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METABOLISM OF [4-^{14}C]LEVULINIC ACID BY ETIOLATED AND GREENING LEAVES OF *HORDEUM VULGARE*

PIERRE J. LEVASSEUR and MERRILL L. GASSMAN

Department of Biological Sciences, University of Illinois at Chicago, Box 4348, Chicago, IL 60680, U.S.A.

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; biochemistry; fatty acid; aminolevulinic acid dehydratase inhibition; levulinic acid; aminolevulinic acid; chlorophyll.

Abstract—When etiolated barley (*Hordeum vulgare* L. var. Larker) shoots are incubated with [4-^{14}C]levulinic acid, $^{14}CO_2$ is evolved, and amino and organic acids are labelled. Respiratory inhibitors and short-chain fatty acids, similar in size to levulinic acid, reduce the production of $^{14}CO_2$ from [4-^{14}C]levulinic acid, while δ-aminolevulinic acid treatment or illuminating the tissue increase $^{14}CO_2$ evolution. The contribution of levulinic acid metabolism to δ-aminolevulinic acid biosynthesis is no greater than that of a general cellular metabolite. The data suggest that fatty acid oxidation and the citric acid cycle are involved in levulinic acid metabolism.

INTRODUCTION

δ-Aminolevulinic acid (ALA) is an important precursor of metalloporphyrins such as heme and chlorophyll (Chl) [1]. The conversion of two molecules of ALA to one molecule of porphobilinogen, a reaction catalysed by the enzyme ALA-dehydratase (EC 4.2.1.24), is inhibited by levulinic acid (LA) [2–4]. When etiolated plant tissues are illuminated in the presence of LA, they accumulate ALA at the expense of Chl [5–7]. This property has led to the use of LA in acquiring information in two areas: (a) the biochemistry of ALA formation, and (b) the role of light in the regulation of ALA and Chl biosynthesis [8].

A 1:1 relationship between the ALA accumulated and the Chl deficit is observed in some LA-treated plants [8], suggesting that LA has no significant effect on metabolism other than to inhibit ALA-dehydratase activity. However, there is evidence which indicates otherwise. For example, some greening organisms accumulate less ALA than can be accounted for by the Chl deficit, indicating that LA inhibits ALA biosynthesis [5, 9–11]. Inclusion of LA in the growth media of the blue–green alga *Agmenellum quadruplicatum* [12] or the diatom *Skeletonema costatum* [13] results in an inhibition of cell growth. Structural differences in the mitochondria of greening bean leaves and the chloroplasts of greening bean and maize leaves have been attributed to LA treatment [7]. Application of LA leads to a reduction in photosystem II activity in greening maize leaves [14]. In etiolated barley leaves, treatment with LA inhibits amino and organic acid metabolism to CO_2 and amino acid uptake and incorporation into protein [9]. In addition, the incorporation of amino acids into thylakoid proteins of greening maize leaves is inhibited [15].

Several types of organisms have been shown to metabolize LA. Bacteria and yeast can utilize LA as a carbon source [16]. Recently, Duggan *et al.* [17] have shown that etiolated and greening barley leaves can metabolize the C-1 of LA to CO_2 and a variety of cellular constituents. The present study examines the metabolism of the C-4 of LA to CO_2 in barley leaves in an attempt to learn (a) how LA is metabolized in this organism, and (b) whether this metabolism can make a significant contribution to the pool of ALA precursors. Preliminary reports on this work have appeared [18, 19].

RESULTS

Kinetics of [4-^{14}C]*LA metabolism*

Etiolated barley shoots evolve $^{14}CO_2$ when incubated with [4-^{14}C]LA (Fig. 1). The kinetics of $^{14}CO_2$ evolution exhibit a lag period of more than 1 hr (Fig. 1); this lag was found to be independent of pH and substrate concentration (data not shown). However, unlike the kinetics of $^{14}CO_2$ evolution, no lag period is observed in [^{14}C]LA uptake (Fig. 1). These data suggest that the uptake of [^{14}C]LA does not limit its metabolism to $^{14}CO_2$.

Sequestering of LA within barley shoots

Barley tissue was incubated with [^{14}C]LA for either 2 or 4 hr and then washed with excess [^{12}C]LA for up to 3 hr. The kinetics of radiolabel loss from the tissue were similar in each case (data not shown).

