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Lipid Biosynthesis, Oxidative Enzyme Activities and Cellular Changes in Growing Olive Fruit

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Fruit of *Olea europea* L. was examined by light and electron microscopy to determine whether commencement of lipid accumulation depended upon the fruit achieving structural maturity. Maturation of fruit develops progressively from the smallest changes towards the largest in cellular structures. Important metabolic and structural changes have been observed: oil body formation, changes in the structural and reserve lipid bioshynthesis and in the fatty acid of total lipid content, as well as in G6PDH and LOX activities. The labelling of fruit lipids by previously incubating the leaves with (1-^{14}C)-acetate and (1,5-^{14}C)-citrate or by putting the labelled subtrates directly on the fruit surface, shows a ^{14}C assimilate derived from acetate greater than that from citrate; the incorporation of the latter is higher in the methanol-water fractions. At the beginning of fruit development the lipid biosynthesis with both substrates is greater in polar lipids; on the contrary, the incorporation of ^{14}C into neutral lipids increases during fruit maturation. Additionally, a maximum of substrate export from leaves to fruit coincides with an increase in the lipoxygenase and, above all, in the glucose-6-phosphate dehydrogenase activities. The transported ^{14}C from leaves begins its activity before the small oil bodies close to the tonoplast can be observed in the fruit, and well before the beginning of maturation. The results suggest that structural development and some other rate controlling metabolic steps can govern the initiation of lipid accumulation in olive fruit.

Key words: Olea europaea, Fruits, Lipid briosynthesis, Glucose 6-phosphate dehydrogenase, Lipoxygenase, Morphology.

The study of structural changes which take place in the olive fruit during its development, may be very important for interpreting the metabolic transformations associated with growth and maturation. Up till now, there has only been a morphological study of this fruit on a light microscopic level and from a histological point of view (9). An ultrastructural examination of other oleaginous fruits and seeds shows a strong formation of new cellular structures in the period before ripening (oil

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bodies or oleosomes), where biosynthesis and accumulation of triglycerides take place (1, 14). From a physiological point of view ripening represents a stage of differentiation and a direct process requiring the synthesis of specific enzymes (18, 20). In this sense, during fruit development a chloroplast membrane degradation takes place (22). With this membrane degradation there is a hydrolysis of lipids and the free fatty acids which are toxic for the cell, are degraded by various ways. For example, the lipoxygenase enzyme catalyzes the oxygenation of polyunsaturated fatty acid as its principal substrate in plants (10). In this respect, it was able to be verified in olive fruit, the *in vivo* oxidation of fatty acid through lipoxygenase action.

Additionally, these structural changes which are produced during the development of the fruit, provoke a modification in the cellular volume, permitting an increase in the transportation of products from the leaf. This transport of foliar material, basically carbohydrates, is very important and appears to be regulated to a great extent by the previously cited intracellular compartmentation and processes in the fruit. On the other hand, it has been observed in many fruits that during the respiratory crisis before ripening there is a change in the pentose phosphate cycle towards glycolysis (13). In castor bean endosperm the former process supplies the carbon for fatty acid biosynthesis and also the required amounts of ATP and reducing power (26). In general, the cytosol and chloroplasts contain the first two enzymes of this cycle, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The former enzyme acts in absence of oxygen and in the presence of NADP, what is more, as is does not use ATP, the cycle should be very active in immature fruits, where the oxydative processes still have no development (2). The capacity of olive fruit to biosynthesize a lot of reserve lipids, has made it possible to define the origin of carbonate energy necessary for such a big lipidic synthesis. In this sense, in previous work an inverse relationship between the amount of Krebs cycle acids in leaf and fruit during its development has been found, suggesting a continuous migration of acids from the leaf (7).

From what has been said one deduces that it is important to study the following objectives during olive fruit development: the lipid biosynthesis and the translocation pattern labelled acids to the fruit following pulse labelling of source leaves with (1-^{14}C) acetate and (1,5-^{14}C) citrate; the determination of the changes in the content of fatty acids from total lipids and in the activity of the G6PDH and LOX enzymes. An examination of the structural development of the mesocarp cells will also be undertaken and an attempt to correlate the observed structures with the biosynthesis and accumulation of lipids.

