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REGULATION OF TRIACYLGLYCEROL SYNTHESIS IN OIL PALM (*Elaeis guineensis*) AND OLIVE (*Olea europaea*) CALLUS CULTURES

UMI SALAMAH, R;* QUANT, P A" and HARWOOD, J L*

We have been using callus cultures as convenient model systems to understand any step where regulation of flux control in the triacylglycerol biosynthesis pathway may be important in two major oil-rich fruits, oil palm (Elaeis guineensis) *and olive* (Olea europaea). Top-down *metabolic control analysis (TDCA) has been used to address the question of where possible control step(s) in the lipid biosynthesis pathway are located. With this method, lipid metabolism is conceptually* divided *into blocks of reactions and these are manipulated to see the effect on a key intermediate.* We *initially measured changes in the overall flux of carbon from [1-^{14}C]acetate as modified by temperature in oil palm and olive callus cultures. A doubling of lipid synthesis with a 10°C rise from 20°C to 30°C did not, however, cause much change in radioactivity incorporation into the acylthioester pools, acyl-CoAs and acyl-ACPs. This suggested that* de novo *fatty acid synthesis reactions exerted higher control than complex lipids assembly via Kennedy pathway.*

Reactions of the Kennedy pathway were examined in more detail in oil palm callus cultures. By using microsomal fractions prepared from such cultures, we showed that radioactivity from [U-^{14}C]glycerol 3-phosphate was effectively incorporated into intermediates of the Kennedy pathway and that the changes in radioactivity caused by temperature manipulation reflected well the endogenous lipid pool levels. Stimulation of triacylglycerol synthesis

* Cardiff School of Biosciences, Cardiff University. Cardiff, CF1 3US. U.K.

** Institute of Child Health. University College London. London WC1N IEH, U.K.

at 30°C was accompanied by slight increases in diacylglycerol and phosphatidic acid. This indicates that at higher rates of triacylglycerol synthesis, specific enzymes of the Kennedy pathway may become more limiting.

INTRODUCTION

Control over lipid biosynthesis is a topic of great importance, especially for effective creation of novel oil crops by genetic manipulation. Crop improvements by modern methods of biotechnology have been used with a variety of plant species to meet the demand of an expanding oil business and changing market requirements. An important aspect which needs consideration and study prior to developing new transgenic oil crops is to determine how their lipid synthesis can be manipulated. However, the overall regulation of lipid metabolism is a relatively uncharted area so it is hardly surprising that isolated attempts to manipulate lipids in transgenic crops often fail (Ohlrogge and Jaworski, 1997). Therefore, studies were initiated to investigate the overall regulation of lipid metabolism in two major oil crops, oil palm and olive through *top-down* metabolic control analysis (TDCA) (Kacser and Burns, 1973; Heinrich and Rapport, 1974).

Economically, olive is one of the most important oil crops in Europe, while oil palm provides a major source of revenue in tropical countries with Malaysia currently providing 60% of the total world production of palm oil (Chow, 1997). In both these plants, palmitate and oleate are the major storage fatty acids although olive contains a higher percentage of the latter. Partly because these two crops contain oil in their fruits, research into their biochemistry has lagged behind other more experimentally convenient crops such as oilseeds. The reason for this could be that fruits at the correct stage of development when the enzymes of the Kennedy pathway are active, are not as readily available as ripening seeds. However, there is considerable interest in developing new oil palm varieties with different oil characteristics (such as high oleate) to extend the conventional use of palm oil and help combat the economic danger from cheap sources of existing varieties and overproduction.

Both olive and oil palm share many features in their lipid metabolism. Furthermore, some useful background information on the biochemistry of olive cultures is available (Williams *et* al., 1993; Rutter *et* al., 1997). Therefore, we used olive cultures for comparative purposes throughout this study.

The principle of TDCA is based on the concept of metabolic control analysis, where control over flux in a metabolic pathway is shared among many steps in a pathway rather than at one particular site (Kacser, 1979). It emphasizes that qualitative terms such as 'rate-limiting' arising from *in vitro* studies of the kinetic properties of individual enzymes are inadequate (Quant, 1993).

Fatty acids, the pathway substrates from which storage lipids such as triacylglycerol are derived, are generated in plastidial compartments and delivered to the endoplasmic reticulum in the form of acyl-CoA thioesters. Application of TDCA involves partitioning the lipid metabolic pathway into two blocks of reactions, so we chose acyl-CoAs as system intermediates. Thus, the first block covers de *novo* fatty acid synthesis while the second comprises all reactions involved in the utilization of acyl-CoAs for complex lipids formation. The overall pathway flux can then be manipulated and changes in the pool sizes of chosen intermediates monitored. Follow up experiments then allow further dissection of each block and measurement of such parameters as flux control coefficients for the individual steps. The big advantage of TDCA is that it can give quantitative measurements of carbon flux control during storage lipid accumulation without the necessity of guessing which enzyme step is the more important or of having specific enzyme inhibitors available.