Replicate washes taken after 20 min were pooled, concentrated and subjected to chromatography. Radiochromatographic analysis revealed that only one labelled compound was present in solution (Fig. 2a). This compound co-migrated with [^{12}C]LA (Fig. 2c). Two labelled compounds other than [^{14}C]LA were found in a combined mixture of the 60-to-180-min washings (Fig. 2b). Assuming that label capable of being leached out of the tissue within the first 20 min is apoplastic and that after 20 min predominately symplastic label is being removed (inferred from the presence of labelled materials in addition to [^{14}C]LA in the wash solution, Fig. 2b), a rough estimate of the amount of apoplastic [^{14}C]LA can be made. The proportion of apoplastic [^{14}C]LA was

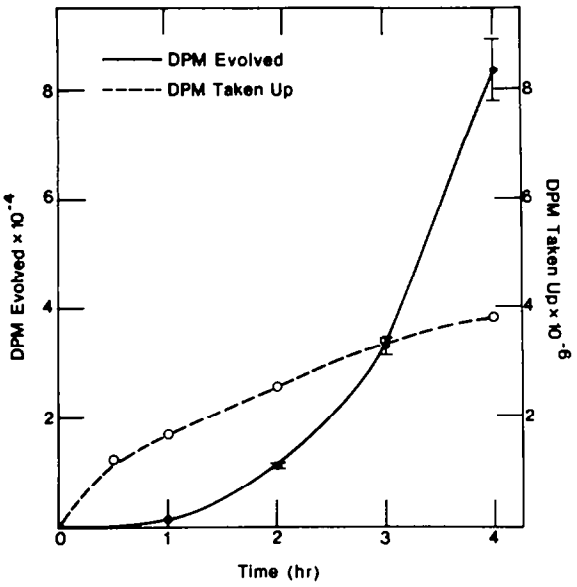

Fig. 1. Evolution of $^{14}CO_2$ and uptake of label by etiolated barley shoot segments incubated with [4-^{14}C]LA in darkness. Evolved $^{14}CO_2$ and the amount of label taken up were determined at the designated points. Values represent the mean of three determinations; s.e. are indicated with bars.

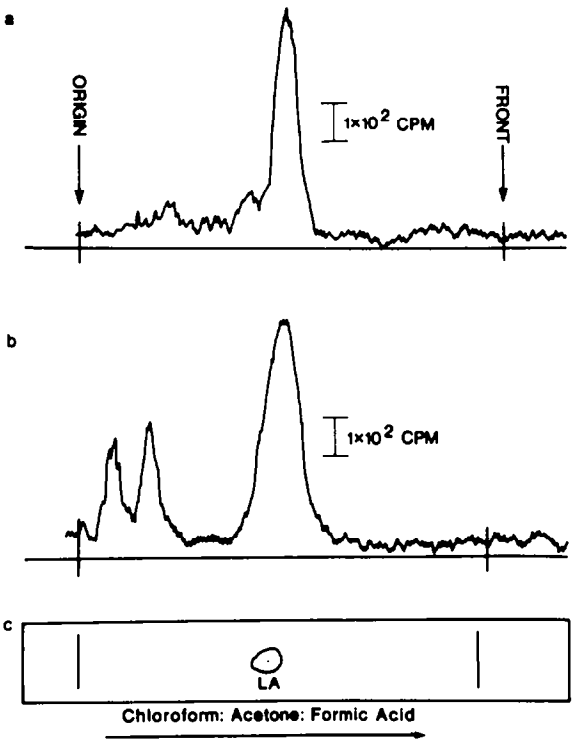

Fig. 2. Thin-layer radiochromatograms of material leached from etiolated barley shoot segments by [^{12}C]LA treatment. (a) Thin-layer radiochromatogram of the first 20-min wash. (b) Thin-layer radiochromatogram of the pooled wash solutions taken between 60 to 180 min. (The results obtained with etiolated barley shoots incubated with [^{14}C]LA for 2 hr were essentially the same as for the 4 hr incubation.) (c) Thin-layer radiochromatogram of authentic [^{12}C]LA.

determined by dividing the total amount of radioactivity in the 20-min wash solution by the amount of [^{14}C]LA absorbed by the tissue. Using this approach, apoplastic [^{14}C]LA was estimated to comprise 14% of the label taken up after 2 hr and 8.6% after 4 hr: the 4-hr estimate is lower than the 2-hr estimate because more [^{14}C]LA is taken up by the tissue during a 4-hr incubation.

Chemical and environmental effects on [4-^{14}C]LA catabolism

Treatments which block respiration, e.g. malonic acid, sodium arsenite, sodium azide and anaerobiosis, profoundly reduced $^{14}CO_2$ production from [4-^{14}C]LA (Table 1). Citric acid cycle intermediates also diminished $^{14}CO_2$ evolution but to a lesser extent than the respiratory antagonists (Table 1).

The catabolism of isoleucine, leucine and valine to CO_2 is inhibited by LA [9]. For this reason, and since LA is itself an aliphatic acid, it seemed plausible that these compounds might have catabolic steps in common with LA. However, of these three amino acids, only isoleucine diminished $^{14}CO_2$ production from [4-^{14}C]LA (Table 1). Catabolic intermediates derived from these amino acids inhibited to varying degrees (Table 1). Those intermediates more metabolically removed showed the greatest effect, viz. propionate, methyl malonate and tiglate, except for β-aminoisobutyric acid, which showed little effect.

