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THE INCORPORATION OF [1-^{14}C]ACETATE INTO LIPIDS DURING EMBRYOGENESIS IN OIL PALM TISSUE CULTURES

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Abstract—A tissue culture line of oil palm produced embryoids and during the embryogenesis large quantities of lipid were stored in the cells The synthesis of the lipid was monitored by measuring incorporation of [1-^{14}C]acetate, under optimum conditions, into the total lipid and separation by TLC into neutral and polar lipid Both synthesis of triacylglycerol and polar lipid increased during embryoid formation A rapid increase in the formation of polar lipid occurred in the period just before the embryoids became visible and this probably corresponded with the increased rate of cell division that occurred at that time

INTRODUCTION

Undifferentiated callus cultures of oil palm differentiate to form hard globular embryoids [1] These embryoids resemble embryos developed *in vivo* in their ability to deposit triacylglycerols in oleosomes [2] The *in vitro* embryoids may develop root and shoot primordia and in some cases plantlet regeneration from somatic embryoids occurs [3]

The incorporation of radioactive precursors into fatty acids *in vivo* has been widely employed in studies of lipid synthesis in both developing oil seeds and in plant tissue cultures Gurr *et al* [4] showed that, in *Crambe abyssinica* seeds, radioactive acetate was incorporated preferentially into phospholipids, but the proportion of radioactivity incorporated into triacylglycerol increased as the seed developed Almost all the acetate was incorporated into the fatty acyl moieties of the lipids, whereas [U-^{14}C]glucose was incorporated into the glycerol moiety In developing sunflower seeds acetate incorporation into fatty acids increased rapidly from ten days after flowering during the time at which triacylglycerol accumulation began [5,6] Similar results have been obtained with developing seeds of soybean [7,8] and Nasturtium [9] Radioactive acetate is also readily incorporated into lipids of plant tissue cultures [10–15]

During embryogenesis in tissue cultures of oil palm, an increase in lipid content of the cells was observed [16] In culture lines in which embryogenesis did not occur there was no rise in total lipid Electron microscopic examination showed that stored lipid was a specific product of differentiated embryoid cells A rise in the activity of acetyl CoA carboxylase *in vitro* was also correlated with the increase in lipid In this work the incorporation of [1-^{14}C]acetate into lipids *in vivo* during a time course of embryogenesis was studied Acetate was incorporated into phospholipids of oil palm callus and into phospholipids and triacylglycerols of embryoid cells During a time course of embryogenesis the rate of incorporation of acetate into both total lipids and triacylglycerols increased just before the appearance of embryoids in the cultures

RESULTS AND DISCUSSION

Optimisation of incubation conditions for incorporation of [1-^{14}C]acetate into oil palm lipids

The optimum pH of the incubation buffer was determined for maximum incorporation of [1-^{14}C]acetate A mixture of oil palm callus and chopped embryoid tissue was incubated with 10 nmol [1-^{14}C]acetate (21 7 kBq) in buffers at pH between 5 0 and 8 5 for 4 hr at 25° No non-radioactive acetate was included in the incubation mixture The amount of acetate taken up by the cells was determined by sampling the incubation medium before and after incubation (Fig 1a) The amount of radioactivity incorporated into total lipid (chloroform–methanol soluble material, Fig 1b) and into triacylglycerol separated by TLC (Fig 1c) was also measured In these optimisation experiments duplicate samples were assayed and the results did not deviate from the mean value by more than 5%

The amount of acetate taken up by the tissue decreased with increasing pH Incorporation of radioactivity into total lipid increased up to pH 6 5 in MES buffer, but was highest at pH 6 0 in phosphate buffer Acetate incorporation into triacylglycerols was highest at pH 7 3 in phosphate buffer, although only slight differences in incorporation into total lipid or triacylglycerols were observed between pH 6 0 and 7 3 in phosphate buffer Acetate incorporation into lipids was low at pH 8 0–8 5 At pH 6 5 *ca* 25% of the radioactivity taken up by the cells was incorporated into total lipid and of this, 44% was present in triacylglycerols For further experiments 0 7 M phosphate buffer pH 6 5 was used Increasing the concentration of acetate in the external medium resulted in an increase in acetate incorporation into total lipid up to a

