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THE INVOLVEMENT OF AMINO ACIDS IN LATEX LIPID SYNTHESIS IN *EUPHORBIA LATHYRIS* SEEDLINGS

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Key Word Index—*Euphorbia lathyris*; Euphorbiaceae; laticifers; amino acids; triterpenes; phytosterols.

Abstract—The breakdown of triglycerides and proteins in the endosperm of *Euphorbia lathyris* was assayed in a 14 day germination period. Six days after germination, the average daily production was 2.7 μmol of amino acids. Arginine, glutamine, asparagine and glutamic acid accounted for 53% of the total amino acids. Excised cotyledons with 1 cm hypocotyls were used for amino acid uptake and their involvement in terpenoid synthesis was studied. Glutamine and aspartate were hardly involved in apolar lipid synthesis. Leucine, isoleucine, valine and threonine were mainly incorporated into the triterpenes in the laticifers. Alanine and serine were also involved in phytosterol synthesis in the adjacent tissue. In the 14 day germination period, *ca* 3% of the daily yield of latex triterpenes may be synthesized from a variety of amino acids.

INTRODUCTION

In the fatty seeds of the caper spurge or gopher plant (*Euphorbia lathyris*), the storage reserves are present in the embryo-surrounding endosperm. During germination, the triglycerides in this storage tissue are converted into soluble carbohydrates, which in turn are rapidly taken up by the cotyledons and translocated to the growing hypocotyl and root. In the seedlings of *Euphorbia lathyris* a laticiferous system is developed at germination [1], and the occurrence of triterpenes in the laticifers can be demonstrated from the sixth day after germination [2]. In a previous paper it was shown that sucrose (produced in the endosperm) was the most efficient precursor of latex triterpenes [3]. Quantitative data obtained in incorporation experiments using [^{14}C]sucrose and excised cotyledons showed that synthesis from this sugar could account for most of the daily synthesis of latex triterpenes in a growing seedling [4].

Proteins are also quantitatively important reserve substances in these fatty seeds. In the germination period, these macromolecules are hydrolysed and the resulting amino acids are taken up simultaneously with the soluble carbohydrates by the cotyledons. In the last decade, several papers have been published on the involvement of various amino acids in terpenoid synthesis [5–9]. This paper deals with the contribution of amino acids from the endosperm to triterpenoid synthesis in the laticiferous system and adjacent tissue of a growing seedling of *Euphorbia lathyris*.

RESULTS AND DISCUSSION

The dry seeds of *Euphorbia lathyris* were found to contain 16.3 ± 0.6 mg of triglycerides and 3.5 ± 0.25 mg of protein per endosperm. Compared with the storage constituents of *Ricinus communis* (300 mg of fat and 40 mg of protein per endosperm [10]), these seeds of the caper spurge are relatively rich in protein. After homogenization of the endosperm of dry seeds and subsequent centrifugation as described by Youle and Huang [11], a bottom fraction was obtained in which many protein bodies were observed: they resembled in shape and size those described for *Ricinus* [11]. Under the conditions employed, the endosperm is completely digested by the fourteenth day of germination (Fig. 1). At that time only the outer papery integuments remain, accounting for the difference between the dry weight and the fat and protein content of a dry endosperm. After 5–6 days, a net loss of triglycerides and protein could be measured and a concurrent yield of amino acids was observed. After 7 days the average amino acid content (μmol) was 2.9 ± 0.2 (four determinations) and the composition of the mixture is given in Table 1. As no drastic alterations were observed from day 6 to day 11, this value was used a a reference. In this mixture 2.9 μmol of amino acids is equivalent to *ca* 400 μg of amino acids or *ca* 350 μg of protein. In a 9 day period *ca* 3.3 mg of protein per endosperm is digested and this gives an average daily yield of *ca* 2.7 μmol of amino acids. The carbon skeletons of some amino acids may be converted to sugars with remarkable efficiency, as was demonstrated in *Ricinus* [10]. The scale of this conversion has not been measured in *Euphorbia lathyris* endosperms, as the point of emphasis here is the involvement of amino acids in lipid synthesis after uptake by the cotyledons.