Materials and Methods

Plant Material. Olive trees 40-50 years old (*Olea europaea*, cv. Marteño) were used. The sampling was made as described in previous work (8). Fruit samples were collected at 10-day intervals from the 1st June to December 1981 and again 1982 and all the following steps were carried out.

Light and Electron Microscopy. Small pieces of the fruit tissues were fixed in 6 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 for 6 hours at

Abbreviations: G6P = Glucose-6-phosphate; G6PDH = Glucose-6-phosphate dehydrogenase; LOX = Lipoxygenase; PPC = Pentose-phosphate cycle.

4° C and postfixed with 2 % buffered osmium tetroxide for 1 hour at 4° C. Samples were dehydrated through a graded ethanol series and embedded in ERL (23). Sections were cut on an LKB III ultramicrotome and stained with Toluidin blue for light studies. For the electron microscopy the sections were double stained using 2 % uranyl acetate and Reynold's lead citrate and examined with a Zeiss EM-952 electron microscope.

Preparation of Enzyme Extracts. Glucose-6-phosphate dehydrogenase and lipoxygenase were extracted from olive tree fruits on each day of the experiments. Fruits (ca. 20 g fresh weight) were homogenized with 7 vols. of 0.1M Tris-HCl buffer, pH 7.5 contaning 1 % Triton X-100, 2 % insoluble polyvinylpyrrolidone, for 2 min at top speed (Sorvall Omnimixer) at 0° C. The homogenate was filtered through three layers of nylon cloth and the filtrate was centrifugated at 27,000 × *g* for 20 min. The extract was used immediately as the enzyme source.

Enzyme Assay and Determination of Protein. The activity of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate NAD(P) oxidoreductase, E.C.1.1.1.49) G6PDH was measured spectrophotometrically by following the initial rate of changes in absorbance at 340 nm. The standard reaction mixture contained the following components in a final volume of 2.5 ml: 0.1 M Tris-HCl buffer pH 8.0, 0.01 M $NADP^+$, 0.03 M G6P and 0.15 M $MgSO_4$. The reaction was started by the addition of 1.0 mg of crude enzyme. Under the condition of the assay the NADPH formation was proportional to time. The enzyme activities were expressed in terms of units; one unit of G6PDH activity was defined as a change of 0.1 absorbance unit x min^{-1} at 30° C.

Lipoxygenase activity (Linoleate: O_2 oxidoreductase, E.C.1.13.11.12) LOX was determined at 25° C using a Clark O_2 electrode (Gilson Oxygraph, model KM). The incubation medium contained in a final volume of 3.5 ml, 0.05 M phosphate buffer pH 7.0 and different quantities of enzymatic protein. The oxygen was measured in the presence of 1.0 mM linoleic acid as substrate.

The proteins were determined by the method of MARKWELL *et al.* (17).

Pulse Labelling Experiments Using (1-^{14}C) acetate and (1,5-^{14}C) citrate. For *in vivo* synthesis of lipids, four 30-40 cm long branches with fruits and leaves were taken and their basal ends immersed in flasks with water. In the first and second branches leaves close to the fruits were selected, and (1-^{14}C) acetate (specific activity 60 mCi × $mmol^{-1}$) and (1,5-^{14}C) citrate (specific activity 27 mCi × $mmol^{-1}$) was put on 10 leaves, as aqueous microdroplets (10 microliter of a 50 nCi × ml^{-1} solution of acetate on each leaf of the first flask and 10 microliter of a 60 nCi × ml^{-1} solution of citrate on each leaf of the second flask). The same quantity of labelled acetate and citrate was put on the surface of 10 fruits on the third and fourth branches respectively. The branches were kept in a growth chamber at 22° C under continuous light (12.9 w × m^{-2}). After 24 h incubation time the adjacent fruits of labelled leaves (first and second flasks) and labelled fruits of the third and fourth flasks, were cut off for analysis.

Analysis of Lipids. Total lipids were obtained according to BLIGH and DYER (6). The separation of polar and neutral lipids was carried out by thin layer chromatography on Silicagel G plates (12). Fatty acid methyl esters from total lipids were prepared according to LECHEVALLIER (15) and determined by

gas chromatography on a column of 15 % diethylene glycol succinate on Chromosorb W, 80-100 mesh at 180° C using a flame ionization detector (Hewlett-Packard model 5730). Methyl heptadecanoate was included in the samples as an internal standard.