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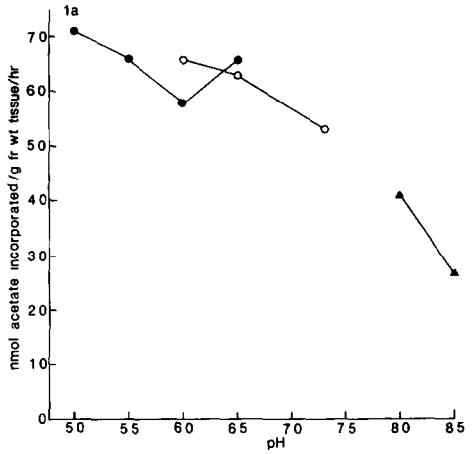

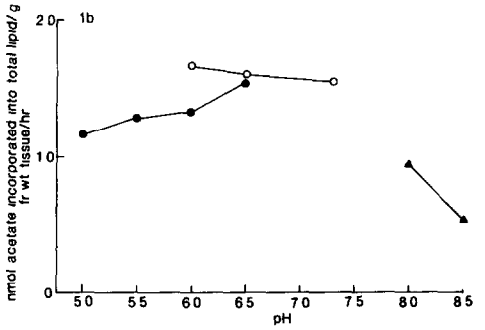

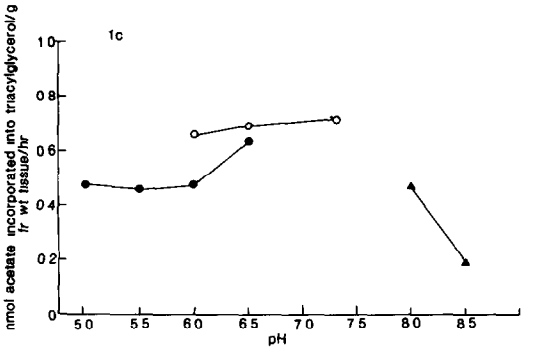

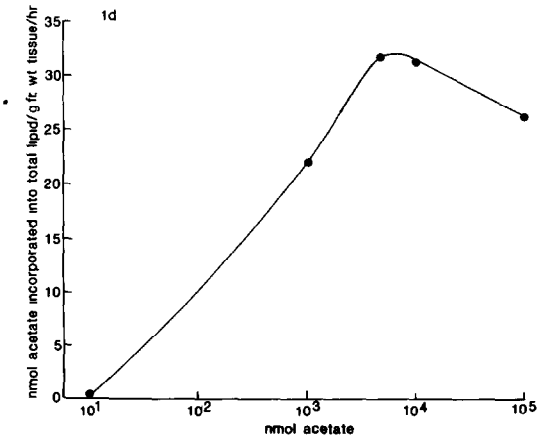

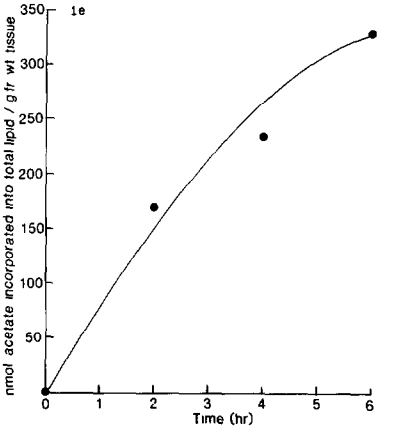

Fig 1 Optimisation of incubation conditions for incorporation of [1-^{14}C]acetate into lipids in oil palm cultures Effect of incubation buffer pH on [1-^{14}C]acetate uptake by cells (a), and incorporation into total lipids (b) and triacylglycerols (c) (●——●, 0 07 M MES buffer, ○——○, 0 07 M sodium phosphate buffer, ▲——▲, 0 07 M Tris–HCl buffer) Effect of acetate concentration in incubation mixture on rate of incorporation into total lipids (d) and time course of incorporation (e)

substrate concentration of 5 mM acetate (Fig 1d) Above this concentration the incorporation began to decline slowly A substrate concentration of 5 mM was therefore used in subsequent experiments