In a previous paper it was shown [2] that the cotyledons are a major site of synthesis of the apolar latex lipids from sugars. Optimal ^{14}C-incorporation was obtained if labelled substrates were taken up by pairs of cotyledons with 0.5–1.0 cm of hypocotyl attached. Therefore, similar tissues were supplied with a mixture of 15 labelled amino acids which was diluted with various concentrations of the amino acid mixture found in the endosperm. Table 2 shows that substrate uptake was virtually complete at all the concentrations of the mixtures tested. In all cases *ca* 0.3% of the ^{14}C from this complex incubation mixture was recovered from the unsaponifiable lipid fraction, in which 65–85% of the radioactivity co-chromatographed

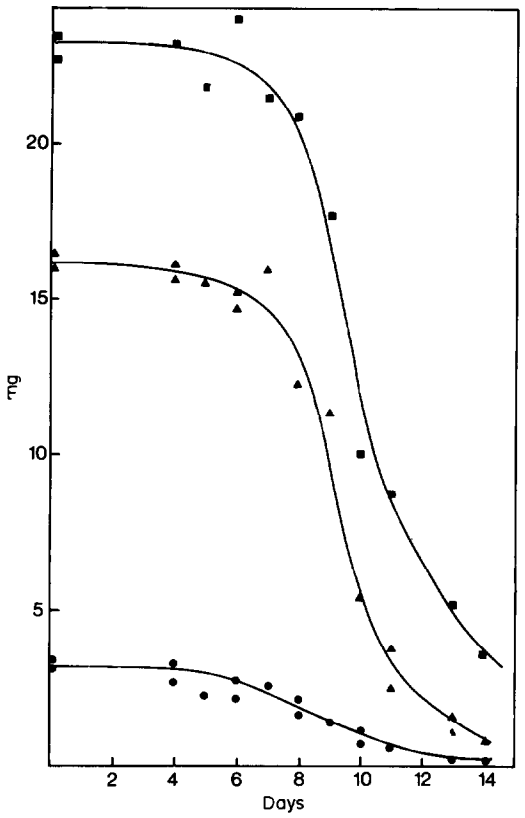

Fig. 1. Decrease of dry weight (■) and breakdown of triglycerides (▲) and proteins (●) in the endosperm of a *Euphorbia lathyris* seedling during germination at 25°.

Table 1. Free amino acids in a single endosperm after 7 days of germination

	nmol	%
Arg	465	16.2
Gln	461	16.0
Asn	334	11.6
Glu	295	10.2
Ile	147	5.1
Ser	141	4.9
Asp	140	4.9
Val	131	4.6
Phe	124	4.3
Leu	121	4.2
Thr	109	3.8
Pro	99	3.4
Ala	87	3.0
Tyr	75	2.6
His	74	2.1
Gly	26	0.9
Lys	18	0.6
γ-Abu	17	0.6
Met	10	0.3
		99.3

Table 2. Incorporation of ^{14}C from a uniformly labelled amino acid mixture* (0.108 μCi) into unsaponifiable lipids after 40 hr of incubation (a); b–f have been supplemented with various amounts of the amino acid mixture (^{12}C-mix) as assayed in the endosperm (Table 1). Data refer to a single pair of cotyledons

	Amino acid supply (nmol)		Uptake by cotyledons		Unsaponifiable lipids
	^{14}C-mix	^{12}C-mix	(nmol)	(μCi)	(dpm)
a	0.016	—	0.0148	0.100	717
b	0.016	+ 90	88.5	0.106	701
c	0.016	+ 180	175	0.105	588
d	0.016	+ 375	356	0.103	745
e	0.016	+ 750	708	0.102	566
f	0.016	+1500	1482	0.107	740

*Composition is given in the Experimental.

with the 4,4-dimethyl sterols (triterpenols), 4α-methyl sterols and 4-demethyl sterols as judged by TLC.

To investigate the contribution of individual amino acids in this lipid biosynthesis, eight different amino acids were supplied in various concentrations. Results of these experiments are summarized in Table 3. Up to *ca* 2 μmol of glutamine was taken up, and irrespective of the concentration used, *ca* 0.2% of the supplied ^{14}C was recovered from the unsaponifiable fraction. The branched-chain amino acids gave a better yield of ^{14}C-labelled lipids: up to 2.6% of the L-[U-^{14}C]leucine taken up (180 nmol) was recovered from this fraction after 2 days. This value is of the same order of magnitude as the daily leucine uptake, but increased levels of leucine produced a higher absolute lipid yield. Similar results were obtained for valine and isoleucine.

Optimal incorporations were obtained with L-[U-^{14}C]threonine if 300–600 nmol was taken up by a pair of cotyledons. After uptake of greater amounts of this substrate, a reduction in ^{14}C-labelled lipid yield was observed. This decrease in ^{14}C-incorporation was not found when serine or alanine was used as a substrate. Both amino acids gave a relatively low incorporation of ^{14}C (0.5–1.0%) in the unsaponifiable fraction. Aspartic acid proved to be the least effective substrate. Glycerol, which is liberated from the triglycerides in the germination process, was used for comparison and was taken up in considerable amounts. About 1% of the ^{14}C from C-1 was recovered in the unsaponifiable fraction, irrespective of the concentration used.

