | 3070 | C-2, 664 |
| Derived tetradecanoic acid | 2406 | C-3, 180 |
| Derived tridecanoic acid | 2226 | C-4, 325 |
| Derived dodecanoic acid | 1901 | |

TABLE VI

SPECIFIC RADIOACTIVITY OF THE FIRST TWO CARBON ATOMS OF BIOSYNTHESIZED STEARIC ACID

| *Acid* | *Counts/min/mmole* ($\times 10^{-3}$) | *Counts/min/mmole* ($\times 10^{-3}$) |
|---|---|---|
| Diluted stearic acid | 31.9 | C-1, 0.9 |
| Derived heptadecanoic acid | 31.0 | C-2, 7.1 |
| Derived hexadecanoic acid | 23.9 | |

TABLE VII

SPECIFIC ACTIVITY OF MONO- AND DICARBOXYLIC ACIDS PRODUCED BY OXIDATION OF UNDILUTED OLEIC ACID

| *Acid* | *Specific activity (counts/min/μmole* $\times 10^{-3}$) |
|---|---|
| $C_9$ Monocarboxylic acid | 43 |
| $C_9$ Dicarboxylic acid | 151 |

*Aerobic and anaerobic conditions*

At present there are two mechanisms for the unsaturation process, both proposed by BLOCH[22]. One involves an aerobic NADPH–$O_2$ system which catalyzes the unsaturation of a long-chain acyl CoA to form a single double bond acyl CoA. Thus stearoyl CoA in the presence of NADPH and oxygen is converted to oleyl CoA and palmityl CoA is converted to palmit-oleyl CoA. The second process is anaerobic and apparently is the mechanism for the synthesis of unsaturated fatty acids by anaerobic organisms. This mechanism involves the condensation of malonyl CoA to a $C_8$ or $C_{10}$ acid with the formation of a $\beta$-keto acyl CoA derivative and the reduction of the $\beta$-keto derivative to a $\beta$-hydroxy acyl derivative and its subsequent dehydration to form the required double bond. Additional malonyl CoA units are added to complete the molecule. JAMES[1] has presented evidence in support of the aerobic mechanism in intact leaf tissue by feeding a series of fatty acids to intact leaf preparations. However, with isolated chloroplast preparations, the incorporation of shorter chain fatty

acids into oleic acid is low. Moreover, since this system does not require oxygen, this process should proceed when the reaction is run under anaerobic conditions. In Table VIII is reported an experiment which had as its gas phase either air or nitrogen Under these conditions oleic acid formation is completely suppressed by the omission of oxygen in the reaction medium; in its presence oleic acid is formed. Since stearic acid is not converted to oleic acid by chloroplast preparations, it would appear that in the chloropast as in avocado particles[23], oxygen is required for the unsaturation process. The detailed mechanism of this system is as yet not known.

TABLE VIII

INCORPORATION OF [$^{14}$C]ACETATE BY CHLOROPLASTS UNDER AEROBIC AND ANAEROBIC CONDITIONS

See Table I for details of reaction mixtures.

| *Chloroplast fatty acids* | *Distribution of radioactivity as per cent total fatty acid activity* | |
|---|---|---|
| | *Aerobic* | *Anaerobic* |
| $C_{10}$ | 0.8 | 1.3 |
| $C_{12}$ | 1.7 | 3.3 |
| $C_{14}$ | 9.3 | 9.7 |
| $C_{16}$ | 58.5 | 61.2 |
| $C_{18}$ | 9.1 | 23.0 |
| $C_{18:1}$ | 20.7 | 0 |

*Distribution of $^{14}$C in chloroplast lipids*

The total lipid isolated from chloroplasts incubated with [$^{14}$C]acetate was placed on a silicic acid column and eluted by the procedure of FREEMEN *et al.*[12]. Three main fractions were obtained depending on the eluting solvents: (a) the hexane (60–80°)-eluted fraction contains steroids and hydrocarbons such as carotenes; (b) the chloroform-eluted fraction contains free fatty acids and mono-, di-, and triglycerides; and (c) the methanol-eluted fraction contains phospholipids and glycolipids. 70% of the total incorporated radioactivity was found in the chloroform fraction and the remaining 30% in the methanol fraction. No radioactivity was found in the hexane fraction. In the chloroform fraction 70% occurred as free long-chain fatty acids and 30% as a neutral lipid, presumably a mixture of mono-, di-, and triglycerides.

The movement of radioactivity into these three major fractions as a function of time is summarized in Fig. 10. There is little, if any, incorporation into the caro-

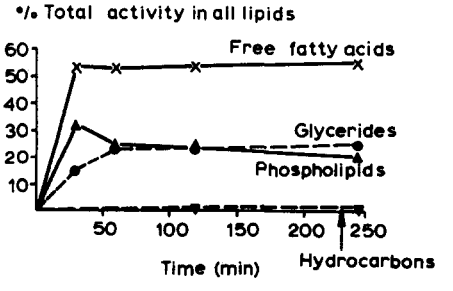



Fig. 10. The movement of radioactivity into various lipid fractions as a function of time.

tenoids or steroids. The phospholipid and glycolipid fractions do not show any significant trend. Since free fatty acids are the major end products and since phospholipases and lipases are known to occur in chloroplasts, it is probable that the fatty acids are first esterified to a glyceride derivative but are rapidly hydrolyzed to free fatty acids which then accumulate. Presumably some of the complex lipid derivatives are not susceptible to the action of the hydrolytic enzymes. Hence the newly incorporated fatty acids are not hydrolyzed and remain associated with that fraction. This would explain the failure of the complex derivative curve to decrease with time. This relationship is depicted as follows:

$$\text{Acetate} \longrightarrow \text{long-chain acyl CoA} \longrightarrow \text{complex derivatives} \xrightarrow[\text{rapid}]{H_2O} \text{free fatty acids} + \text{acceptors}$$

## DISCUSSION

Isolated chloroplasts are thus capable of carrying out the syntheses of long-chain fatty acids and complex lipids observed in the intact leaf with the exception of the conversion oleic $\longrightarrow$ linoleic $\longrightarrow$ linolenic acid. Whether this is due to damaging of the enzyme complex during isolation of the chloroplasts or whether this particular enzyme system is located elsewhere in the leaf, is impossible to say at this stage. During time experiments the specific activity of the oleic acid is found to rise steadily, whereas in the intact leaf an initial rise is followed by a steady fall as the oleic acid is converted to linoleic acid. The poor conversion of the $C_8$, $C_{10}$, $C_{12}$ and $C_{14}$ acids may be due to inadequacy of the initial activation stage in the isolated chloroplasts.

The cofactor requirements for fatty acid synthesis by the chloroplast are the same as found in other systems. The effect of light is explicable in terms of the generation of ATP and NADPH and there seems to be little evidence to support SMIRNOV's contention[3] of a specific photoactivation step in fatty acid synthesis by chloroplasts.

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