A time course of acetate incorporation into total lipid is shown in Fig 1e An incubation time of 2 hr was chosen for time course experiments

Analysis of radioactive lipids

Callus or embryoid tissues were incubated separately in 0 07 M phosphate buffer, pH 6 5, containing 10 nmol [1-^{14}C]acetate for 4 hr at 25° No non-radioactive acetate was included in the incubation buffer The extracted lipids were analysed by TLC and the amount of radioactivity incorporated into the different lipid classes determined Radioactive profiles of the chromatograms of callus and embryoid lipids run in heptane–diethyl ether–acetic acid and chloroform–methanol–water are shown in Figs 2 a–d In callus tissue 82% of the radioactivity incorporated into lipid remained at the origin when heptane–ether–acetic acid was used, this probably represented incorporation into polar lipids Only 8% of applied radioactivity cochromatographed with triacylglycerols Recovery of radioactivity from the TLC plate was 78% of the total radioactivity applied When chloroform–methanol–water was used 76% of the applied radioactivity cochromatographed with phosphatidyl choline and other polar lipids

Chromatography, using heptane–ether–acetic acid, of radioactively labelled lipid from embryoid tissue showed that 40% of the radioactivity remained at the origin and a further 40% cochromatographed with triacylglycerols When analysed with chloroform–methanol–water, 58% of the total radioactivity cochromatographed with neutral lipids

Samples of lipid from callus and embryoids were saponified and rechromatographed with heptane–ether–