Radioactive compounds were localized on the plates by autoradiography (Valca X-ray film). The radioactivities of the chloroform fractions obtained according to BLIGH and DYER, and the ^{14}C labelled products separated on the plates were measured with a liquid scintillation counter (Packard Tri-Carb) in a scintillation mixture consisting of 5 g PPO and 0.3 g dimethyl-POPOP × l^{-1} toluene. For methanol-water soluble compounds, a mixture of 10.5 g PPO, 0.45 g POPOP, 15 g naphthalene, 1,500 ml dioxane and 300 ml water was used.

Results

Morphological study. From a very early stage, the mesocarp of olive fruit is composed by isodiametric parenchyma cells with the normal complement of cell organelles: a large central vacuole surrounded by cytoplasm and the nucleus located usually in the peripheral region (plate A, 1). Mitochondria, plastids and rough endoplasmic reticulum are observed in a cytoplasm rich in ribosomes. However, dyctiosomes as much as Gorgi vesicles are scarce. Small grey bodies can also be observed in the peripheric cytoplasm (plate A, 2, 3). As the fruit grows larger, a greater number of these bodies are observed in the cytoplasm and also in the vacuole, frenquently in direct contact with the tonoplast (plate A, 4). In a more advanced stage of fruit development the grey bodies in the vacuole fuse, thus forming large oil droplets during the fruit maturation (plate A, 5; plate B, 6). During fruit development an important change in the content of the vacuole also takes place, which is at first electron-transparent (plate A, 1) but later evolves becoming fibrillar (plate A, 5). Simultaneously, invaginations of the tonoplast are seen, which give rise to spheres of variable diameter with a simple unit membrane whose contents, coming from the hyaloplasm, become analogous to the large vacuole but denser, and are incorporated into the latter compartment (plate B, 7).

In the mesocarp cells the morphology of plastids depends on their cellular localization, according to whether they are more or less superficial and to the stage of fruit development. When the mesocarp cell is more external and when the fruit is younger, the plastids present the characteristic features of a chloroplast (plate A, 2, 4; plate B, 7). When the cells are more internal or when fruit development is more advanced, the plastids undergo a gradual decrease in their internal membranes and a parallel increase in their content of starch granules (plate B, 8-10). Finally, when the fruit is near ripening a large grey body occupies the totality of

Plate A. *Formation of the oil bodies in mesocarp cells during olive fruit development.*
1: Mesocarp cells with large central vacuole whose content is electron-transparent, in an early stage of fruit development (× 3,000). 2-3: Grey bodies are observed in the cytoplasm at an early stage of fruit development; the content of the vacuole beginning becomes fibrillar (arrow) (× 20,000). 4: Grey bodies are also observed between the cytoplasm and the vacuole (× 20,000). 5: A large grey body (O) is localized in the vacuole. The content of the vacuole present a fibrillar aspect (*) (× 4,000). Abbreviations: V: vacuole; N: Nucleus; P: Plastid; CW: Cell Wall; IS: Intercellular spaces; ER: Rough Endoplasmic Reticulum; M: Mitochondria; St: Starch; ob: Oil body (grey body); O: Large oil body.

