Cette différence se maintient au-delà de la huitième heure pour des quantités moindres de diholoside. Parallèlement, la quantité d'UDPG se stabilise dans les graines fourragéres à un niveau supérieur à celui des graines sucrières.

Ces différents dosages ne permettent de mesurer que le résultat d'un équilibre dynamique entre biosynthèse et dégradation des composés étudiés. Remarquons toutefois que, dans la graine sèche, les nucléosides tri-phosphates non adénylîques constituent la principale source de composés riches en énergie, utilisée en partie immédiatement après l'imbibition quelle que soit la variété de la semence. Au cours de la germination, les deux types de graines entretiennent des 'pools' de nucléosides tri-phosphates identiques et nous avons pu vérifier que, à partir de la huitième heure, la totalité des composés étudiés se trouve dans l'embryon. Par contre les métabolismes du saccharose et de l'UDPG semblent commandés par des mécanismes génétiques capables de s'exprimer très précocement, et il est intéressant de noter que les embryons de Betterave sucrière ont la possibilité d'entretenir un 'pool' de saccharose plus important que celui des embryons de Betterave fourragère tout en disposant d'un 'pool' d'UDPG plus petit. Il sera nécessaire, dans l'avenir d'expérimenter sur un nombre plus important de variétés pour vérifier si les résultats exposés ici peuvent être utilisés pour rendre compte de la qualité sucrière d'un cultivar.

PARTIE EXPERIMENTALE

Matériel végétal. Les graines (Betterave sucrière: Cerès, lot 904.48: Betterave fourragère: Monoval) sont extraites de leurs glomérules et mises à germer en boîtes de Pétri sur papier filtre imbibé d'eau distillée à 23° et à l'obscurité.

Extraction. Les nucléotides et le saccharose sont extraits par la méthode de ref. [2]. Cette méthode permet de recueillir des quantités du diholoside identiques à celles que fournit une extraction alcoolique à chaud.

Dosage des nucléotides. Le dosage enzymatique de l'ATP en présence du système luciférine–luciférase est réalisé d'après Pradet [3]. La quantité de nucléosides tri-phosphates non adényliques est déterminée par la mesure de l'augmentation de la teneur en ATP de l'extrait en présence de nucléoside diphosphate kinase et d'un excès d'ADP [4]. L'UDPG est dosé suivant le même principe d'après l'augmentation de la teneur en nucléosides tri-phosphates non adényliques de l'extrait à la suite d'une incubation en présence d'UDPG-pyrophosphorylase et d'un excès de pyrophosphate [4].

Dosage du saccharose. Il est basé sur la mesure de la consommation d'ATP nécessaire, en présence d'hexokinase, à la phosphorylation des hexoses provenant de l'hydrolyse totale du saccharose par l'invertase. La réaction catalysée par l'invertase est effectuée dans un vol. final de 50 µl. A 5 µl de tampon acétate de Na 0,1 M, pH 5,0, sont ajoutés 5 µl d'une solution d'invertase dans ce même tampon (3 µg/µl) et 40 µl d'extrait biologique dilué de manière à contenir une quantité de saccharose inférieure à 250 p mol. L'ensemble est maintenu pendant 30 min à 37°. Le dosage des hexoses libérés au cours de la réaction est réalisé sur 10 µl de ce milieu. Les témoins suivants sont toujours réalisés: milieu réactionnel sans invertase; milieu réactionnel additionné de 250 p mol de saccharose. La réaction catalysée par l'hexokinase est faite dans un vol. final de 200 µl. A 10 µl du milieu précédent sont ajoutés 100 µl de tampon Tris–H_2SO_4 0,04 M, pH 7,5, Mg SO_4 0,0035 M, K_2SO_4 0,025 M, EDTA 0,00055 M, 10 µl d'une soln d'ATP (50 p mol/µl), 10 µl d'une soln d'hexokinase de levure (0,1 µg/µl) et 70 µl H_2O. L'ensemble est placé pendant 20 min à 25°. L'ATP restant est alors dosé selon la technique habituelle. Il a été vérifié que la réaction de phosphorylation est complétement terminée au bout de 20 min, alors que l'activité ATPasique [5] de l'hexokinase reste négligeable. De plus, la quantité d'ATP consommée correspond stoechiométriquement à la quantité d'hexoses provenant de l'hydrolyse du saccharose. L'ensemble de chaque expérimentation est répété indépendamment au moins quatre fois et les écarts observés par rapports à une valeur moyenne ne dépassent pas 4% pour les dosages de différents métabolites.