This report describes an initial stage, in our effort to generate essential data for analysis of carbon flux control for the lipid biosynthesis pathway in oil palm and olive callus cultures. We have been using callus cultures as convenient model systems which provide year-round materials. They can also be easily manipulated

and this makes them an attractive system to study the regulation of oil synthesis. We changed the overall flux of carbon through lipid synthesis by temperature manipulation. As a follow up experiment, we examined in more detail the reactions in the Kennedy pathway using microsomal fractions prepared from oil palm callus cultures. Again, by using temperature, triacylglycerol synthesis was manipulated and the glycerdlipid intermediates produced analysed to see which step might be important in controlling the flux of carbon through the Kennedy pathway. By such means, we intend to elucidate the control of carbon flux in lipid metabolism for oil palm and olive callus tissues.

MATERIALS AND METHODS

Chemicals

[U-^{14}C]Glycerol 3-phosphate (specific radioactivity 5.44Gbq mmol^{-1}) and [1-^{14}C]acetate (specific radioactivity 1.85Gbq mmol^{-1}) were purchased from Amersham (Bucks, U.K.). All other lipids and assay biochemicals were of the highest purity available.

Callus Cultures

Olive callus cultures were established as described by Williams et al. (1993). The calli were subcultured four-weekly intervals on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with an auxin-cytokinin combination (2,4-dichlorophenoxyacetic acid [2,4-D] 12µM and 6-benzylaminopurine [BAP] 5.6µM). The cultures were incubated at 25°C with a 12hr light/dark cycle. Oil palm calli were maintained on Murashige and Skoog medium (Murashige and Skoog, 1962) in the presence of 10ppm napthaleneacetic acid (NAA) at 30°C in the dark. Subculturing was performed at four-weekly intervals.

Radiolabelling of Callus Cultures with [1-^{14}C]Acetate

Callus cultures were selected for uniformity of weight and physiological appearance. [1-^{14}C]Acetate diluted in 100mM sorbitol was added to the surface of the callus which was then incubated for 24hr at 20°C and 30°C. At the end of the incubation, the calli were rinsed briefly with distilled water to remove any unimbibed radiolabelled acetate and incorporation of radioactivity was terminated by addition of *iso*-propanol (1.25ml) followed by a two-phase lipid extraction according to Garbus et al. (1963) as modified for plant tissues (Smith et al., 1982). Acyl-CoAs and acyl-ACPs were purified from the aqueous phase obtained from total lipid extraction through SEP-PAK C18 columns according to Stymne and Glad (1981).

Preparation of Oil Palm Callus Microsomes

Microsomal fractions were prepared from oil palm callus according to the previously published method for olive callus (Rutter et al., 1997). All the steps were carried out on ice or at 4°C, unless otherwise stated. Oil palm callus, 20-25 days old after subculturing, was used to prepare microsomal fractions. The tissues were immediately homogenized in a cold buffer containing 50mM Hepes (pH 7.2), 330mM sorbitol, 1mM $MgCl_2$, 3mM EDTA, 5mM β-mercaptoethanol, 0.1% BSA (fraction V-fatty acid-free), 0.2% ascorbate and 1% PVPP at a callus tissue:buffer (g ml^{-1}) ratio of 1:10 using a pre-cooled domestic blender. The homogenate was filtered through a layer of miracloth and centrifuged at 5000xg for 10min. The resulting supernatant was recentrifuged at 18 000xg for 20min. The supernatant was then ultracentrifuged at 105 000xg for 75min. The microsomal pellet was gently resuspended in 1-2ml 50mM Hepes (pH 7.2), 330mM sorbitol, 1mM DTT using a pre-cooled glass homogenizer. Aliquots were kept at -70°C, where activity was stable, for at least four weeks.

Protein Estimation

Protein concentrations were estimated by the method of Bradford (1972) using BSA as standard.

Incubation of Microsomal Fractions

Triacylglycerol synthesis was investigated in oil palm callus microsomes using [U-^{14}C]glycerol 3-phosphate as precursor. The

incubation mixture (total volume 1.0ml) contained as final concentrations: 35mM Hepes-NaOH (pH 7.2), 300mM sorbitol, 0.5% BSA (fraction V-fatty acid-free), [U-^{14}C]glycerol 'J-phosphate (0.1µCi, 150nmol), 0.5mM dithiothreitol, 100µM palmitoyl-CoA and 100µM oleoyl-CoA. Any variations are as detailed in the legends of Figures and ***Tables.*** Standard incubation conditions were for 30min and 30°C with constant shaking. Incubations were initiated by addition of microsomal proteins and terminated by addition of 1ml 0.15M acetic acid.

Lipid Extractions

Several methods of lipid extraction have been reported. However, the method adopted from Bjerve et al. (1974) was chosen in this study due to its ability to ensure quantitative extraction of lysophosphatidate. A one-phase solution mixture containing 1.0ml butan-1-ol, 0.95ml distilled water and 0.05ml acetic acid was added to the incubation solution (2.0ml). The mixture was vortex-mixed followed by addition of 2ml butan-1-ol. Phase separation was aided by spinning at 1500xg for 10min. The upper butanol phase, containing lipid products, was transferred to a clean centrifuge tube before further extraction of the bottom non-lipid phase with an equal volume of artificial upper phase. Finally, the combined upper butanol phases were evaporated to dryness under N_2, redissolved in a small volume of chloroform and kept for analysis.