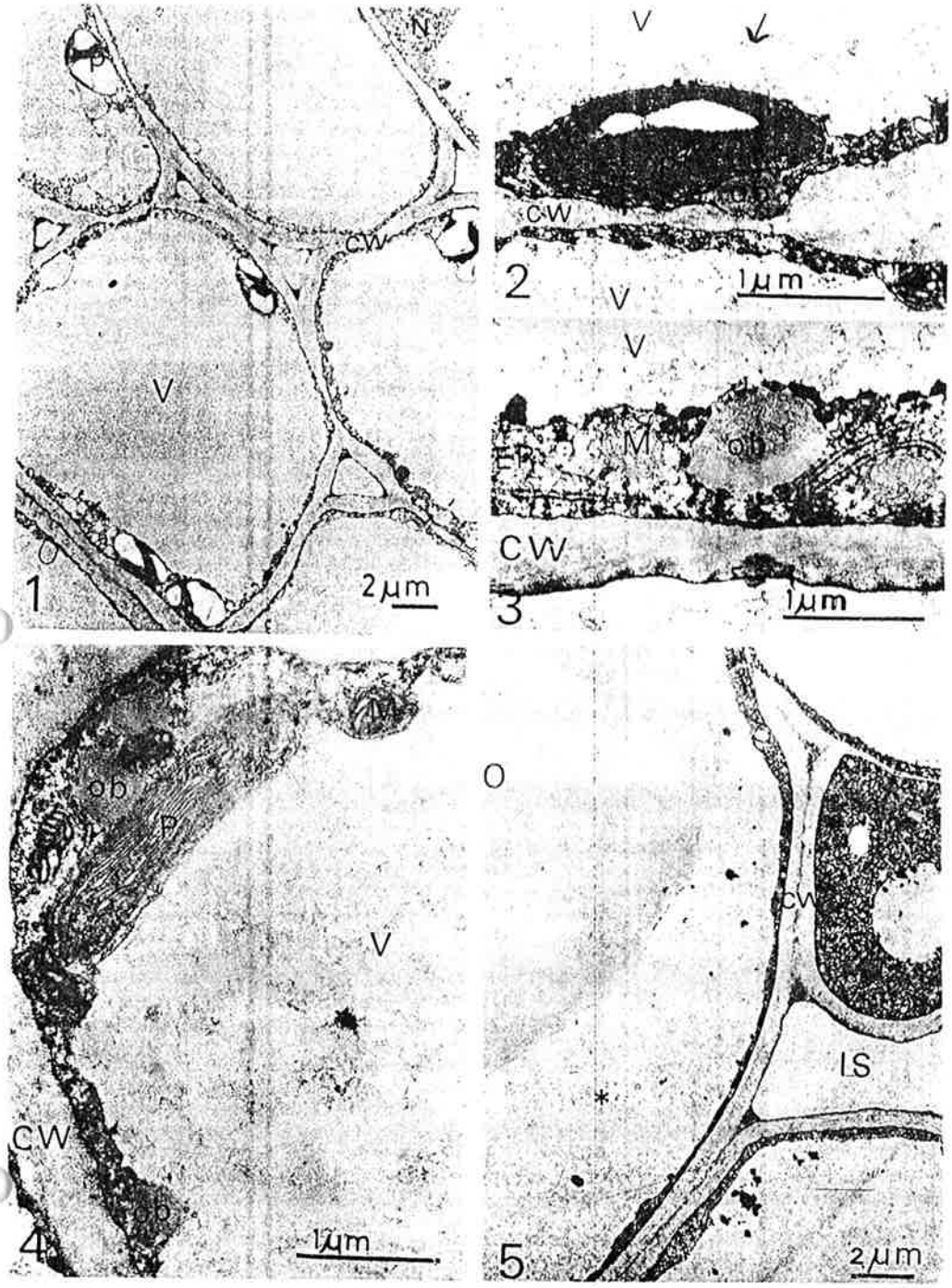

PLATE A

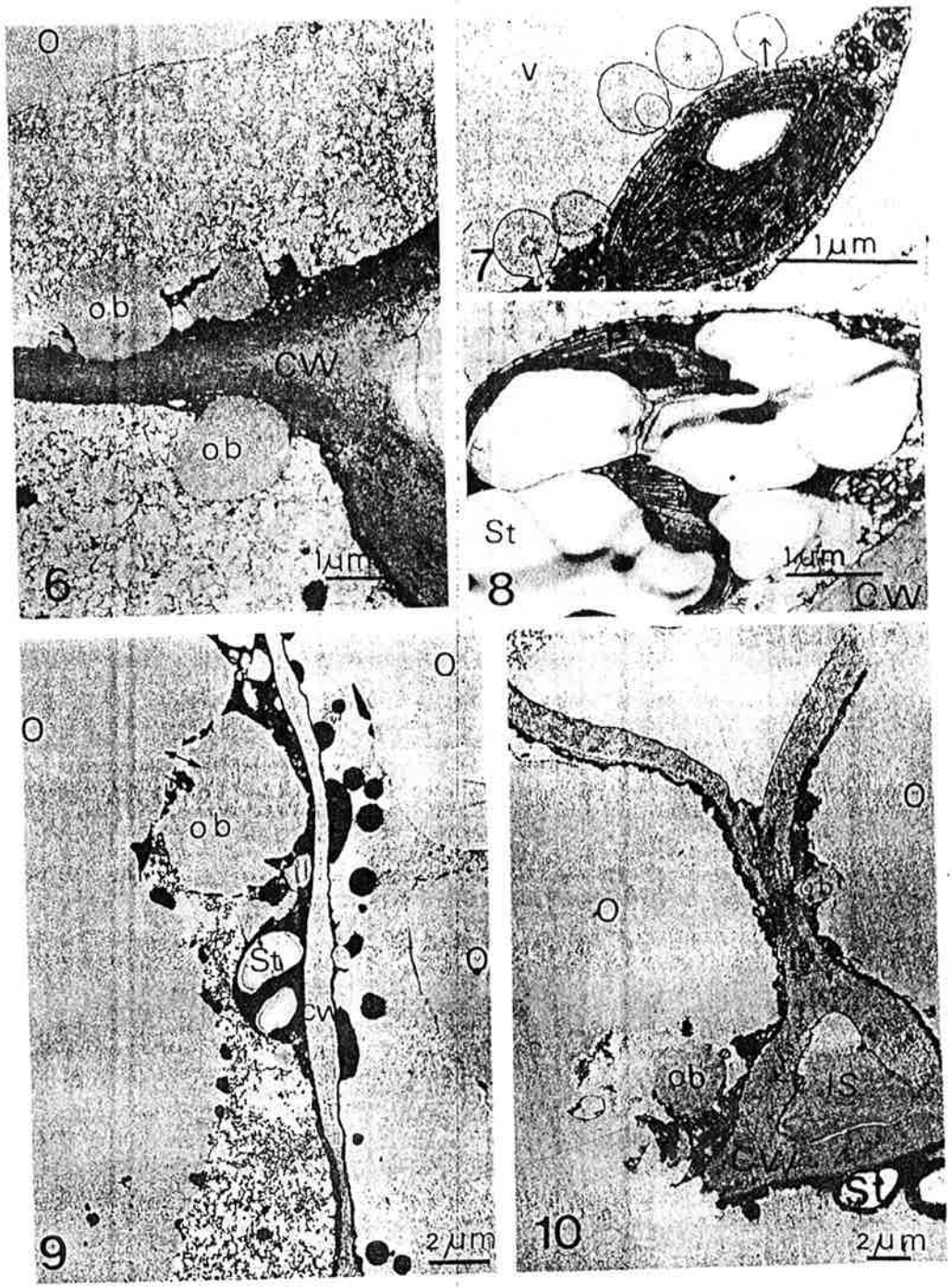
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PLATE B

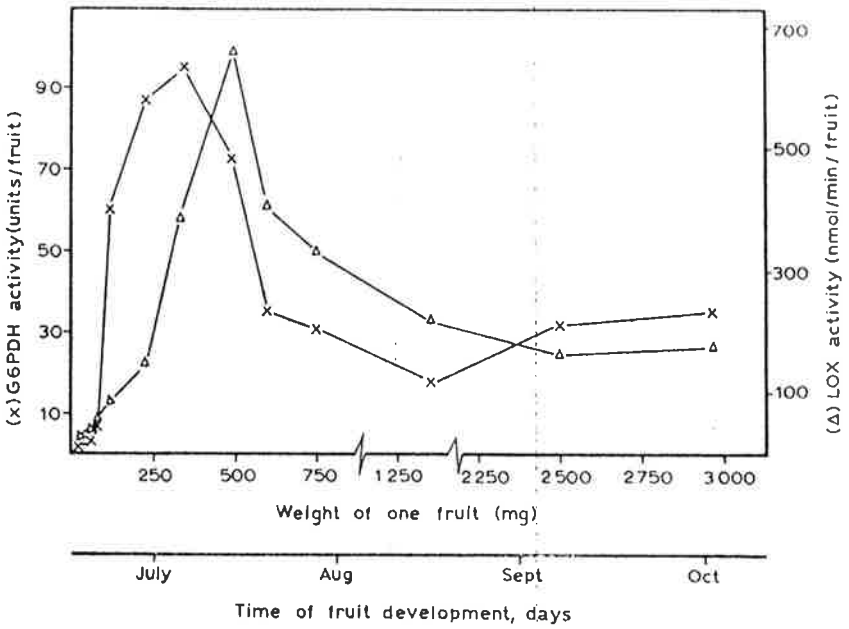

Fig. 1. *Changes in the G6PDH (x) and LOX (Δ) activities of olive fruit during development.* Results are expressed as enzyme units/fruit (G6PDH) and as nmol O_2 consumption/min/fruit (LOX). The values are the average from four independent experiments not differing by more than 10 % (G6 PDH) or 5 % (LOX) from the mean.

the vacuole (plate B, 9) and senescence phenomena are observed: rupturing of the cell walls and cellular degradation in the cytoplasm (plate B, 10); the lipid contents from adjoining cells are mixed and a larger lipid bag is formed in the fruit, which is limited on the outside by the exocarp and on the inside by the lignificated endocarp.