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REFERENCES

1. Jumelle, H. (1910) in *Les Plantes à Tubercules Alimentaires*, p. 287. Douin.
2. Keppler, D., Rudigier J. et Decker, K. (1970) *Analyt. Biochem.* **38**, 105.
3. Pradet, A. (1967) *Physiol. Vég.* **5**, 209.
4. Gendraud, M. (1977) *Physiol Véa.* **15**, à paraître.
5. Kaji, A. et Colowick, S. P. (1965) *Biol. Chem.* **240**, 4454.

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TRANSESTERIFICATION OF FARNESOL MEDIATED BY A LIPASE FROM *ANDROGRAPHIS* TISSUE CULTURES

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Key Word Index—*Andrographis paniculata*; Acanthaceae; tissue culture; lipase; transesterification; farnesyl oleate; farnesyl palmitate.

Abstract—A cell-free system from *Andrographis paniculata* tissue cultures catalysed the transesterification of administered *cis,trans*-farnesol-[1-$^3H_2$] with (glyceryl) oleate and palmitate present in the coconut water that forms part of the culture medium.

Recent experiments in which *cis,trans*-farnesol-[1-$^3H_2$] was incubated with a cell-free extract from hypocotyl tissue cultures of *Andrographis paniculata* [1–3] have uncovered the efficient incorporation of radioactivity

into two previously unsuspected metabolites in the ratio of 4:1. The major metabolite (80%, 13–17% incorporation of radioactivity) appeared to have a MW of 450–500 based upon its GC–RC behaviour. Its efficient labelling suggested that it was a simple derivative of farnesol rather than a more distant biosynthetic product such as a triterpenoid. This was confirmed when K_2CO_3-promoted methanolysis (1 hr, 20°, occasional shaking) converted the new material into farnesol (GC–RC) indicating a farnesyl ester as the metabolite. Consideration of possible acyl sources and the MS determined MW suggested farnesyl oleate as a likely candidate.

Synthetic farnesyl oleate had an identical R_f value (0.6, C_6H_6 on precoated Si gel plates) and GLC RI [4] (3518–20 on 1% OV-17, 3314–18 on 1% OV-1, 250°) indicated the identity of the synthetic and enzymatically produced materials. Since the OV-17 column is capable of resolving the *trans,trans* and *cis,cis* isomers of farnesyl oleate from the *cis,trans–trans,cis* mixture, it seems unlikely that the natural material can be other than farnesyl oleate. Significant quantities of other C_{18} farnesyl esters, which would have been readily resolved from farnesyl oleate on this column, were not observed.

Further confirmation came from GC–MS analysis. A M^+ at *m/e* 486 was observed along with peaks at *m/e* 69, 93 and 205 corresponding to cleavage of the farnesyl residue and its further fragmentation [5]. The absence of a peak at *m/e* 264, corresponding to a McLafferty rearrangement, is not unexpected since this process produces ions in very low abundance with fatty esters of long chain alcohols [6].

The minor product (20%) has RI 3332 (OV-17) and 3144 (OV-1) suggesting that it is an ester of farnesol and a C_{16} acid, probably palmitic. This was supported by the isolation of oleic and palmitic acids as the only fatty acids from hydrolysis of the neutral lipids of coconut water used as a major constituent (15%) of the culture medium. That coconut water was the source of the esterifying oleic and palmitic acids was demonstrated as follows. It was first shown that *Andrographis* tissues do not synthesize oleate or palmitate. Thus, Na acetate-[2-^{14}C] was incubated for one week with *Andrographis* suspension cultures at a point in their growth cycle (3 weeks after transfer from surface culture) when secondary metabolism is at a maximum.

The homogenised tissues were extracted with cold methanolic KOH and both the free fatty acids and the fatty acids obtained from hydrolysis of the neutral fraction were converted into Me esters with CH_2N_2 and examined by GC–RC. In neither case could radioactivity be detected. Coconut water was similarly extracted and there was no evidence of free fatty acids or of Me esters in the range C_{14}–C_{20} but hydrolysis of the neutral fraction and methylation of the acids afforded Me oleate and Me palmitate (2:1), which were identified by GLC and MS comparison with authentic materials.

The fact that the palmitate and oleate esters present in the neutral fraction were too involatile to be detected by GC–RC and the observed transesterification, implicating a lipase, make it seem very probable that the esters present in coconut water are glyceryl palmitate and oleate.

