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GLYCEROLIPID SYNTHESIS BY MICROSOMAL FRACTIONS FROM *OLEA EUROPAEA* FRUITS AND TISSUE CULTURES

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Key Word Index—*Olea europaea*; olive; glycerolipid; microsomal fraction; callus culture; lipid synthesis.

Abstract—Glycerolipid synthesis in olive, *Olea europaea*, was investigated using both olive fruits and tissue cultures. Microsomal fractions were isolated from fruits and tissue cultures and incubation conditions with [^{14}C]glycerol 3-phosphate were optimised. Characteristics of these incubations, including the incorporation of radioactivity into intermediates of the triacylglycerol synthetic pathway, were compared. The effect of temperature on triacylglycerol synthesis via the Kennedy pathway was also examined. Increasing the temperature of the incubations from 20 to 30°, but not from 30 to 40°, increased the rates of triacyglycerol synthesis for microsomes from both fruits and tissue cultures. In the case of olive callus cultures, microsomes prepared from callus tissue cultured at 35° displayed greater levels of triacylglycerol synthesis than microsomes prepared from 25° maintained cultures. Possible reasons for these differences are discussed. © 1997 Elsevier Science Ltd

INTRODUCTION

The world production of edible oils has doubled in the last twenty five years, to over fifty million tons per annum. This has been achieved through improved agricultural practices and the development of new varieties of oilseeds through plant breeding. Olive oil can be consumed without any refining and it differs from other major edible oils in two other important ways. First, it has a high percentage of oleic acid together with significant natural antioxidants and a low percentage of saturated fats which give the oil good oxidative stability whilst still being desirable nutritionally. Second, it has high concentrations of minor flavour compounds which give olive oil its characteristic fragrance and taste. Since no other edible vegetable oil has similar characteristics, olive oil commands a high price in the marketplace. This has introduced a temptation for adulteration in order to gain more profit [1].

Most of the research on glycerolipid biosynthesis in oil-rich tissues has been done with seeds [2]. Although avocado and olive fruits have been used in the past, and recent utilisation of such tissues has increased [3, 4], we still do not have much knowledge of fruit metabolism in comparison to seeds. A reason for this resides in the fact that fruits at the right stage of development, when the enzymes involved in lipid biosynthesis are most active, are not as readily available as seeds. This problem has been addressed in recent years where glycerolipid synthesis in olives, including the acyltransferase enzymes of the Kennedy pathway, has been studied [4]. The enzymes involved in triacylglycerol formation and acyltransferase reactions are located on the endoplasmic reticulum. Thus, microsomal fractions can be prepared, stored and used subsequently to study the formation of complex lipids *in vitro*.

One way around the problem that active developing fruits are not available year-round is to try and use tissue cultures from such crops. In comparison to the level of research using tissue cultures from oilseed rape and palm, other oil-producing crops such as olive have been neglected. This has been redressed somewhat with a recent study of lipid metabolism in tissue cultures of *Olea europea* [5]. Olive callus cultures have proven to be very stable [6] and, therefore, are a suitable supply of experimental material for biochemical studies. The advantages of a tissue culture system are that a year-round supply of tissue is provided and it is relatively easy to test individual parameters on tissue cultures with regard to possible effects on lipid synthesis. Furthermore, in many cases enzyme preparations from cultures are more stable than those from fruits, possibly because of the lower levels of phenolics and/or proteinases in cultures.

The underlying theme of this study was to study

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glycerolipid synthesis in olives. In the work reported here we have compared the ability of microsomal preparations from olive cultures with those from olive fruits to synthesise glycerolipids. The data show that microsomes from callus cultures are capable of satisfactory rates of glycerolipid, including triacylglycerol, synthesis. Together with their other advantages, referred to above, this makes them a suitable and convenient system for further studies on lipid biosynthesis.