All the ^{14}C-samples of the unsaponifiable lipid fractions obtained with various amounts of amino acids were combined and separated by TLC. In all cases over 50% of the radioactivity co-chromatographed with the 4-demethyl sterols, 4α-methyl sterols and the 4,4-dimethyl sterols. Results in Table 4 show that serine can be considered as one of the most efficient precursors in 4-demethyl sterol synthesis. Alanine and threonine were also converted into these lipids to a considerable extent, but the branched-chain amino acids appeared to be hardly involved; nor was glycerol. The radioactivity of these substrates was mainly detected in the triterpenol fraction after 46 hr of incorporation. The triterpenes of *Euphorbia lathyris* seedlings occur mainly in the laticifers, which contain 4-demethyl sterols in trace amounts only [3]. The

Table 3. Uptake of uniformly labelled amino acids and subsequent ^{14}C-incorporation in the unsaponifiable lipids by a single pair of cotyledons after 46 hr of incubation. A similar experiment was done with [1-^{14}C]glycerol for comparison

(dpm × 10^3)	Substrate concentration (M)	Uptake % of ^{14}C fed	Uptake nmol	^{14}C-Incorporation into unsaponifiable lipids % ^{14}C-recovery
Gln	0.15	98	1840	0.19
(213)	0.05	98.4	620	0.20
	0.0002	99	2.6	0.27
Asp	0.038	96.7	520	0.10
(215)	0.019	99.5	270	0.10
	0.0065	99.8	185	0.16
	0.0007	99.7	9.7	0.15
Ala	0.1	98.1	1400	1.36
(212)	0.05	98.8	700	0.99
	0.033	98.8	460	0.63
	0.0167	96.9	230	0.51
	0.0084	98.8	117	0.63
	0.0007	99.1	9.5	0.57
Ser	0.1	93.3	1330	0.41
(284)	0.05	95.2	680	0.47
	0.033	96.1	450	0.61
	0.016	96.9	230	0.60
	0.008	97.3	110	0.72
	0.0009	97.4	12	0.70
Thr	0.3	72.3	2740	0.42
(202)	0.15	69.8	1300	0.74
	0.10	86.0	1075	1.16
	0.05	95.8	600	2.30
	0.03	97.0	360	2.39
	0.0002	97.7	0.4	1.25
Val	0.25	73.9	2300	0.35
(138)	0.125	95.9	1490	0.36
	0.083	95.3	990	0.84
	0.042	97.2	500	1.28
	0.020	97.9	250	1.31
	0.0002	97.7	0.3	1.18
Ile	0.05	98.2	620	0.46
(185)	0.025	97.4	310	0.86
	0.016	98.0	210	1.24
	0.008	98.5	100	1.12
	0.0002	96.2	0.2	1.09
Leu	0.05	63.0	790	1.24
(116)	0.025	83.2	520	1.45
	0.017	86.9	360	1.80
	0.008	89.7	180	2.67
	0.004	95.2	99	2.45
	0.0001	85.2	0.2	1.88
[1-^{14}C]Glycerol	0.6	67.4	5055	1.04
(125)	0.4	74.9	3750	1.07
	0.2	84.6	2120	1.25
	0.1	87.4	1090	1.30
	0.025	87.9	270	1.34
	0.00025	88.0	2.7	1.40

radioactive triterpenols, however, may be formed in the laticifers and/or belong to intermediates in 4-demethyl sterol synthesis in the adjacent tissue. To investigate where the labelled triterpenols had been synthesized, the labelled products were acetylated and then separated by argentation TLC into two distinct groups. One had the same R_f (0.46) as 24-methylene cycloartanyl acetate and the other triterpene acetates ran with R_f values of 0.55–0.61, the latter being typical for lanosteryl acetate. These constituents, free and esterified, are the major triterpenoids in

Table 4. ^{14}C-Incorporation from various uniformly labelled amino acids and [1-^{14}C]glycerol into 4-demethyl sterols and triterpenes by a single pair of cotyledons after 46 hr of incubation