The results of our investigation of the time course of *cis,trans*-farnesol incorporation into the esters are presented graphically in Fig. 1. Apparently, the steady

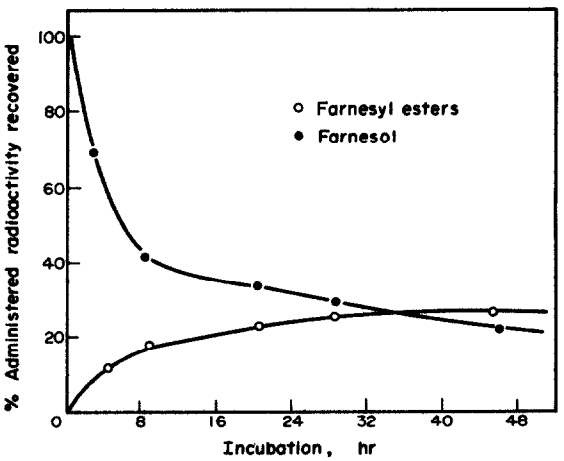

Fig. 1. Time-course of the incorporation of *cis,trans*-farnesol-[1-$^3H_2$] into farnesyl esters by a cell-free system from *A. paniculata* tissue cultures. Incorporations were measured by method 2 (see Table 1).

state ratio of activity for ester to free farnesol (1:1) is reached after 20–28 hr. After this the total radioactivity in hexane-soluble farnesyl derivatives diminishes as a result of further metabolic processes.

The results of some other incorporation experiments are collected in Table 1. The incorporation of *cis,trans* farnesol into esters is *ca.* 3–4 times more efficient than that of *trans,trans*-farnesol. Farnesol biosynthesized *in situ* from mevalonate is also esterified, although to a much smaller extent, as would be expected both because of the longer pathway involved and the fact that the *trans,trans*-farnesol initially produced is less efficiently esterified.

Table 1 also contains the results of some experiments with a selection of dispersants; the sensitivity of enzymatic activity to dispersants is a characteristic of lipases [10, 11].

Lipases are a broadly distributed class of enzymes whose normal function is the hydrolysis of glycerides [10]. However, their normal site of action is a hydrophobic interface where the local concentration of water may be restricted to the extent that acyl transfer reactions become important. The present case clearly exemplifies

Table 1. Incorporation experiments with a cell free system from *A. paniculata* tissue cultures

Substrate	Dispersant	% incorporation into farnesyl esters*
cis,trans-Farnesol [1-$^3H_2$]	Tween-80 (0.025%)	16–21
cis,trans-Farnesol [1-$^3H_2$]	sodium deoxycholate (0.85 mM)	29
cis-trans-Farnesol [1-$^3H_2$]	dimethyl sulphoxide (5%)	25–31
cis,trans-Farnesol [1-$^3H_2$]	buffer and cofactors†	10
boiled extract		0.5
trans,trans-Farnesol [1-$^3H_2$]	Tween 80 (0.025%)	5
Mevalonate-[2-^{14}C]		1–2

* Incorporations were measured in two ways. (1) TLC of hexane soluble fraction followed by elution of ester band (R_f 0.6) and scintillation counting or (2) scintillation counting of aliquot portion of hexane solution and GC–RC analysis. Agreement of the order indicated in the Table was obtained in different expts. Incubations for 17–21 hr.

† Substrate sonicated in buffer–cofactor solution [1, 2] (1 ml), without added dispersant.

such transfer of oleate and palmitate from glycerol to farnesol. Related studies with enzymes from broccoli leaves [12] and potato tubers [13] have been reported recently, and analogous processes are known to be important in wax ester biosynthesis [7–10]. However, we are unaware of a previous example involving a terpenoid alcohol and an enzyme system derived from a plant tissue culture. Coconut milk cannot be the source of lipase activity, since the medium is routinely autoclaved prior to use. Given the broad distribution of lipases [14, 15] and their apparent lack of significant selectivity among primary alcohols [12, 13] and fatty acids [16] it is to be anticipated that transesterifications of the kind here reported may be fairly common. Since this will usually represent a diversion of labelled materials from the pathway under study, experiments involving the incubation of long chain alcohols with cell free and similar systems should be planned and interpreted accordingly.

EXPERIMENTAL

GC–MS analyses were performed on a LKB 9000 chromatograph, GLC with a radio-gas detector; system operated in ^3H mode. PMR were recorded at 60 MHz. *cis,trans-* and *trans, trans*-farnesols-[1-$^3H_2$] were prepared and purified by standard procedures [1, 2, 16].