Lipid Analysis

Lipids were analysed by thin layer chromatography using pre-coated silica gel G plates activated before use for one hour at 110°C. TLC was performed using a double development system to separate both polar and neutral lipids on a single plate. Polar lipids were first separated using chloroform/methanol/acetic acid/water (170:30:20:7, by vol.) as solvent system to within 5cm from the top of the plate. The solvent was allowed to evaporate from the plate and a second development (principally to separate neutral lipids better) was used with petrol (60°C-80°C b.p.)/diethyl ether/acetic acid (80:20:2, by vol.). Plates were sprayed with 8-anilino-1-naphthalene sulphonic acid (0.02% in dry methanol) and the lipid bands visualized under UV light.

RESULTS AND DISCUSSION

Temperature Manipulation of Lipid Biosynthesis

The callus cultures of both olive and oil palm, used in this study, contained palmitate (C16:0) and oleate (C18:1) as major constituents with olive containing a higher percentage of the latter. Thus, both cultures presented strong similarities to their respective fruits in regard to their lipid compositions. Therefore, together with their demonstrated high metabolic activities, the cultures could be used conveniently for study of storage oil quality. Initial flux control experiments were carried out using temperature to manipulate the overall flux of carbon through lipid metabolism in oil palm and olive callus cultures with [1-^{14}C]acetate as carbon precursor. Some representative data are shown in ***Tables 1*** and 2 (see also Ramli et al., 1998). Changes in the total flux of carbon through the system and specific lipid class labelling as well as the intermediate pools of acyl-ACPs and acyl-CoAs were determined. As expected, in both cultures, total lipid lahelling was enhanced on raising the incubation temperature from 20°C to 30°C with a temperature coefficient of approximately 2 ***(Table*** I). This result showed that a rise in temperature increased the flux of carbon through fatty acid synthesis and the Kennedy pathway, thus, resulting in an increase in total lipid synthesized. Non-lipid (aqueous1 phases contained very low radioactivity suggesting that [1-^{14}C]acetate when taken up was efficiently used for lipid synthesis.

In order to generate data for measurement of flux control coefficients, we analysed the acylthioesters, as appropriate intermediate pools. As shown in ***Table 1,*** no changes in either acyl-CoA or acyl-ACP pools were observed. Thus, the results indicated that the overall flux of carbon was not limited by the sizes of the pools, and hence, there is no major constraint to the use of these acylthioesters by

TABLE 1. MANIPULATION OF LIPID SYNTHESIS BY TEMPERATURE IN OLIVE AND OIL PALM CALLI

°C	Olive callus			Oil palm callus		
	Lipids (d.p.m.x10^{-5})	Acyl-ACPs (d.p.m. x 10^{-4})	Acyl-CoAs (d.p.m. x 10^{-3})	Lipids (d.p.m.x 10^{-5})	Acyl-ACPs (d.p.m. x 10^{-4})	Acyl-CoAs (d.p.m. x 10^{-3})
20	2.2 ± 0.4	8.7 ± 2.1	7.0 ± 2.0	1.9 ± 0.8	4.3 ± 2.0	2.4 ± 1
30	5.4 ± 0.6	9.2 ± 1.4	7.3 ± 1.0	4.2 ± 1.0	5.0 ± 2.8	2.5 ± 1

Values are means ± standard deviations, where n = 9. (see Ramli et al., 1998 for details). Labelling was from [1-^{14}C]acetate. (see Materials and Methods).

TABLE 2. EFFECT OF TEMPERATURE ON THE RELATIVE LABELLING OF LIPID CLASSES FROM [1-^{14}C]ACETATE IN OLIVE AND OIL PALM CALLI

Olive (°C)	Relative labelling (% total)					
	DAG	NEFA	TAG	PtdCho	PtdEtn	PtdOH
20	10 ± 2	7 ± 1	12 ± 1	32 ± 4	15 ± 2	24 ± 3
30	14 ± 2	3 ± 1	21 ± 2	35 ± 3	10 ± 1	17 ± 2

Oil palm (°C)	DAG	NEFA	TAG	PtdCho	PtdEtn	PtdOH
20	8 ± 1	6 ± 1	15 ± 2	43 ± 3	17 ± 2	11 ± 1
30	5 ± 1	3 ± 1	23 ± 2	60 ± 4	6 ± 1	3 ± 0

Values are means? standard deviations, where n = 9. Abbreviations: DAG, diacylglycerol; NEFA. non-esterified fatty acid; TAG, triacylglycerol; PtdCho. phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdOH, phosphatidic

enzymes such as acyltransferases. However, we observed some changes in the labelling of acyl lipid classes which indicated some modification of the end-products of lipid metabolism (Table 2). For olive, the increase in diacylglycerol when triacylglycerol formation was stimulated at 30°C suggests flux control at the level of DAG acyltransferase. This was previously proposed from the results of other labelling experiments with olive callus (Rutter et al., 199'7). Oil palm callus, on the other hand, showed less label in diacylglycerol when triacylglycerol was stimulated at 30°C, suggesting that DAG acyltransferase exerted less control in these cultures.