RESULTS AND DISCUSSION

The preparation of active subcellular fractions from olive fruits has proved to be extremely difficult and, for a number of years, such preparations were of limited utility. Cell extracts rapidly turned brown, probably due to oxidation reactions of polyphenols present in the fruits. This problem has been partly overcome by Sanchez *et al.* [4] who developed a procedure for preparing active subcellular fractions which allowed the assay of glycerolipid synthesis in fractions from maturing olives using [^{14}C]acyl-CoA substrates. Using this procedure [4], we isolated microsomes from ripening olive fruits but the preparations did not incorporate any appreciable radioactivity from [^{14}C] glycerol 3-phosphate into glycerolipids. It was originally considered that the time taken for the olives to arrive in Cardiff from Seville (three to four days) was the limiting factor in that the enzyme activity could have been lost due to deterioration of the fruits during transit. This idea was dispelled when microsomes were prepared in Seville within hours of harvesting and still no incorporation of radioactivity from [^{14}C]glycerol 3-phosphate was observed. However, such preparations could still incorporate radioactivity from [^{14}C] acyl-CoA substrates, as originally reported. Therefore, it seemed that one or more of the enzymes of the Kennedy pathway for triacylglycerol synthesis (such as glycerol 3-phosphate acyltransferase) was inactive in such microsomal preparations from olive fruits, whereas other acyltransferases (capable of using acyl-CoAs) were still active.

As mentioned above, extracts from olive fruits rapidly turned brown due to the oxidation of polyphenols and Sanchez *et al.* [4] overcame this problem by including suitable protectants in the homogenisation buffer. Thus, for example, in order to observe fatty acid synthetase activity in olive extracts, it was necessary to pass the cell extract through a Sephadex G-25 column to remove any endogenous low molecular weight inhibitors [7]. This procedure was tested also with microsomal fractions from olive fruits but still no activity with [^{14}C]glycerol 3-phosphate was observed. The problem was overcome finally by increasing the ratio of homogenisation buffer to weight of olive fruits to 10:1 and by including defatted bovine serum albumin in the incubations because it can bind acyl-CoA esters thus preventing them from forming micelles and also making them more readily available to the acyltransferases. (For some enzymes, acyl-CoAs are inhibitory because of their 'detergent' properties and binding to bovine serum albumin was thought to decrease such effects.) Different concentrations of bovine serum albumin were added to the microsomal incubations to determine the optimal incubation concentration, bearing in mind that bovine serum albumin can interfere with some lipid extractions [8]. It was found that 0.5% bovine serum albumin was optimum for the incorporation of radiolabel into total lipids (results not shown). The preparation of active microsomal fractions from olive callus tissue proved less of a problem than the preparation of microsomes from olive fruits. This is probably due to the fact that callus tissue does not have a cuticular surface layer, like the olive fruit. The absence of this layer with its attendent phenolics and other toxic compounds, makes the preparation of active subcellular fractions easier. Bovine serum albumin was included in the incubation at a concentration of 0.5% in order to obtain optimal activity (results not shown) but the high dilution factor employed in the preparation of fruit microsomes was not required.

The ability of microsomal preparations from olive fruit or from olive callus to utilise [^{14}C]glycerol 3-phosphate in the Kennedy pathway and, hence, synthesise triacylglycerol was optimised with regard to substrate concentrations and other conditions of the assay. The starting point of the Kennedy pathway is glycerol 3-phosphate acyltransferase; its apparent K_m for fruit microsomes was calculated to be 1.37 mM and V_{max} as 94.5 ± 9.4 nmol h^{-1} mg^{-1} protein using Michaelis–Menten kinetics [Fig. 1(a)]. Similar experiments with microsomal fractions from avocado mesocarp indicated an apparent K_m of 0.4 mM and V_{max} of 150 nmol h^{-1} mg^{-1} protein [3]. With microsomes prepared from olive callus microsomes the reaction also showed Michaelis–Menten kinetics [Fig. 1(b)] which gave an apparent K_m of 0.1 mM and a V_{max} of 26.3 ± 6.6 nmol h^{-1} mg^{-1} protein. Clearly this K_m value is an order of magnitude lower than that calculated for olive fruit microsomes. It would be premature to speculate in detail as to the reason for this difference because of the crude nature of microsomal fractions. However, the higher K_m in microsomes from olive fruits could relate to the presence of a competitive inhibitor and/or conformation changes in the glycerol 3-phosphate acyltransferase caused by chemicals or proteinases in the fruits.