Farnesyl oleate. Oleic acid (290 mg, 1.03 mmol) and $SOCl_2$ (1 ml) were maintained at 100° for 15 min. Excess reagent was removed in a N_2 stream with the aid of dry C_6H_6, the residue dissolved in dry Pyr (15 ml) and farnesol (222 mg, 1 mmol; mixture of 4 isomers, Koch–Light) added. The initially clear brown mixture became warm and deposited needles. It was stirred for 16 hr, then dild with petrol (20 ml), washed successively with H_2O, satd $CuSO_4$, H_2O and aq. NaCl, and dried ($MgSO_4$). The residue after solvent removal *in vacuo* was purified by TLC (C_6H_6, Si gel). PMR δ 0.88 (3H, *t*, —C$\underline{H}_3$), 1.27 (22H, *bs*, —C$\underline{H}_2$—), 1.58, 1.68 (6H each, *s*, =C—C$\underline{H}_3$), 2.04 (12H, *bs*, =C—C$\underline{H}_2$—), 2.31 (2H, *s*, COC$\underline{H}_2$), 4.58 (2H, *d*, J = 7 Hz, —O—C$\underline{H}_2$—), 5.12–5.36 (5H, *bs*, =C—$\underline{H}$). TLC (C_6H_6, Si gel) showed one spot and GLC (1% OV-17; 1% OV-1/250°) 90% volatile material in one peak (RI 3518–20; 3314–18). [Found: M^+ 486.4430. Calc. for $C_{33}H_{58}O_2$ 486.44366].

Experiments with tissue cultures. Techniques for maintenance of tissue cultures, prepn of cell-free extracts and anaerobic incubations have been described previously [1, 2]. For time-course incorporation studies, Warburg flasks were maintained under positive N_2 press. (balloon) and samples periodically withdrawn by syringe via a rubber septum cap.

Analysis of Andrographis *cultures and coconut water for free fatty acids and glycerides.* Na acetate-[2-^{14}C] (3.6 μCi) was distributed into 5 flasks of Ag II suspension cultures (3 weeks after transfer of the callus from solid agar into suspension culture) and incubated for 1 week at 27°. Cells were separated from the nutrient medium and homogenized in a blender with KOH in MeOH–H_2O (250 ml, 0.05 M in MeOH–H_2O 1:1). The resulting slurry was centrifuged 1.5 hr at 0°, 2500 rpm. The supernatant was concentrated *in vacuo* to remove MeOH and this was replaced by H_2O. Neutral lipids were extracted into EtOAc (4 × 50 ml) from the alkaline extracts, and after acidification (dil HCl to pH 2) the free fatty acids were similarly extracted. Neither fraction showed radioactivity when examined by GC–RC. The neutral lipids were saponified by heating with 2 M KOH at 80° for 3 hr. The fatty acids, obtained in the usual way, were methylated with CH_2N_2–Et_2O. The Me ester fraction did not show any radioactivity although there were peaks at RI 2020 and 2210, corresponding respectively, to Me palmitate and Me oleate (comparison with authentic samples) presumably originating from the coconut water of the nutrient medium. Coconut milk (200 ml; as used for the preparation of tissue culture medium) was made alkaline by addition of solid KOH to 0.05 M. Methylation of the acid fraction obtained from alkaline hydrolysis of the neutral fraction afforded Me pamitate and Me oleate (1:2) but no other Me esters in the range C_{14}–C_{20}; identifications were made by GLC and GC–MS.

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REFERENCES

1. Overton, K. H. and Roberts, F. M. (1974) *Phytochemistry* **13**, 2471.
2. Roberts, F. M. and Overton, K. H. (1974) *Biochem. J.* **144**, 585.
3. Overton, K. H. and Picken, D. J. (1976) *J. Chem. Soc. Chem. Commun.* 105.
4. Kovats, E. (1965) *Advan. Chromatog.* **1**, 229.
5. Enzell, C. R., Appleton, R. A. and Wahlberg, I. (1972) *Biochemical Applications of Mass Spectrometry* (Waller, G. R. ed.) p. 358. Wiley Interscience, New York.
6. Odham, G. and Stenhagen, E. (1972) Ref. 5, p. 232.
7. Harwood, J. L. (1975) *Recent Advances in the Chemistry and Biochemistry of Plant Lipids* (Galliard, T. and Mercer, E. I., eds) Ch. 3. Academic Press, London.
8. Long, C. (1961) *Biochemists Handbook* p. 987. Spon, London.
9. Gunetileke, K. E. and Laurentius, S. F. (1974) *J. Food Sci.* **39**, 230.
10. Desnuelle, P. (1972) *The Enzymes* (Boyer, P. D. ed.) 3rd ed. Vol. VII, Ch. 3. Academic Press, London.
11. Galliard, T. (1975) Ref. 7. Ch. 11.
12. Kollatukudy, P. (1967) *Biochemistry* **6**, 2705.
13. Dennis, S. and Galliard, T. (1974) *Phytochemistry* **13**, 2469.
14. Wills, E. D. (1965) *Advan. Lipid Res.* **3**, 197.
15. Wardale, D. E. and Galliard, T. (1975) *Phytochemistry* **14**, 2323.
16. Cornforth, J. W., Cornforth, R. H., Donninger, C. and Popjak, G. (1966) *Proc. Roy. Soc.* (*B*) **163**, 492.