Enzyme activities. An increase in the G6PDH activity is observed (fig. 1) from the first period of fruit formation up till beginning of endocarp differentiation. The LOX activity is very reduced during the whole cycle, although as the G6PDH also shows a maximum during the same period of development (weight of fruit from 50 to 500 mg). A strong

Plate B. *Formation of the oil bodies in mesocarp cells during olive fruit development.*
6: A large oil body (O) is present in the vacuole and small grey bodies (ob) are also observed in contact with the peripheric cytoplasm (× 8,600). 7: In the vacuole are present invagination of the tonoplast (arrow) and spheres (*) with a single unit-membrane. The content of the latter structures are analogous to the vacuole but with a greater density (× 19,000). 8: Plastid of internal mesocarp cells at a young stage of fruit development. A great number of starch granule and a scarce internal membrane system can be observed (× 14,000). 9: The grey bodies (ob) in the vacuole apparently fuse (arrow) and a large oil body (O) occupies the totality of this compartment (× 4,000). 10: Rupturing of the cell walls and mixing of the lipid content from adjacent cells (X 3,000). Abbreviations as in Plate A.

Table I. *Changes in the content of fatty acids from total lipids of olive fruit during development.* Each value is the average of three independent experiments, not differing by more than 10 % from the mean. Results in percentages of fatty acids in total lipids.

Weight of one fruit (mg)	% of fatty acids 16:0	18:0	18:1	18:2	18:3
5	45.02	0.82	17.56	14.95	21.45
9	40.87	1.73	19.73	17.24	20.43
20	31.51	1.00	19.42	20.53	27.55
33	36.25	2.02	22.95	16.88	21.90
124	30.40	1.70	27.24	16.34	24.32
222	24.02	0.50	24.98	21.58	28.72
331	30.10	3.47	25.98	22.47	17.98
570	29.15	2.57	27.19	22.96	18.13
750	28.00	2.00	39.88	17.20	12.92
1350	25.12	2.72	51.16	12.33	8.78
2500	17.91	3.24	60.27	6.58	7.21
2900	15.90	2.52	80.05	0.71	0.81

diminution in these activities was observed afterwards.

Analysis of Lipids. Table I shows the changes in the percentages of fatty acids from total lipids in olive fruit. A parallelism is observed between increase in the percentage of oleic acid and the diminution of linoleic acid. This fact coincides with a maximum in the lipoxygenase activity.

Lipid Biosynthesis. The labelling of fruit lipids after feeding source leaves with (1-^{14}C)acetate and (1,5-^{14}C)citrate, which were also put directly on the surface of the fruit during its development are shown in tables II and III. At the beginning of fruit development ^{14}C assimilates derived from (1-^{14}C)acetate is greater in lipid fractions; on the contrary, methanol-water fractions show a higher ^{14}C incorporation during ripening. In general, limited incorporation occurred in different fractions of fruit derived from (1,5-^{14}C)citrate, but with higher concentration of ^{14}C assimilates in the methanol fractions (table II). It can be seen from table III the percentages (1-^{14}C)acetate and (1,5-^{14}C)-citrate incorporated to different lipid categories. At the beginning the biosynthesis is greater in polar lipids; on the contrary, neutral lipids show a higher percentage ^{14}C incorporation during ripening, although important biosynthesis of polar lipids was also observed during this period.