The first two reactions of the Kennedy pathway involve the successive transfer of acyl groups from acyl-CoA esters to the glycerol backbone. Because palmitate and oleate are the two most abundant fatty acids in olives, these were used in the incubations (as their acyl-CoA esters). Furthermore, general conclusions from measurements of acyltransferases in many extra-chloroplastic plant preparations suggest that palmitate is an appropriate substrate for the first acyltransferase reaction and oleate for the second [2]. In the case of palmitoyl-CoA, an optimal concentration was about 0.2 mM for microsomes from

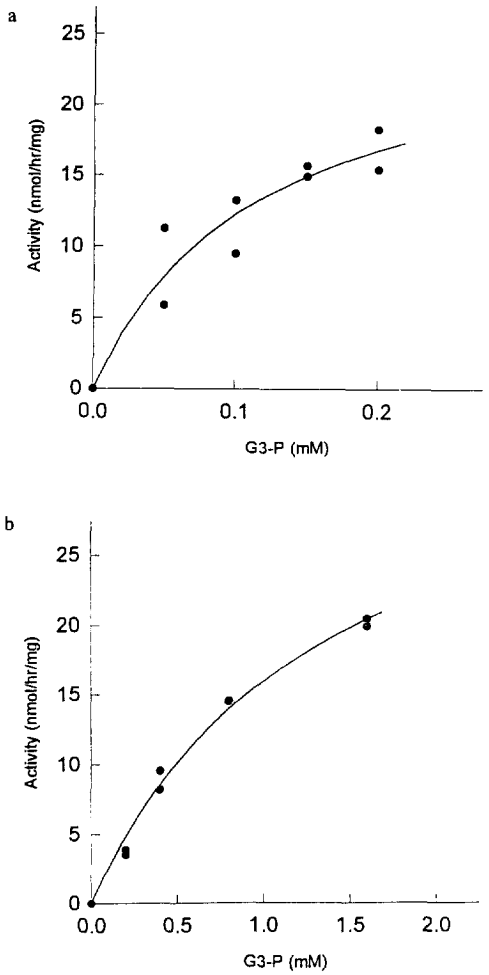

Fig. 1. The effect of glycerol 3-phosphate concentration on the incorporation of radioactivity from [^{14}C]glycerol 3-phosphate into lipids by: (a) olive callus and (b) olive fruit microsomes. Activity is expressed as [^{14}C]glycerol 3-phosphate incorporated into glycerolipids (nmol h^{-1} mg^{-1} microsomal protein). Individual duplicate measurements are shown. Incubations were carried out under standard conditions (see Experimental). The curves were fitted using Michaelis–Menten kinetics and Sigmaplot Scientific Graph System.

fruits, above which inhibition was found (Table 1). Increasing concentrations of palmitoyl-CoA caused rather little change in the pattern of labelling of intermediates (Table 1), except that, at the highest concentration tested, there was a significant reduction in triacylglycerol labelling. In similar preparations the optimal concentration of oleoyl-CoA in the incubation was also found to be 0.2 mM. It was noted that increasing concentrations of oleoyl-CoA caused a progressive increase in the relative labelling of phosphatidate, partly at the expense of lysophosphatidate. This result is in keeping with the preferred use of oleate by the second acyltransferase of the Kennedy pathway. As with palmitoyl-CoA, there was also a reduction in further flux through the pathway at higher oleoyl-CoA concentrations. Thus, diacylglycerol and triacylglycerol seemed less well labelled at high oleoyl-CoA levels (Table 1).

It is important to reiterate the importance of including bovine serum albumin in the incubations. At neutral pH, the critical micellar concentration of palmitoyl-CoA is approximately 4 μM [9]. The concentration of acyl-CoA esters found optimal in these experiments is greatly in excess of this but the inclusion of bovine serum albumin should help prevent the formation of micelles by binding the esters to albumin. Thus the acyl-CoA substrates should be presented appropriately to the acyltransferases. Moreover, harmful detergent effects of acyl-CoAs should be reduced. However, the inhibition of total incorporation at higher acyl-CoA concentrations may indicate that, at these high levels, not all such thioesters were effectively bound to the bovine serum albumin.

With regard to microsomal preparations from olive callus tissue, the optimal concentrations of palmitoyl-CoA and oleoyl-CoA were found to be about 0.075 mM, above which no significant increase in activity was found (Table 2). Increasing the concentration of palmitoyl-CoA in the incubation, up to 50 μM, increased the flux of radiolabel through the Kennedy pathway as indicated by accumulation of radioactivity in triacylglycerol (Table 2). The label in triacylglycerol accounted for around 50% of the total at optimal radiolabelling rates (i.e. at 75 μM palmitoyl-CoA: Table 2). Increasing the oleoyl-CoA concentration, up to 100 μM, also produced good accumulation of radiolabel in triacylglycerol, again with about 50% of the total radiolabel being found there. Compared to experiments with microsomes derived from olive fruits (Table 1), those from callus cultures accumulated much more radioactivity in triacylglycerol. Presumably, this reflected the relative activities of the four enzymes of the Kennedy pathway in the two types of microsomal preparations.