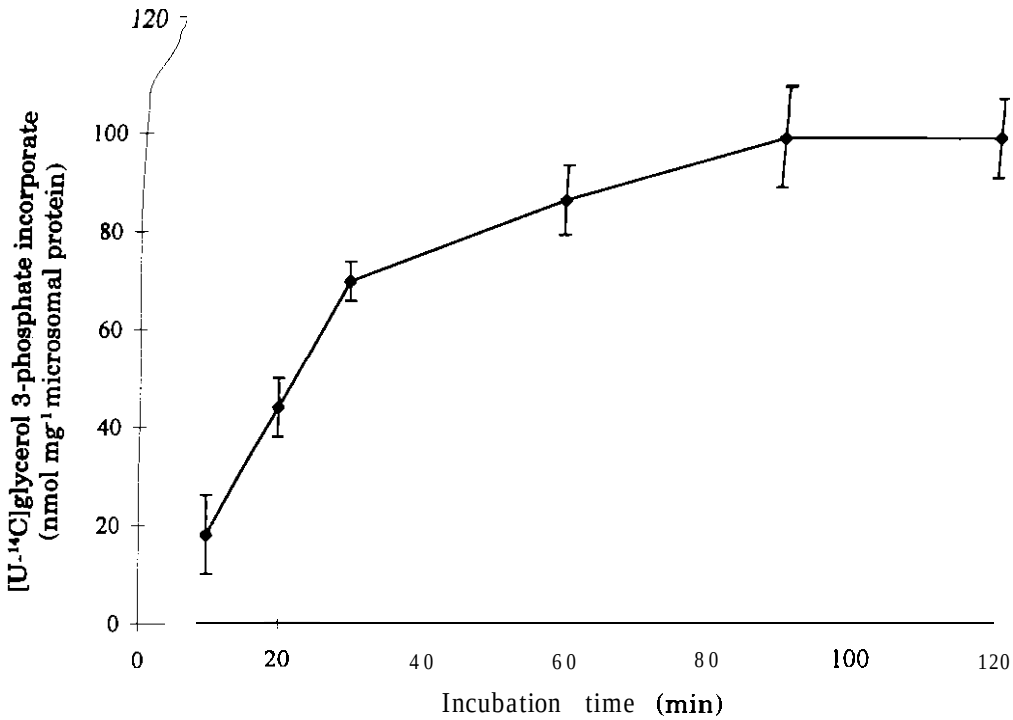
We observed that radiolabelling into plastidial sulpholipid and phosphatidylglycerol only accounted for less than 5% of the total lipid labelling (data not shown). For plants, such as oil palm and olive which are '18:3'-plants, acyl-ACPs are used by plastidial acyltransferase for sulpholipid and phosphatidylglycerol biosynthesis and by acyl-thioesterase(s) for export to the cytosol. Acyl-CoAs are formed on the plastid envelope and used by extra-plastidial acyltransferases (Browse and Somerville, 1991). Therefore, we concluded that fatty acids synthesized in the plastids of both olive and oil palm cultures are mainly (>95%) channelled into the extra-plastidial compartments for lipid assembly. We then attempted to obtain more information concerning the reactions of the Kennedy pathway leading to storage lipid accumulation by utilizing microsomal fractions prepared from oil palm callus cultures.

Glycerolipid Synthesis by Oil Palm Callus Microsomes

Previous attempts to prepare active subcellular fractions of microsomal membranes from several plant tissues (Sanchez et al., 1992; Eccleston and Harwood, 1995) have made experiments possible on the regulation of triacylglycerol synthesis. Recent studies by Rutter et al. (1997) have shown that microsomal fractions prepared from both olive fruits and callus cultures were capable of good rates of triacylglycerol synthesis. Furthermore, it is believed that the absence of cutin and phenolics in callus tissue makes the calli less of a problem for preparation of active microsomal fractions than the fruits.