Substrate	Uptake (dpm × 10^3)	Uptake (μmol)	[^{14}C]Demethyl sterols (dpm)	[^{14}C]Triterpenes (dpm)
Gln	209	1.23	28	144
Asp	209	0.33	32	116
Ala	209	0.58	243	890
Ser	231	0.56	403	753
Thr	170	1.42	252	993
Val	128	1.11	72	909
Leu	97	0.39	103	995
Ile	182	0.26	89	1250
Glycerol	102	2.46	121	1163

the latex of this plant [12]. The radioscan of the argentation TLC plate revealed two distinct ^{14}C-peaks co-chromatographing with these triterpenyl acetates. Results in Table 5 show that *ca* 30% of the ^{14}C in the triterpene fraction co-chromatographed with 24-methylene cycloartanol after alanine, threonine, isoleucine, leucine and glycerol incorporation: the remaining 70% was found in the lanosterol fraction. This ^{14}C-distribution is the same as that typically found in the triterpenes of the latex after [^{14}C]sucrose incorporation.

A different ^{14}C-distribution was found after serine incorporation. Most of the radioactivity co-chromatographed with 24-methylene cycloartanol. In this case the occurrence of labelled latex triterpenes in these fractions cannot be ascertained since a considerable proportion might be intermediates in phytosterol synthesis in the adjacent tissue. The recovery of ^{14}C in the triterpene fraction after aspartate and glutamine uptake was too low for accurate ^{14}C-assay in both groups of acetylated triterpenes.

The branched-chain amino acids are mainly incorporated into the triterpene fraction of the apolar lipids of the latex. To calculate the actual contribution of these substrates to latex lipid synthesis, a detailed biochemical pathway from the amino acid in question to an intermediate in sterol synthesis is required. In a previous paper it was demonstrated that the conversion of sucrse via pyruvate, acetyl-CoA, mevalonate and isopentenyl pyrophosphate to a triterpene is a major route of lipid synthesis inside a laticifer [2, 3]. Isoleucine and leucine may be converted to mevalonate or dimethylallyl pyrophosphate and lose one carbon atom in isopentenyl pyrophosphate synthesis. No carbon aoms are lost in the conversion of valine into dimethylallyl pyrophosphate [6]. Threonine is the main substrate in isoleucine synthesis. Serine and alanine are supposed to be converted into pyruvate, which in turn can be used in mevalonate synthesis via acetyl-CoA. Isoleucine synthesis from threonine requires one molecule of pyruvate and therefore alanine and serine might be partially involved in latex lipid synthesis via isoleucine.

Table 5. ^{14}C-Distribution between two groups of triterpenols formed from various uniformly labelled amino acids and [1-^{14}C]glycerol after 46 hr of incubation. Data refer to a single pair of cotyledons

Substrate	^{14}C-Distribution (%) 24-Methylene cycloartanol fraction	Lanosterol fraction
Ser	61	39
Ala	29	71
Thr	30	70
Leu	28	72
Ile	31	69
Glycerol	29	71

Summarizing, it can be stated that the amino acids which are biochemically linked to mevalonate or isopentenyl pyrophosphate are mainly involved in triterpene synthesis inside the laticifers: these substrates are apparently easily translocated to this specific compartment. The amino acids used from the pyruvate family are also effectively used in phytosterol synthesis outside the laticifers. Nevertheless, a considerable part of the supplied alanine and threonine (or their metabolites) can reach the laticifers after uptake by the cotyledons and can subsequently be used in triterpene synthesis. The site of the triterpenes produced from serine remains obscure.

From the results in Table 1 and Fig. 1 an average daily uptake of *ca* 110 nmol of leucine, 140 nmol of isoleucine, 120 nmol of valine, 100 nmol of threonine and 80 nmol of alanine can be calculated. If a loss of carbon atoms as described above is taken into account, a daily production of 0.3–0.35 μg of latex triterpenes from amino acids is to be anticipated. In a previous paper we reported a daily production of 18 μg of triterpenes in the laticifers [4]. Therefore, *ca* 2% of these polar lipids may be synthesized from amino acids in the second half of the germination period.

EXPERIMENTAL

Plants. Seeds of *Euphorbia lathyris* L. were soaked in H_2O for 6 hr and transferred to a moistened filter paper in a Petri dish. After 5 days of incubation in the dark at 25°, the germinated seeds were selected for the incorporation expts.