In potassium phosphate buffer the pH optimum of the incorporation of total radioactivity from [^{14}C]glycerol 3-phosphate into glycerolipids by olive fruit microsomes was 7.6. This pH was also optimum for the percentage labelling of diacylglycerol and triacylglycerol (Table 3), suggesting that the enzymes phosphatidate phosphohydrolase and diacylglycerol acyltransferase were close to their pH optima. With microsomes prepared from callus tissue, a broad pH optimum was found (Table 3) and in the range pH 7.2–8.0 there was little change in total activity. Examination of the reaction products (Table 3) showed that labelling of diacylglycerol and triacylglycerol was also good within this range, presumably, again reflecting the pH optima of the enzymes, phosphatidate phosphohydrolase and diacylglycerol acyltransferase, in the callus culture microsomes. With olive fruit microsomes, the incorporation of radioactivity from [^{14}C]glycerol 3-phosphate into glycerolipids remained linear for at least 90 minutes (Table 4), using 0.4 mg microsomal protein in the presence of optimal glycerol 3-phosphate, palmitoyl-CoA and oleoyl-CoA. The

Table 1. The effect of acyl-CoA concentration on the total and relative labelling of Kennedy pathway intermediates from [^{14}C]glycerol 3-phosphate by olive fruit microsomes

Acyl-CoA (mM)	Incorporation (nmol h^{-1} mg^{-1} protein)	Lipid class labelling (%) LysoPA	PA	DAG	TAG
Palmitoyl-CoA					
0.1	3.2±0.1	42.0±1.6	34.2±2.6	17.2±1.4	6.6±0.3
0.2	6.1±0.1	35.9±1.3	39.7±1.1	18.3±1.3	6.3±1.2
0.3	3.3±0.2	30.2±1.4	40.4±4.1	23.3±7.5	6.1±2.0
0.4	1.4±0.4	23.8±6.9	53.4±7.4	22.4±0.1	0.5±0.5
Oleoyl-CoA					
0.1	1.0±0.1	38.3±3.9	29.0±9.8	27.3±11.3	5.6±5.6
0.2	5.5±0.4	37.0±1.3	39.4±1.8	16.5±1.0	7.1±0.5
0.3	3.3±0.1	32.2±2.0	49.5±7.0	14.6±2.2	3.9±2.8
0.4	0.7±0.1	23.1±4.5	61.6±8.6	14.2±4.8	1.2±0.6

Data are expressed as means of duplicate incubations ± maximum/minimum of duplicate values. Incubations were carried out under standard conditions (see Experimental) except that palmitoyl-CoA concentration was varied in the presence of 200 μM oleoyl-CoA, and likewise, the oleoyl-CoA concentration was varied in the presence of 200 μM palmitoyl-CoA.

Abbreviations: LysoPA, lysophosphatidate; PA, phosphatidate; DAG, diacylglycerol; TAG, triacylglycerol.

Table 2. The effect of acyl-CoA concentration on the total and relative labelling of Kennedy pathway intermediates from [^{14}C]glycerol 3-phosphate by olive callus microsomes

Acyl-CoA (μM)	Incorporation (nmol h^{-1} mg^{-1} protein)	Lipid class labelling (%) LysoPA	PA	DAG	TAG
Palmitoyl-CoA					
0	20.8±6.6	29.2	24.7	15.8	30.3
25	30.7±6.7	27.4	21.6	8.3	32.8
50	39.9±1.9	18.6	19.6	5.8	56.1
75	46.4±3.8	21.6	17.6	8.5	52.3
100	47.5±1.3	21.5	22.1	9.6	46.9
125	48.0±0.9	29.5	33.1	9.1	29.2
Oleoyl-CoA					
0	9.7±3.3	43.9	16.6	12.1	27.4
25	23.4±1.1	21.8	22.7	17.4	38.0
50	52.8±7.2	12.9	26.1	19.1	42.0
75	82.2±15.0	17.0	24.4	15.7	42.9
100	98.2±8.3	11.8	25.7	16.1	46.4
125	114.7±10.4	19.0	26.2	15.0	39.8