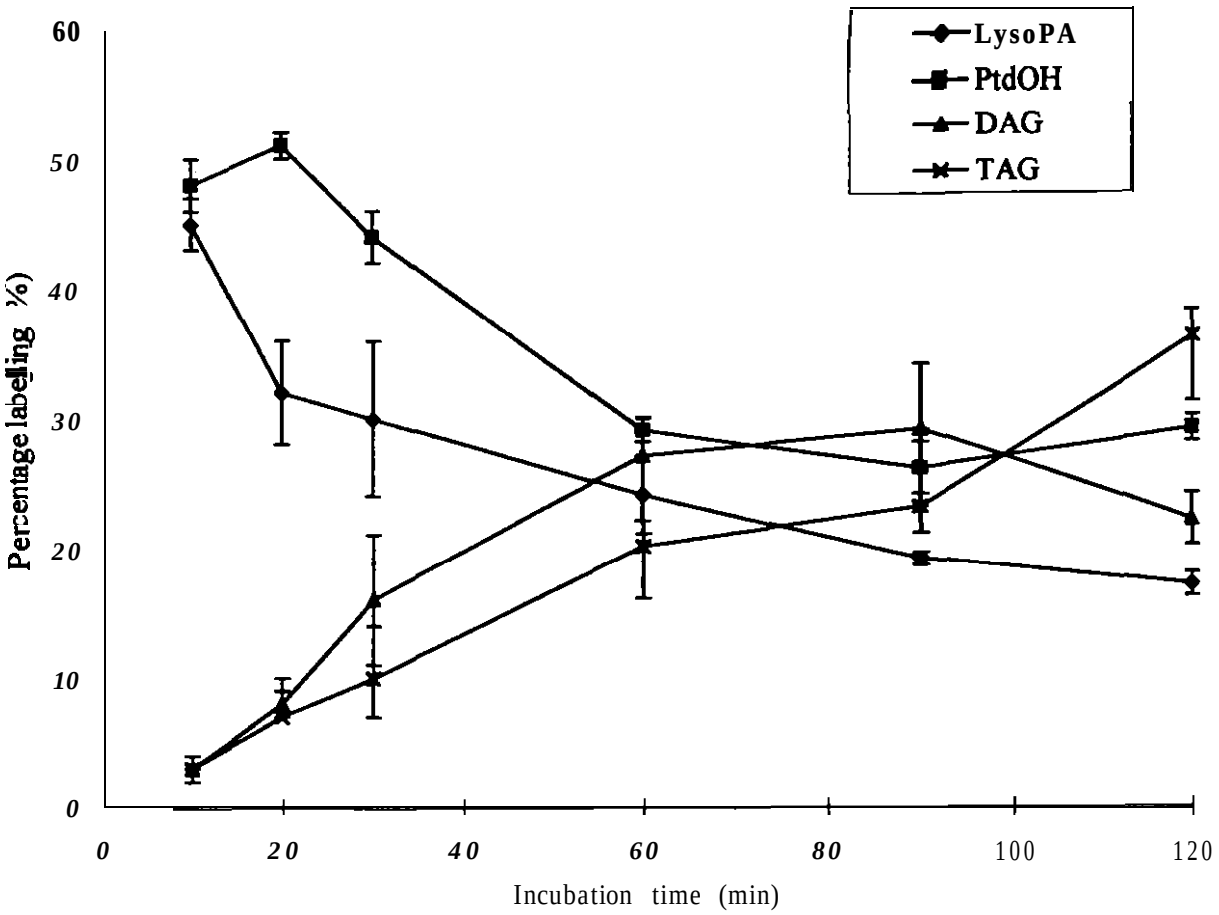
We used oil palm callus cultures to prepare microsomes using the method described for olive callus (Rutter et *al.*,1997). The ability of microsomal preparations to synthesize triacylglycerol was optimized with regard to substrate concentrations and other conditions of the assay. Once optimum conditions were obtained, we used temperature to manipulate the Kennedy pathway to obtain an immediate overview for the overall control of carbon flux to triacylglycerol synthesis.

Our results showed that triacylglycerol was actively synthesized by oil palm callus microsomes and analysis of lipid products revealed that only the Kennedy pathway intermediates were labelled (*i.e.*, LysoPA, PtdOH, DAG and TAG) from [^{14}C]glycerol 3-phosphate. The incorporation of radiolabel into total lipids remained linear for at least 30min and a plateau was reached after 90min (Figure *1a*). As expected, the percentage distribution of radiolabel in lipid products varied with incubation time (*Figure 1b*). Initially (up *to* 30min),



Values are means of triplicate incubations. Error bars represent standard deviations. Incubations were carried out under standard conditions (see Materials and Methods).

Figure la. Tim course Of incorporation Of radioactivity from [^{14}C]glycerol 3-phosphate into total glycerolipids by oil palm callus microsomes.



Values are means **of** triplicate incubations. **Error** bars **represent standard deviations. (Abbreviations: LysoPA, lysophosphatidate;** PtdOH, **phosphatidate; DAG, diacylglycerol; TAG, triacylglycerol.)**

Figure lb. Time course** of **incorporation of** radioactivity from [^{14}C]glycerol 3-phosphate **into Kennedy pathway intermediates by oil palm callus microsomes.

radiolabel was mainly channelled into lysophosphatidic acid and phosphatidic acid, the first two intermediates of the Kennedy pathway. With increasing time, there was an increase in diacylglycerol and triacylglycerol with concomitant decreases in the relative labelling of lysophosphatidic acid and phosphatidic acid. The pattern of intermediates generally stabilized after 60min but with a continuous rise in triacylglycerol seen. These results were expected for a flux of carbon through the Kennedy pathway, with sequential acylation of glycerol 3-phosphate to yield LysoPA, PtdOH, DAG and, finally, TAG. Our results indicated that triacylglycerol synthesis was occurring satisfactorily *in vitro* and, therefore, the microsomal system was suitable for more detail investigation of triacylglycerol metabolism. Another fact worth noting is that phosphatidic acid was always better labelled than its precursor, lysophosphatidic acid, at all time intervals which indicates the high activity of lysophosphatidate acyltransferase in oil palm, as generally accepted in other plant tissues (Stymne and Stobart, 1987).

Effect of Acyl-CoA

In general, for many plant preparations, the first acylation step on the *sn*-1 position of glycerol backbone by glycerol 3-phosphate acyltransferase involves saturated fatty acids (normally from palmitoyl-CoA), whereas unsaturated fatty acids (normally oleoyl-CoA) are

used for attachment to position *sn*-2 by lysophosphatidate acyltransferase (Stymne and Stobart, 1937). Based on this evidence (and also the fact that both palmitate and oleate are the most abundant fatty acids in palm oil), these fatty acids (as acyl-CoA thioesters) were used in the incubations with oil palm microsomes to study triacylglycerol synthesis. *Tables* 3 and 4 show that maximal incorporation of radiolabel into total lipids occurs with the combination of 100μM oleoyl-CoA and 100μM palmitoyl-CoA.