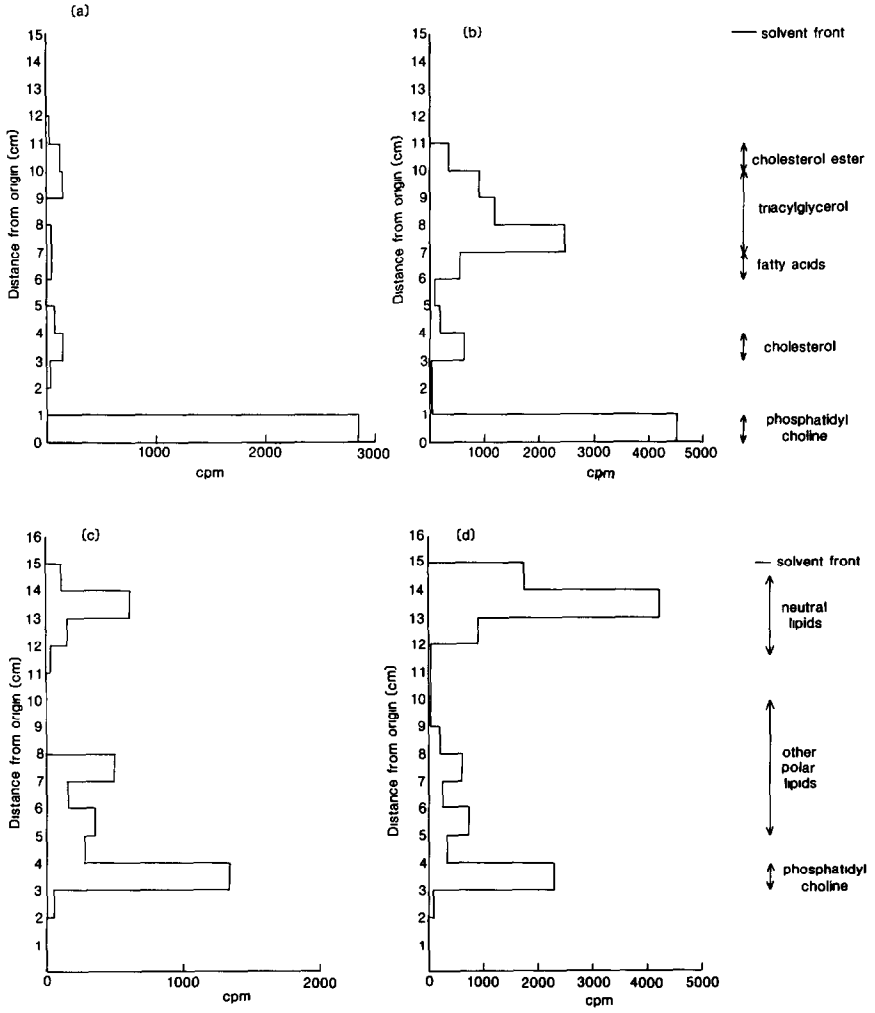

Fig 2 Radioactivity profiles of thin layer chromatograms of oil palm callus and embryoid lipid samples (a) Callus, heptane–diethyl ether–acetic acid (15 10 1), (b) Embryoid, TLC solvent as (a), (c) Callus, chloroform–methanol–water (65 25 4), (d) Embryoid, TLC solvent as (c) The positions of marker lipid samples are indicated on (b) and (d)

acetic acid (Fig 3a, b) The majority of the radioactivity ran at the position of free fatty acids The ratio of radioactivity present in the chloroform extract compared to the aqueous phase after saponification was 11 1 for callus and 135 1 for embryoid tissue This indicated that the radioactivity from [1-^{14}C]acetate was incorporated mostly into the fatty acyl chains of the lipid and not into glycerol moieties The recovery of radioactivity after saponification was 94% (callus) and 116% (embryoids)

Incorporation of [1-^{14}*C*]*acetate into lipids during a time course of embryogenesis of oil palm tissue culture line JH*

Callus of line JH when grown on medium containing 2 5 mg 3-napthyl acetic acid/l spontaneously differentiated to give rise to embryoids During a time course of growth of 63 days, samples of tissue (0 5 g) were incubated in 1 0 ml 0 07 M phosphate buffer pH 6 5 containing 30 μmol potassium hydrogen carbonate, 5 μmol sodium acetate and 10 nmol [1-^{14}C]acetate (21 7 kBq) for 2 hr at 25° Incorporation of radioactivity into total lipid (chloroform–methanol soluble material) and triacylglycerols was measured (Fig 4) At each time point two tissue samples were assayed separately The results did not deviate from the mean value by more than 10% Embryoids were visible in the cultures from 35 days

Over the time course of 63 days changes in the rate of incorporation of [1-^{14}C]acetate into total lipids and triacylglycerols were observed After an initial drop in the rate of acetate incorporation into both triacylglycerols and total lipid, there was a rapid increase from day 21 to day 35 There was 3 4-fold increase in incorporation into total lipid over this period and the incorporation into triacylglycerols increased 5-fold, the incorporation then declined The peak of incorporation into triacylglycerol or total lipid coincided with the time at which embryoids became visible in the cultures

Although increases in acetate incorporation into both total lipid and triacylglycerol occurred, the incorporation into total lipid always remained much higher than in-