Incubations were carried out as described in Experimental. For abbreviations see legend to Table 1.

pattern of labelling of Kennedy pathway intermediates is shown in Table 4. The intermediates phosphatidate and diacylglycerol and the product triacylglycerol were labelled at increasing rates with time with the former two reaching a plateau after 60 minutes. Given the generally high activity of lysophosphatidate acyltransferase in plant tissues [2], then it was not surprising that phosphatidate was better labelled than its precursor, lysophosphatidate, at most time intervals. We interpret the build up of radiolabel in lysophosphatidate between 60 and 90 minutes as being due to a reduction in the activity of lysophosphatidate acyltransferase, possibly by end-product inhibition. In general, the results are consistent with the operation of the normal Kennedy pathway in the olive fruit microsomes although, as mentioned before, these preparations do not accumulate high levels of triacylglycerols.

For microsomal fractions prepared from olive callus tissue, the incorporation of radiolabel was linear for 30 minutes (results not shown) using 0.1 mg microsomal protein. An analysis was made of intermediates beyond this time when radiolabelling began to decline [Fig. 2(a)]. Lysophosphatidate was best labelled at short incubation times and showed a decline in labelling after 60 minutes. Phosphatidate and diacylglycerol showed very similar time-courses of labelling, as they had with the fruit microsomes (Table 4) but with the difference that, in callus microsomes, diacylglycerol was well labelled. Accumulation of label in triacylglycerol occurred throughout the time-course. As with diacylglycerol, triacylglycerol was

Table 3. The effect of pH on the total and relative labelling of Kennedy pathway intermediates from [^{14}C]glycerol 3-phosphate by olive fruit or olive callus microsomes

pH	Incorporation (nmol h^{-1} mg^{-1} protein)	Lipid class labelling (%) LysoPA	PA	DAG	TAG
Olive fruit					
6.8	1.4±0.1	19.4±1.1	75.0±0.6	3.4±1.8	0.3±0.3
7.2	3.2±0.1	22.3±2.3	62.9±1.7	6.9±0.5	5.2±1.0
7.6	4.1±0.1	27.6±0.9	47.5±0.5	10.6±0.2	15.2±0.1
8.0	2.2±0.1	22.8±3.1	57.6±8.3	7.7±0.9	6.1±5.2
Olive callus					
6.8	22.9±0.2	49.1±0.3	45.5±0.5	2.4±0.2	3.2±0.1
7.2	34.8±2.6	29.8±12.8	50.2±7.2	5.7±1.3	14.4±4.2
7.6	32.4±3.8	34.9±3.0	39.6±2.9	9.0±0.2	17.2±0.6
8.0	32.2±4.1	39.3	35.2	10.5	15.1

Data are expressed as means of duplicate incubations ± maximum/minimum of duplicate values. Incubations were carried out as described in Experimental. For abbreviations see legend to Table 1.

Table 4. Time course of incorporation of radioactivity from [^{14}C]glycerol 3-phosphate into lipids by olive fruit microsomes

Time (mins)	Incorporation (nmol mg^{-1} protein)	Lipid class labelling LysoPA	PA	DAG	TAG
15	2.7±0.3	0.6	1.6	0.5	n.d.
30	4.4±0.4	1.0	2.4	0.8	0.1
45	5.9±0.5	1.7	3.0	1.0	0.1
60	9.9±0.9	2.9	4.7	1.7	0.4
90	13.2±0.1	6.0	4.9	1.7	0.6

Activity is expressed as [^{14}C]glycerol 3-phosphate incorporated into glycerolipids (nmol mg^{-1} protein). Incubations were carried out as described in Experimental. For abbreviations see legend to Table 1 and n.d., none detected.

well-labelled in callus microsomes showing that these preparations contained much more active phosphatidate phosphohydrolase and diacylglycerol acyltransferase enzymes than fruit microsomes. Again, the overall pattern of labelling of intermediates was consistent with the operation of the Kennedy pathway for triacylglycerol formation.