Discussion

From morphologic study it can be established that the grey bodies observed in mesocarp cells from a very early period of olive fruit development, correspond to the oleosomes or oil bodies that are described by authors in other origins (1, 27). The results that have been obtained in this work do not permit us to maintain without reservation any of the existing hypothesis in relation with how the oil bodies originate in higher plants. In this respect, FREY-WISSLING *et al.* (11) suggest an origin form endoplasmic reticulum and

YATSU and JACKS (28) proposed that the oil bodies are bound by a half unit membrane. PARKER and MURPHY (19) found that the oleosomes are bordered with an osmiophilic layer rather than a unit-membrane and BERGFELD *et al.* (5) had already suggested and origin from plastids. From these results obtained in olive fruit it is not possible to assure that the grey bodies which are initially found in the cytoplasm and later in the vacuole, are chemically similar, as in the vacuole, at first electron-transparent and later becoming fibrillar; apart from the grey bodies, there are also spheres of variable diameter and unknown chemical composition. All this suggests the possibility that the mesocarp olive vacuoles besides accumulating the reserve lipids, can also contain the necessary substrates and enzymes for these lipid biosynthesis.

An objective of this work was to study lipid biosynthesis fluctuations in olive fruit and to attempt to relate this information to the translocation of substrates from leaves. Pulse feeding of source leaves with (1-^{14}C)acetate and (1,5-^{14}C)citrate have permitted this study. Considering the period from the beginning of fruit formation up to maturity, it was apparent that the initial period placed a heavy demand on current photosynthate from the leaf. In this sense, at an early stage the fruit may have to compete with the leaves for the available substrate; aftewards, when the fruit had become ripe, the plant showed a rapid decline in translocated ^{14}C to the fruit. The labelled lipid came principally from acetate. A low level of labelled lipid fractions derived from citrate is observed, despite the high level of endogenous organic acids, principally malic and citric (7). It can be seen that the biosynthesis of polar lipids is greater at the beginning of olive fruit development than during ripening, although an important biosynthesis of these lipids in the later period, necessary for the formation of new membranes and also as a substrate in the triglyceride biosynthesis, is also observed (25).

From the enzymatic activities measured, it appears that the G6PDH enzyme may contribute to determining the participation of PPC in the G6P metabolism during the growth of olive fruit. This estimate is important with respect to our understanding the carbohydrate metabolism in this fruit. In this sense, previous works have shown a strong increase in soluble sugars and malate/citrate ratio value in a later period after maximum activity G6PDH, before the beginning of maturation (7). These results suggest that the sugar metabolism in long chain fatty acids in this fruit, requires the pentose-phosphate pathway intermediate enzymes, as a means in order to provide the reducing power for biosynthesis. Up to the moment the biochemical and physiological functions which the lipoxygenase effects in superior plants is unknown (4, 24). In olive fruit a diminution of linolenic acid coincides with a maximum in the lipoxygenase activity. In this sense, SÁNCHEZ-RAYA *et al.* (21) have verified an increase in the ethylene evolution in the same period as the life of this fruit. Taking into account that the linolenic acid may act as a natural ethylene precursor, above all, during fruit maturation (3, 16), it is possible that during this stage an induction of the biosynthesis of ethylene takes place in this fruit through lipoxygenase action.

In conclusion, in a period of olive fruit development that should coincide with the beginning of maturation, important metabolic and structural changes have been observed: oil body formation and thylakoid membrane degradation; changes in the structural and reserve lipid biosynthesis and in the fatty acid of total lipids content, the same as G6PDH and LOX activities. All this suggests an

intense metabolism and important cellular transformation during this period of fruit development.

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Resumen

Se estudian, en la aceituna, algunos cambios estructurales y metabólicos que tienen lugar en relación con la maduración: formación de cuerpos grasos, biosíntesis de lípidos de reserva y de membrana, cambios en las actividades glucosa-6-P-deshidrogenasa y lipoxigenasa. La incorporación de C^{14} a los lípidos del fruto, incubando previamente las hojas con (1-C^{14})acetato y (1,5-C^{14}) citrato o depositando los substratos marcados directamente sobre la superficie de los frutos, es superior a partir de acetato que de citrato. En la etapa inicial del desarrollo del fruto es mayoritaria la biosíntesis de lípidos polares; por el contrario, durante la maduración la síntesis se dirige, principalmente, a los lípidos neutros. Un máximo en la cantidad de substrato marcado exportado desde la hoja, coincide con un incremento en la actividad lipoxigenasa del fruto. El transporte de C^{14} desde la hoja comienza antes de que en el fruto se detecten cuerpos grasos de pequeño tamaño próximos al tonoplasto. Los resultados sugieren que los cambios metabólicos y estructurales observados pueden determinar el inicio de la acumulación de lípidos en la aceituna.

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