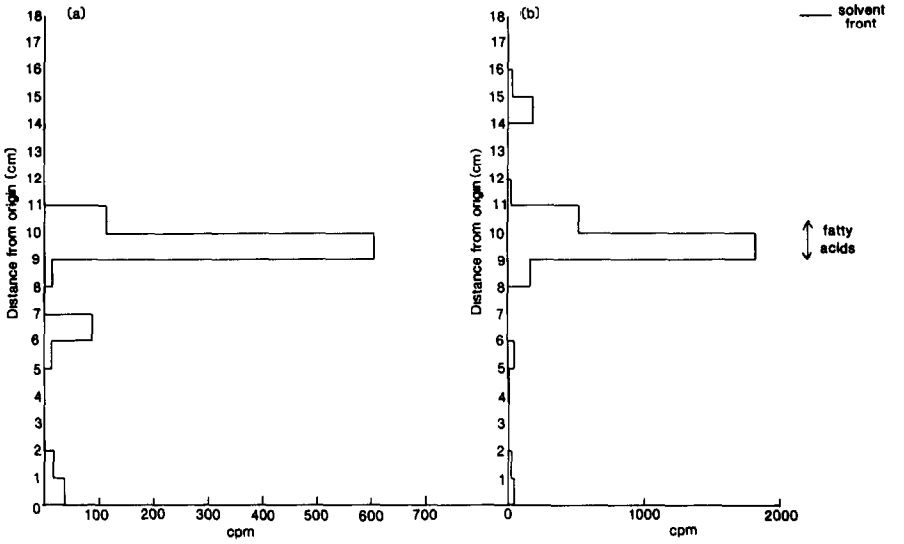

Fig 3 Radioactivity profiles of thin layer chromatograms of oil palm callus and embryoid lipid samples after saponification (a) Callus, heptane–diethyl ether–acetic acid (15 10 1), (b) Embryoid, TLC solvent as (a) The position of a marker fatty acid sample is indicated on (b)

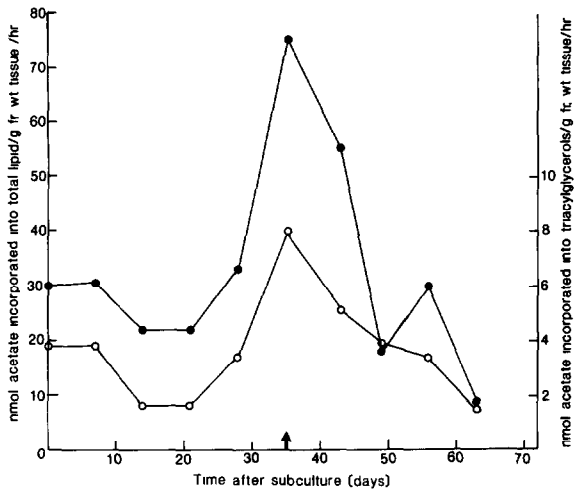

Fig 4 Changes in the rate of incorporation of [1-^{14}C]acetate into total lipids (●——●) and triacylglycerols (○——○) during a time course of embryogenesis of oil palm tissue cultures Embryoids became visible in the cultures on day 35 (arrowed)

corporation into triacylglycerol Incorporation of acetate into other lipid classes e g polar lipids, also occurred in oil palm tissue cultures and the incorporation into these lipids increased during the time course However, the proportion of radioactivity from [1-^{14}C]acetate incorporated into triacylglycerol compared to other lipid classes varied during the time course, from 7% at day 21 to 11% at day 35, with a further increase to 22% at day 49, and 17% at day 63 This reflects an increasing synthesis of triacylglycerol compared to other lipids as embryogenesis occurred

The prominent lipids of most heterotrophic cell cultures are sterols, steryl esters and steryl glycosides [17] Phospholipids are synthesized at a high rate during periods of rapid cell division [18] Triacylglycerols are usually present at a level of 5% or less [19, 20] During *in vitro* embryogenesis of oil palm cultures, storage lipid is deposited in the embryoid cells [2] This deposition of triacylglycerol was preceded by an increase in the activity of acetyl CoA carboxylase in the tissue cultures [16] The results presented here demonstrate that the incorporation of labelled acetate into lipids *in vivo* increased concomitant with embryoid appearance However, the increase in lipid synthesis was not due entirely to increases in triacylglycerol synthesis A rapid increase in incorporation of radioactive acetate into other lipid classes, especially polar lipids, occurred just before embryoids became visible Turnham and Northcote [16] showed that an increase in the rate of cell division and primary wall deposition occurred, together with the appearance of embryoids in the tissue cultures and the rise in acetate incorporation into polar lipids shown here may reflect increases in cell membrane phospholipid synthesis during this phase of rapid cell division As the embryoids developed in the tissue cultures and increased in size from 35 days onwards, lipid synthesis declined but the proportion of radioactive acetate incorporated into triacylglycerols compared to other lipid classes increased, probably reflecting the continuing deposition of storage triacylglycerol in the embryoid cells