One of the most important environmental parameters known to control the quality of edible oils is the environmental temperature. Olive fruit microsomal fractions were incubated at different temperatures to investigate the effect of this parameter on lipid biosynthesis. The temperatures were chosen to represent the physiological range to which ripening fruits are exposed. Increasing the incubation temperature from 20 to 30° dramatically increased the total activity of the enzymes of the Kennedy pathway (Table 5). This change in temperature not only altered total flux through the pathway but also increased the relative labelling of diacylglycerol and triacylglycerol. Increasing the incubation temperature further to 40° did not change the total labelling rate much, but there was a significant decline in the relative labelling of triacylglycerol. The commensurate rise in diacylglycerol labelling which accompanied the above change might be indicative of limiting activity of diacylglycerol acyltransferase at 40°.

Microsomes prepared from olive callus cultures maintained at 25° were also incubated at different temperatures (Table 6). An approximate doubling of incorporation of radiolabel was seen on raising the temperature from 20 to 30° (i.e. a Q_{10} of 2). There was no further increase in total incorporation or a change in the labelling pattern of metabolites when the incubation temperature was raised from 30 to 40°. In contrast, a rise in temperature from 20 to 30° increased the flux of carbon through the Kennedy pathway with almost a doubling in the proportion of radiolabel accumulated in triacylglycerol. Because of the increase in overall labelling at 30°, then triacylglycerol at this temperature was four-fold better labelled than at 20°. As noted above (Tables 1 and 2), microsomes from olive callus accumulated significantly more radiolabel in triacylglycerol (Table 6) during the incubation period than those from olive fruits (Table 5). Thus, carbon flux through the entire Kennedy pathway was faster for callus microsomes, presumably because of a high activity of diacylglycerol acyltransferase relative to preparations from fruits.

The effects of temperature during incubations

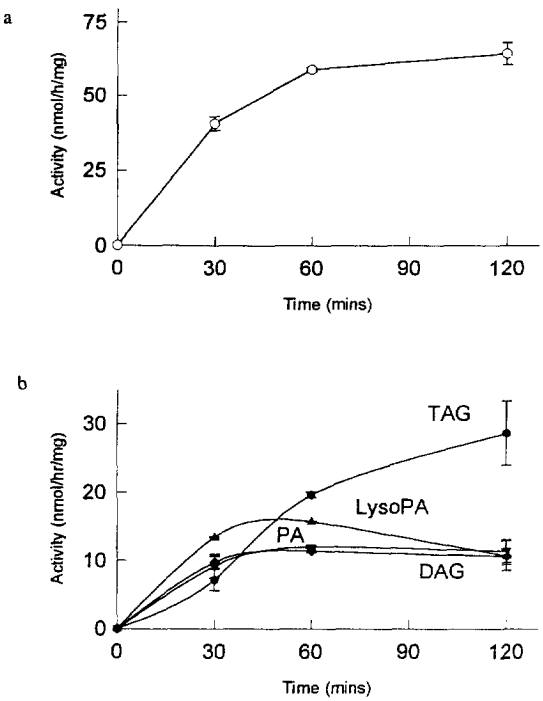

Fig. 2. Time course of incorporation of radioactivity from [^{14}C]glycerol 3-phosphate into (a) total glycerolipids and (b) Kennedy pathway intermediates by olive callus microsomes. Activity is expressed as [^{14}C]glycerol 3-phosphate incorporated into glycerolipids (nmol h^{-1} mg^{-1} microsomal protein). Data are expressed as means of duplicate measurements and error bars show maximum/minimum of duplicates where these exceed the dimensions of the symbols. Incubations were carried out under standard conditions (see Experimental).

(above) may reflect changes in the properties of the individual enzymes of the Kennedy pathway, their spatial relationship to each other and/or the accessibility (and physical state) of lipid substrates. *In vivo*, it is also known that environmental temperature can cause changes in gene expression [9], apparently because of alterations in membrane fluidity [10]. Moreover, the temperature at which the olive calli were maintained has previously been shown to affect the lipid composition of cultures [6]. Therefore, the effect of culture temperature on the subsequent products of microsomal incubations was also investigated. Olive callus was maintained at two different temperatures (25 and 35°) which were both within the normal environmental range for ripening olive fruits. Microsomal fractions prepared from such cultures were incubated at the same, intermediate (30°), incubation temperature. Under such conditions, microsomes from callus tissue cultured at the higher temperature incorporated more radiolabel into lipids on a protein basis, namely 54.4 compared to 34.3 nmol h^{-1} mg^{-1} protein (Fig. 3) and, furthermore, were also able to synthesise proportionally more triacylglycerol. The simplest interpretation of this result is that the levels of synthetic enzymes, particularly diacylglycerol acyltransferase, were enhanced compared to other proteins during growth at the higher temperature. Since it seems unlikely that the callus membranes undergo a significant change in their fluidity in the range 25–35°, then if, indeed, gene expression is changed then it seems unlikely that the only signal perceived for gene regulation is plasma membrane fluidity [10]. It is possible that factors controlling the stability of the different mRNAs may also be important.