Total labelling was reduced by increasing concentrations of oleoyl-CoA, possibly because of detergent effects at high concentrations. Alternatively, at higher concentrations of acyl-CoA substrates, micelles may be formed. Thus, increasing concentrations of substrate above the critical micelle concentration (CMC) will reduce the amount of acyl-CoA capable of interacting with acyltransferases. Zahler *et al.* (1968) provided evidence that palmitoyl-CoA forms micelles in an aqueous phase with a critical micelle concentration (CMC) of 3-4μM. However, additional evidence by Powell et al. (1981) demonstrated that the CMC of physiologically-significant fatty acyl-CoA molecules exceeds 30 to 60μM. Thus, the concentration of acyl-CoAs used was theoretically enough for micelles to be formed. However, we included 0.5% BSA (which was found to be the optimal concentration; data not shown) to help prevent formation of micelles as the binding of acyl-CoAs to BSA would make them more freely available for the acyltransferases. The inclusion of BSA should also help prevent the known detergent action of acyl-CoA's on microsomal enzymes.

TABLE 3. EFFECT OF OLEOYL-CoA CONCENTRATION ON INCORPORATION OF RADIOACTIVITY FROM [^{14}C]GLYCEROL 3-PHOSPHATE INTO TOTAL LIPIDS AND KENNEDY PATHWAY INTERMEDIATES BY OIL PALM CALLUS MICROSOMES

Oleoyl-CoA (μM)	Total incorporation (nmol min^{-1} mg^{-1})	Lipid class labelling (% total)			
		LysoPA	PtdOH	DAG	TAG
25	4.5 ± 0.9	37 ± 5	29 ± 3	19 ± 2	15 ± 5
50	7.1 ± 0.6	28 ± 4	38 ± 5	22 ± 4	12 ± 2
75	11.2 ± 1.4	23 ± 3	47 ± 5	22 ± 3	8 ± 1
100	13.7 ± 0.8	16 ± 4	56 ± 7	18 ± 2	8 ± 1
125	6.4 ± 0.7	16 ± 2	59 ±10	19 ± 5	6 ± 1

Values are means of triplicate incubations ± standard deviations. Incubations were carried under standard conditions (see Materials and Methods). For abbreviations, see legend in *Figure 1b.*

TABLE 4. EFFECT OF PALMITOYL-CoA CONCENTRATION ON INCORPORATION OF RADIOACTIVITY FROM [^{14}C]GLYCEROL 3-PHOSPHATE INTO TOTAL LIPIDS AND KENNEDY PATHWAY INTERMEDIATES BY OIL PALM CALLUS MICROSOMES

Palmitoyl-CoA (μM)	Total incorporation (nmol min^{-1} mg^{-1})	Lipid class labelling (% total)			
		LysoPA	PtdOH	DAG	TAG
25	2.9 ± 0.5	17 ± 5	51 ± 4	24 ± 5	8 ± 5
50	4.3 ± 0.6	30 ± 4	41 ± 7	21 ± 3	8 ± 3
75	7.3 ± 0.4	30 ± 6	41 ± 8	19 ± 5	10 ± tr
100	10.2 ± 0.6	35 ± 8	34 ± 3	15 ± 3	16 ± 3
125	9.4 ± 0.7	38 ± 5	37 ± 4	13 ± 2	12 ± 2

Values are means of triplicate incubations ± standard deviations. tr (trace) = <1%. Incubations were carried under standard conditions (see Materials and Methods). For abbreviations, see legend in *Figure 1b.*

The use of higher concentrations of oleoyl-CoA resulted in even higher incorporation of radiolabel in phosphatidic acid at the expense of lysophosphatidic acid, implying that microsomes of oil pahn callus contained a highly active lysophosphatidate acyltransferase which preferred oleoyl-CoA as substrate. Increasing concentrations of palmitoyl-CoA, on the other hand, enhanced lysophosphatidic acid labelling. The latter is in keeping to the well known characteristic of 'C18:3' plants in which the *sn*-1 position is usually occupied by a saturated fatty acid whereas sn-2 contains an unsaturated fatty acid. It also agrees with the high concentration of palmitate at the sn-1 position of pahn oil. When the acyl-CoAs were tested singly, higher amounts of triacylglycerol were formed with palmitoyl-CoA (*Tables* 3 and 4). This result implies that diacylglycerol acyltransferase also preferred palmitoyl-CoA as substrate. The data do not indicate a substrate preference for phosphatidate phosphohydrolase since the total amounts of diacylglycerol and triacylglycerol labelled with each acyl-CoA substrate arc similar.

Effect of Microsomal Concentration and Incubation pH

The incorporation of radioactivity from [U-^{14}C]glycerol 3-phosphate into total glycerolipids increased linearly with increasing amounts of microsomal protein up to 100μg (*Table* 5). There was little difference in the pattern of labelling of Kennedy pathway intermediates, except tbat triacylglycerol labelling was increased with higher microsomal protein concentrations. In sll other experiments described, microsomal protein was included in the incubations at about O.lmg.

Glycerolipid synthesis wasmeasured over a range of different pHs (*Table 6*). Incorporation of radioactivity from [U-^{14}C]glycerol 3-phosphate was favoured at pHs between 7.2 to 7.6 with a maximum at co. 7.4. Diacylglycerol and triacylglycerol syntheses were both stimulated in the pH range 7.4 to 7.6 whereas counts in LysoPA were increased at lower pHs. These results probably reflect the different pH optimum for the four enzymes of the Kennedy pathway.