EXPERIMENTAL

All chemicals used were of A R quality wherever possible Murashige and Skoog medium was obtained from Flow Laboratories, Irvine, Ayrshire, Scotland Agar Noble and casamino acids were from Difco Laboratories, Detroit, MI, USA [1-^{14}C]Acetic acid, sodium salt (sp radioactivity 2 17 GBq/mmol) was purchased from Amersham International,

Amersham, Bucks, U K

Growth of oil palm tissue cultures Tissue cultures of oil palm (*Elaeis guineensis* Jacq) line JH were obtained from Unilever Research, Sharnbrook, Bedford, U K The cultures were maintained on half-strength Murashige and Skoog medium [21] with the addition of 1 g casamino acids/l, 2 5% sucrose and 2 5 mg 3-napthylacetic acid/l The medium was solidified by the addition of 1% Agar Noble The cultures were maintained at 25° and illuminated at a light intensity of 100 lux with an 18 hr photoperiod They were routinely subcultured every 8–10 weeks All manipulations were carried out on a sterile laminar air-flow bench Media and instruments were sterilized by autoclaving at 120° and 103 kPa for 20 min Under these culture conditions, cultures of oil palm line JH differentiated spontaneously to produce small hard globular embryoids

Incubation of tissue cultures with [1-^{14}C]acetate Samples of oil palm cultures were incubated with [1-^{14}C]acetate *in vivo*, followed by extraction and analysis of lipids The optimum conditions for the incorporation of acetate into lipids were determined The standard incubation mixture contained 0 07 mmol NaPi buffer, pH 6 5, 30 μmol $KHCO_3$, 5 μmol NaOAc and 10 nmol [1-^{14}C]acetate (21 7 kBq) in a total vol of 1 0 ml Tissue (*ca* 500 mg) was added and the samples incubated at 25° for up to 6 hr At the end of the incubation the tissue was removed and the incubation medium sampled to determine the amount of radioactivity remaining The tissue was washed with buffer and lipids extracted

Lipid extraction and analysis At the end of the incubation, the tissue was homogenized in propan-2-ol at 80° (20 ml/g tissue) to inhibit lipase activity After filtration the residue was extracted overnight at 4° with $CHCl_3$–MeOH (3 2) (20 ml/g tissue) and then refiltered The propanol extract was rotary evaporated to dryness, taken up in $CHCl_3$–MeOH (3 2) and recombined with the first $CHCl_3$–MeOH (3 2) extract The extracted lipids were purified by washing by the method of Folch *et al* [22], rotary evaporated to dryness and taken up in a small vol of $CHCl_3$

Samples of the extracted lipids were saponified The $CHCl_3$ solvent was removed by evaporation and the lipids redissolved in 10% KOH in 90% EtOH followed by incubation at 75° for 2 hr The samples were acidified to pH 1 0 using HCl and free fatty acids extracted into $CHCl_3$ The $CHCl_3$ extract was washed with an equal vol of 1% HOAc plus 1% malonic acid

Samples of saponified or unsaponified lipids were analysed by TLC on silica gel plates in heptane–Et_2O–HOAc (15 10 1) or in $CHCl_3$–MeOH–H_2O (65 25 4) Both solvent systems contained 0 005% butylated hydroxytoluene to prevent lipid oxidation Lipids were visualized by exposure of the air-dried plates to I_2 vapour Lipid markers were run in parallel with the samples

The TLC plates were cut into 1 cm strips and radioactivity determined in the strips using a Searle Mark III liquid scintillation counter, after immersion of the strips in scintillation fluid (1 5 l toluene, 0 75 l Triton X-100, 6 g 2,5-diphenyloxazole)

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