Incorporation expts. After 11 days of germination the endo-

sperm was removed from the etiolated seedlings and 8 pairs of cotyledons (with 1 cm of hypocotyl attached) were excised and immersed immediately in 100 μl of incubation mixture. The tissue was incubated in the dark at 25°. After incubation, the tissue was rinsed in a few ml of H_2O and the washings were combined with the remains of the incubation mixture for uptake assay. The tissue was frozen and extracted with Me_2CO for 2 hr at 50°. The extract was then diluted with an equal amount of petrol (40–60°) and a few drops of H_2O added to obtain 2 layers. The petrol fraction was saponified (5% KOH in MeOH–C_6H_6, 9:1; 2 hr at 90°) and the non-saponifiable matter extracted twice with petrol. The combined extracts were evapd under N_2 and separated on silica gel G TLC plates developed in cyclohexane–EtOAc (4:1). The bands of the 4,4-dimethyl sterols (triterpenols), 4α-methyl sterols and the 4-demethyl sterols were scraped off after detection with a radiochromatogram scanner and eluted with a mixture of petrol (40–60°)–Me_2CO (1:1). After evapn of the solvent, the fractions were acetylated for 6 hr at 80° in a mixture of C_5H_5N–Ac_2O (2:1). The acetates were extracted with petrol after dilution with 4 vols. of H_2O and separated by $AgNO_3$–silica gel (1:6.6) TLC in toluene–petrol (1:1). Radioactivity was detected as above and the labelled material scraped off and eluted with toluene for ^{14}C-assay in a liquid scintillation counter. Similar (non-radioactive) lipid fractions from seedlings and latex were used as reference in TLC. They were visualized after chromatography by spraying with chlorosulphonic acid–HOAc (1:2) and heated to 90° for 3 min.

Radioisotopes. [1-^{14}C]Glycerol, L-[U-^{14}C]serine and L-[U-^{14}C]alanine were obtained from the Radiochemical Centre, Amersham. L-[U-^{14}C]Glutamine, L-[U-^{14}C]leucine, L-[U-^{14}C]isoleucine, L-[U-^{14}C]valine, L-[U-^{14}C]aspartic acid and L-[U-^{14}C]threonine were products of New England Nuclear; as was the amino acid mixture (NEC-445E) containing 15 uniformly labelled L-amino acids: ala (172, 80), arg (336, 70), asp (229, 80), glu (267, 125), gly (117, 40), his (336, 15), ile (360, 50), leu (355, 140), lys (345, 60), phe (536, 80), pro (283, 50), ser (162, 40), thr (210, 50), tyr (499, 40), val (286, 80). [The first number in parentheses refers to the sp. act. (μCi/μmol), the second represents the amount in μCi per mCi of mixture.]

The triglycerides in the endosperm were assayed with the colorimetric glycerol assay described in ref. [13]. The analytical data for the simple mixture of fatty acids in these seeds [14] were then used to calculate the absolute triglyceride content. Proteins were determined as described by Bradford [15]. Amino acids were extracted with 80% aq. EtOH and assayed according to ref. [16]. The composition of the amino acid mixture extracted from the endosperm was obtained using an amino acid autoanalyser (Biotronic LC699E)

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REFERENCES

1. Schullerus, J. (1882) *Abhandl. Bot. Ver. Prov. Brandenburg.* **24**, 27.
2. Groeneveld, H. W., Peters, E., Kroese, G. and Papenhuyzen, C. (1983) *Ann. Botany* **51**, 307.
3. Groeneveld, H. W. and Roelvink, P. W. (1982) *Planta* **154**, 347.
4. Groeneveld, H. W. and Roelvink, P. W. (1982) *Planta* **156**, 264.
5. Loomis, W. D. (1967) in *Terpenoids in Plants* (Pridham, J. B., ed.), p. 65. Academic Press, London.
6. Suga, T., Hirata, T., Shishibori, T. and Tange, K. (1974) *Chem. Letters* 189.
7. Suga, T., Hirata, T. and Tange, K. (1975) *Chem. Letters* 243.
8. Tange, K., Hirata, T. and Suga, T. (1979) *Chem. Letters* 269.
9. Suga, T., Tange, K., Icchô, K. and Hirata, T. (1980) *Phytochemistry* **19**, 67.
10. Stuart, C. R. and Beevers, H. (1967) *Plant Physiol.* **42**, 1587.
11. Youle, R. J. and Huang, A. C. H. (1978) *Plant Physiol.* **58**, 703.
12. Nielsen, P. E., Nishimura, H., Liang, Y. and Calvin, M. (1979) *Phytochemistry* **18**, 103.
13. Foster, L. B. and Dunn, R. T. (1973) *Clin. Chem.* **19**, 338.
14. Kleinmann, A. (1965) *J. Am. Oil Chem. Soc.* **42**, 169.
15. Bradford, M. (1976) *Analyt. Biochem.* **72**, 248.
16. Rosen, H. (1957) *Arch. Biochem. Biophys.* **67**, 10.