Manipulation of Triacylglycerol Biosynthesis in Oil Palm Callus Microsomes by Temperature

As mentioned earlier, a ten degree rise in incubation temperature approximately doubled total lipid labslling from [1-^{14}C]acetate by intact oil palm callus cultures. Furthermore, we observed that triacylglycerol labelling was increased when total lipid synthesis was stimulated, suggesting that flux of carbon through the Kennedy pathway was also stimulated by temperature. To investigate whether triacylglycerol synthesis in microsomal fractions could be manipulated by temperature and to obtain an overall view of the properties of individual. enzymes in the Kennedy pathway, we incubated microsomal fractions from oil palm callus

TABLE 5. EFFECT OF MICROSOMAL PROTEIN CONCENTRATION ON INCORPORATION OF RADIOACTIVITY FROM [^{14}C]GLYCEROL 3-PHOSPHATE INTO TOTAL LIPIDS AND KENNEDY PATHWAY INTERMEDIATES BY OIL PALM CALLUS MICROSOMES

Microsomes (Mg ml^{-1})	Total incorporation (nmol min^{-1} mg^{-1})	Lipid class labelling (% total)			
		LysoPA	PtdOH	DAG	TAG
0.025	6.4 ± 0.5	19 ± 5	45 ± 6	27 ± 4	9 ± 1
0.05	11.6 il.2	17 ± 3	40 ± 5	29 ± 3	14 ± 3
0.1	15.6 f2.4	22 ± 4	38 ± 4	22 ± 5	18 ± 3
0.2	18.0 f4.3	17 ± 3	40 ± 3	23 ± 2	20 ± 3

Values are means of triplicate incubations ± standard deviations. Incubations were carried out under standard conditions (see Materials and Methods) except that they were for 60min.

TABLE 6. EFFECT OF pH ON INCORPORATION OF RADIOACTIVITY FROM [^{14}C]GLYCEROL 3-PHOSPHATE INTO TOTAL LIPIDS AND KENNEDY PATHWAY INTERMEDIATES BY OIL PALM CALLUS MICROSOMES

pH	Total incorporation (nmol min^{-1} mg^{-1})	Lipid class labelling (% total)			
		LysoPA	PtdOH	DAG	TAG
6.5	6.4 ± 2.3	43 ± 7	35 ± 5	18 ± 3	4 ± 2
1.2	14.3 ± 3.5	34 ± 6	34 ± 7	23 ± 5	9 ± 3
7.4	15.3 ± 1.4	30 ± 8	35 ± 4	23 ± 8	12 ± 6
1.6	12.5 ± 2.7	26 ± 2	34 ± 3	27 ± 2	13 ± 6
8.0	8.5 ± 0.6	25 ± 2	44 ± 7	21 ± 3	10 ± 5

Values are means of triplicate incubations ± standard deviations. Incubations were carried out under standard conditions (see Materials and Methods).

TABLE 7. EFFECT OF TEMPERATURE ON INCORPORATION OF RADIOACTIVITY FROM [^{14}C]GLYCEROL 3-PHOSPHATE INTO TOTAL LIPIDS AND KENNEDY PATHWAY INTERMEDIATES BY OIL PALM CALLUS MICROSOMES

Temp. (°C)	Total incorporation (nmol min^{-1} mg^{-1})	Distribution of radioactivity (% total)			
		LysoPA	PtdOH	DAG	TAG
20	3.0 ± 0.6	48 ± 3	42 ± 4	7 ± 1	3 ± tr
30	6.5 ± 0.8	34 ± 5	51 ± 3	9 ± 1	6 ± tr

Values are means of triplicate incubations ± standard deviations. tr (trace) = < 1%. Incubations were carried out under standard conditions (see Materials and Methods).

under standard incubation conditions at 30°C and 20°C, respectively (*Table 7*). As expected, triacylglycerol synthesis was increased on raising the temperature from 20°C to 30°C with a Q_{10} of about 2. This agreed with the results on temperature manipulation in whole cells using [1-^{14}C]acetate as precursor (*Table 1*).

Changes in the incubation products were monitored in order to see the effect on the intermediates and, hence, indirectly, on the four enzymes of the Kennedy pathway. Increases in the carbon flux through the pathway at 30°C resulted in two-fold increase in label in triacylglycerol. In fact, all intermediates after lysophosphatidic acid were relatively better labelled. The increase in phosphatidic acid labelling may indicate that at high rates of triacylglycerol labelling in oil palm microsomes, phosphatidate phosphohydrolase may be slightly limited to the overall carbon flux. This agrees with the results obtained for two other important oil crops – oilseed rape (Harwood and Page, 1993) and olive (Rutter et al., 1997). Diacylglycerol labelling was not significantly affected in agreement with the in *vivo* data (*Table 2*) and in contrast to olive (*Table 2;* Rutter et al., 1997).

CONCLUSION

We have shown that callus cultures can be used for the study of lipid metabolism. Their ease of manipulation plus the convenient availability of such cultures make them suitable model systems to study the regulation of oil biosynthesis. The overall flux through the lipid biosynthesis pathway can be manipulated with temperature. Increases in temperature stimulated carbon entry from [1-^{14}C]acetate to triacylglycerol synthesis but no changes in the acylthioesters pools were observed, suggesting that the major control of lipid synthesis lies at an early step (in fatty acid synthesis) of the pathway. By the use of [U-^{14}C]glycerol 3-phosphate as precursor with microsomal preparations from the appropriate stage of develop-

ment, satisfactory rates of triacylglycerol synthesis were possible for analysis.