Table 5. The effect of incubation temperature on the incorporation of radioactivity from [^{14}C]glycerol 3-phosphate into lipids by olive fruit microsomes

Temp. (°)	Incorporation (nmol h^{-1} mg^{-1} protein)	Lipid class labelling (%) LysoPA	PA	DAG	TAG
20	5.2±0.5	23.7±0.1	66.2±5.6	9.4±4.8	0.7±0.7
30	29.0±0.8	17.9±1.6	65.4±2.1	13.4±0.2	3.4±0.7
40	32.0±0.5	11.8±0.3	70.5±0.9	16.1±0.9	1.6±0.3

Data are expressed as means of duplicate incubations±maximum/minimum of duplicate values. Incubations were carried out as described in Experimental. For abbreviations see legend to Table 1.

Table 6. The effect of incubation temperature on the incorporation of radioactivity from [^{14}C]glycerol 3-phosphate into lipids by olive callus microsomes

Temp. (°)	Incorporation (nmol h^{-1} mg^{-1} protein)	Lipid class labelling (%) LysoPA	PA	DAG	TAG
20	13.3±3.7	47.9	26.3	7.4	18.4
30	24.5±5.9	15.8	25.3	28.5	30.4
40	25.3±1.7	10.3	31.0	25.0	33.7

Data are expressed as means of duplicate incubations±maximum/minimum of duplicate values. Incubations were carried out as described in Experimental. For abbreviations see legend to Table 1.

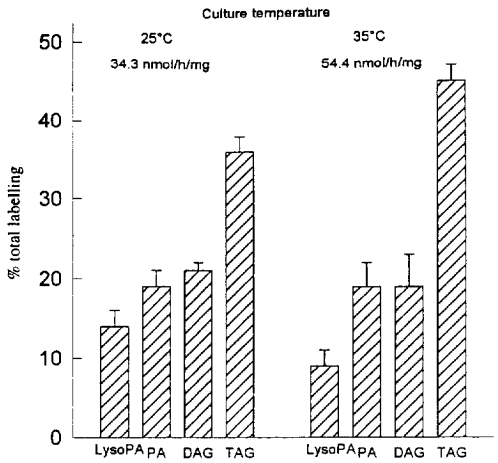

Fig. 3. The effect of growth of olive callus at 25 or 35° on the subsequent incorporation of radioactivity from [^{14}C]glycerol 3-phosphate into lipids by olive callus microsomes incubated at 30°. Activity is expressed as [^{14}C]glycerol 3-phosphate incorporated into glycerolipids (nmol h^{-1} mg^{-1} microsomal protein). Data are expressed as means of duplicate measurements and error bars show maximum/minimum of duplicates. Incubations were carried out under standard conditions (see Experimental).

We have shown that microsomes prepared from both ripening olive fruits and callus cultures are capable of synthesising triacylglycerol by the Kennedy pathway. In particular, the microsomal fractions from olive callus are capable of good rates of triacylglycerol accumulation. This fact, plus the convenient availability of such cultures and their ease of manipulation, makes them an attractive system with which to study the regulation of olive oil biosynthesis.

EXPERIMENTAL

Materials. Olive (*Olea europaea* L., Picual) fruits were obtained from twenty-year-old trees growing near Seville, Spain. During the growing season, olive fruits were picked and sent by air to Cardiff. They were used within three to four days of harvesting.

Generation of olive callus cultures. Olive fruits were obtained *ca* 120 days after flowering. The pericarp was removed from the fruit and broken. The kernel was surface-sterilised with 4% sodium hypochlorite for 20 min. and allowed to imbibe in cold running H_2O for 6 h. The kernels were then rinsed in sterile H_2O and germinated aseptically at 25°. Etiolated embryos were dissected so that the cotyledons were cut transversely into two basal and two apical pieces. The individual segments were incubated on Murashige and Skoog medium [11] supplemented with 12 μM 2,4-dichlorophenoxyacetic acid and 0.56 μM benzylaminopurine riboside. The cultures were incubated at 25 or 35° with illumination (20 $\mu E\ m^{-2}\ s^{-1}$) and a 12 h light/dark cycle. The resultant callus cultures were routinely sub-cultured at 28 day intervals.

Preparation of olive fruit microsomes. Olive fruits were rinsed thoroughly with distilled H_2O and wiped dry with paper tissue. Approximately 50 g of intact fruits were ground for 30 s using a precooled domestic blender in 200 ml of a buffer containing 25 mM HEPES, (pH 7.2), 330 mM sorbitol, 2 mM $MgCl_2$, 20 mM KCl, 10 mM $Na_2S_2O_5$, 2 mM EDTA, 2 mM dithiothreitol, 5 mM glutathione, 12 mM β-mercaptoethanol, 0.1% BSA, 0.2% ascorbic acid, 10% glycerol and 1% polyvinylpyrrolidone. The resultant homogenate was filtered through two layers of Miracloth. The filtrate was centrifuged at 4300 g for 10 min and the floating 'fat' layer formed was removed by careful filtration through a pad of glass wool. This filtrate was centrifuged at 27 000 *g* for 20 min and the resultant supernatant was subsequently centrifuged at 105 000 *g* for 75 min. The microsomal pellet obtained was resuspended in 1–2 ml of 50 mM HEPES (pH 7.2), 330 mM sorbitol, 1 mM dithiothreitol using a pre-cooled glass homogenisier.

Preparation of olive callus microsomes. Olive callus tissue, approximately 20 g, was homogenised in 50 mM HEPES, (pH 7.2), 330 mM sorbitol, containing 1 mM $MgCl_2$, 3 mM EDTA, 5 mM β-mercaptoethanol, 0.1% BSA, 0.2% ascorbate and 1% PVPP, using an Ultra-Turrax homogeniser. The centrifugation procedure was as described above for olive fruits.

Protein estimations. Protein concns were estimated by the method of Peterson [12] using BSA as a standard.

Microsomal incubations. Incubations, (final vol. 1 ml), were carried out in buffered mixts containing 35 mM HEPES, (pH 7.2, or 0.1 M potassium phosphate for the pH optima curves), 0.5% BSA, 300 mM sorbitol, 0.5 mM dithiothreitol in stoppered tubes. Standard incubation conditions were for 1 hr at 30° with constant shaking; with, for olive fruit microsomes [^{14}C]glycerol 3-phosphate (1.28 μCi, 1.6 μmol), 200 μM palmitoyl-CoA and 200 μM oleoyl-CoA and, for olive callus microsomes, [^{14}C]glycerol 3-phosphate (0.15 μCi/150 nmol), 75 μM palmitoyl-CoA and 100 μM oleoyl-CoA. Any variations are described in the legends of figs and tables. For incubations with [^{14}C]oleoyl-CoA, 5 mM $MgCl_2$ was included in the buffer. Incubation reactions were stopped by the addition of 1 ml of 0.15 M acetic acid.

Lipid extraction. A chloroform–MeOH mixt. in the ratio 1:2 (4.5 ml) was added to the 1 ml microsomal incubation soln. After vortexing, the phases were left to separate before vortexing again and left for a further 15 min. Sepn of the phases was aided by centrifugation at 1000 *g* in a bench top centrifuge for 5 min. A further 1.5 ml of chloroform was added, followed by 2.25 ml of H_2O. After vortexing and phase sepn, the aq. phase was discarded and the lower chloroform phase kept for analysis.

Lipid analysis. TLC was performed using precoated silica gel plates activated before use for one hour at 110°. Lipids were sepd using a one dimensional double development technique. Firstly, polar lipids were sepd using chloroform–MeOH–HOAc–H_2O (170:30:20:7) as the solvent which was run to 5 cm

from the top of the plate. Solvent was allowed to evap. from the plate, and then neutral lipids were sepd further using petrol (60–80 bp)–Et_2O–HOAc (80:20:2). Plates were sprayed with 8-anilino-1-naphthalene sulphonic acid (0.2% in dry MeOH) and lipid bands visualized under UV light.

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