Furthermore, only the Kennedy pathway intermediates were significantly labelled during such incubations, making such preparations suitable for quantitative and qualitative experiments on the regulation of oil accumulation. The results using microsomal fractions from oil palm callus were consistent with whole tissues'in showing a doubling of lipid synthesis for a 10°C rise in temperature (i.e. Q_{10} of 2). Our preliminary conclusions are that the first reactions of lipid synthesis (i.e. fatty acid formation) are more important for overall triacylglycerol accumulation. However, at high rates of triacylglycerol biosynthesis, specific enzymes of the Kennedy pathway may become more limited. These conclusions are now being examined by further appropriate flux control analyses.

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REFERENCES

BJERVE, K S; DAAE, L N W and BREMER, J (1974). The selective loss of lysophospholipids in some commonly used lipid-extraction procedures. ***Anal. Biochem.,*** *58:*238-245.

BRADFORD, M M (19721. A rapid and sensitive method for the quantitation of microgramme quantities of protein utilizing the principle of protein-dyebinding. ***Anal. Biochem.,*** *72:*248-254.

BROWSE, J and SOMERVILLE, C R (19911. Glycerolipid synthesis: biochemistry and regulation. ***Ann. Rev. Plant Physiol. Plant Mol. Biol.,*** *42:*467-506.

CHOW, C S (1997). Future trends of crude palm oil production in major producing regions and the relative position of palm oil in the world market of oils and fats. In the 22nd World Congress and Exhibition of the International Society for Fat Research (ISF), September 8-12, 1997, Kuala Lumpur, Malaysia.

ECCLESTON, S V and HARWOOD, J L (1995). Solubilization, partial purification and properties of acyl-CoA:glycerol 3-phosphate acyltransferase from avocado (*Persea americana*) fruit mesocarp. ***Biochim. Biophys. Acta, 1257:*** l-10.

GARBUS, J; DE LUCA, H F; LOOMANS, M and STRONG, F M (1963). The rapid incorporation of phosphatidate into mitochondrial lipids. ***J. Bid. Chem.,*** *238:*59-64.

HARWOOD, J L and PAGE, R A (1993). Biochemistry of oil synthesis. In (ed. Murphy, D J). ***Designer Oil Crops.*** VCH, Weinheim. p. 165-194.

HEINRICH, R and RAPOPORT, T A (1974). ***Eur. J. Biochem.,*** *42:*97-105.

KACSER, H and BURNS, J A (1973). *Symp. Soc. Exp. Biol., 32:*65-104.

KACSER, H (1979). Molecular democracy: who shares the control? ***Biochem. Soc. Trans.,*** *7:* 1149-1161.

MURASHIGE, T and SKOOG, F (1962). Revised medium for rapid growth and bioassay with tobacco tissue cultures. ***Plant Physiol., 15: 473-497.***

OHLROGGE, J B and JAWORSKI, J G (1997). Regulation of fatty acid synthesis. ***Ann. Rev. Plant Physiol. Plant Mol., 48:*** 109-136.

POWELL, G L; GROTHUSEN, J R; ZIMMERMAN, J K, ANDERSON, E C and FISH, W W (1981). A re-examination of some properties of fatty acyl-CoA micelles. ***J. Bid. Chem., 256:***12740-12747.

QUANT, P A (1993). Experimental application of ***top-down*** control analysis to metabolic systerns. ***TIBS., 18:*** 26-30.

RAMLI, U S; QUANT, P A and HARWOOD, J L (1998). Biochemical studies of oil synthesis in olive (*Olea europaea*) and oil palm (*Elaeis guineensis*) callus cultures. *Biochem. Soc. Trans., 26:*S151.

RUTTER, AJ and SANCHEZ, J and HARWOOD, J L (1997). Glycerolipidsynthesis bymicrosomal fractions from *Olea europaea* fruits and tissue cultures. *Phytochemistry, 46*:265-272.

SANCHEZ, J; CUVILLO, T D and HARWOOD, J L (1992). Glycerolipid biosynthesis by microsomal fractions from olive fruits. *Phytochemistry, 31*:129-134.

SMITH, K L; DOUCE, R and HARWOOD, J L (1982). Phospholipid metabolism in the brown alga, *Fucus serratus. Phytochemistry, 21*:569-573.

STYMNE, S and GLAD, G (1981). Acyl exchange between oleoyl-CoA and phosphatidylcholine in microsomes of developing soya bean and its role in fatty acid desaturation. *Lipids, 16*:298-305.

STYMNE, S and STOBART, A K (1987). Triacylglycerol biosynthesis. In (eds. Stumpf, P K and Conn, E E). *Biochemistry of Plants,* Vol. 9. Academic Press, New York. p. 175-214.

WILLIAMS, M; SANCHEZ, J; HANN, A C and HARWOOD, J L (1993). Lipid biosynthesis in olive cultures. *J. Exptl. Botany., 44*:1717-1723.

ZAHLER, W L; BARDEN, R E and CLELAND, W W (1968). Some properties of palmitoylcoenzyme A micelles. *Biochim. Biophys. Acta, 164*:1-11.