These results suggested that reactions similar to those found in fatty acid oxidation may be involved in LA catabolism. Therefore, various short-chain fatty acids, C-4 to C-6 in length (i.e. those which approximate the size and structure of LA), were added to tissue along with [^{14}C]LA. All of the compounds tested effectively reduced $^{14}CO_2$ production from [4-^{14}C]LA (Table 1). Of particular interest was the observation that 4,6-dioxoheptanoic acid (DA), which is a structural analogue of LA and ALA, and, like LA, an inhibitor of the enzyme ALA-dehydratase [9], greatly reduced $^{14}CO_2$ production from [4-^{14}C]LA (Table 1).

Since ALA accumulates in etiolated leaves subjected to LA treatment under illumination, it was of particular interest to determine the effect which ALA treatment and illumination have on LA metabolism in barley leaves. Each of these treatments—ALA at 10 or 15 mM and incubation in the light—leads to substantially increased levels of $^{14}CO_2$ production from [4-^{14}C]LA (Table 1).

Two amino acids, threonine and methionine, were found to slightly stimulate $^{14}CO_2$ production from [4-^{14}C]LA (Table 1).

The fractionation of tissue incubated with [^{14}C]LA

To determine the metabolic fate of LA, etiolated barley shoot segments were incubated with [^{14}C]LA for up to 4 hr under various conditions and then fractionated. The results are shown in Tables 2–4.

LA metabolism during and after the lag period. The lag period observed in the catabolism of [4-^{14}C]LA to $^{14}CO_2$ is between 1 and 2 hr long (Fig. 1). An analysis of LA metabolism during this period could reveal early, i.e. proximal, metabolites. For this reason, etiolated barley leaves were incubated with [4-^{14}C]LA for 90 min and the tissue fractionated. The acidic fraction derived from these leaves contained a significantly greater amount of label

Table 1. The effect of various treatments on the oxidation of the C-4 of LA to CO_2 by etiolated barley shoots

Treatment	Concentration (mM)	% of control*
Malonic acid	15	24
Sodium arsenite	15	8.9
	1.5	25
	0.15	76
Sodium azide	1.5	6.7
Anaerobiosis		6.1
Acetic acid	15	53
Pyruvic acid	15	62
Citric acid	15	80
α-Ketoglutaric acid	15	63
Succinic acid	15	61
Fumaric acid	15	78
L-Malic acid	15	75
Aspartic acid	15	58
Isoleucine	15	74
α-Keto-β-methyl-*n*-valeric acid	15	67
Tiglic acid	15	22
Propionic acid	15	12
	1.5	47
Methyl malonic acid	15	18
	1.5	69
β-Aminoisobutyric acid	15	93
Valine	15	105
β-Ketoisovaleric acid	15	102
Isobutyric acid	15	36
Leucine	15	100
α-Ketoisocaproic acid	15	109
Isovaleric acid	15	16
	1.5	40
β-Hydroxy-β-methyl glutaric acid	15	40
ALA†	10	153
	15	126
DA‡	15	15
	1.5	34
Caproic acid	15	3.4
	1.5	31
Valeric acid	15	2.1
	1.5	23
Butyric acid	15	5.7
	1.5	28
Acetoacetic acid	15	48
	1.5	97
Threonine	15	119
Methionine	15	113
Incubation in the light		167

*The effect of a particular treatment was determined by dividing the ratio of $^{14}CO_2$ evolved to [4-^{14}C]LA taken up for that treatment by the same ratio determined for the control. Values were calculated from the mean of two to three determinations.

†5-Aminolevulinic acid.

‡4,6-Dioxoheptanoic acid.

than after a 4-hr incubation (Table 2); chromatographic analysis revealed that a greater proportion of this label remained in unreacted LA (data not shown). The label content of the other fractions was the same or lower (Table 2).

Effect of anaerobiosis. Incubation of etiolated barley leaves in the absence of O_2 resulted in strong inhibition of the uptake and metabolism of [4-^{14}C]LA (Tables 1 and 2). The acidic and organic fractions derived from these leaves contained a larger proportion of label than from

Table 2. The incorporation of ^{14}C from [4-^{14}C]LA into various chemical constituents by etiolated barley shoots in darkness in the presence or absence of O_2

	Aerobic				Anaerobic	
	1.5-hr incubation Label incorporated		4-hr incubation Label incorporated		4-hr incubation Label incorporated	
	dpm × 10^{-6}*	%	dpm × 10^{-6}	%	dpm × 10^{-6}	%
Total incorporation	3.82	100	4.26	100	2.87	100
$^{14}CO_2$	0.0052	0.14	0.0801	1.9	0.0039	0.14
Basic fraction	0.250	6.5	1.02	24.0	0.025	0.88
Acidic fraction	2.61	68.3	2.23	52.3	1.94	67.5
Neutral fraction	0.0675	1.8	0.130	3.1	0.112	3.9
Organic fraction	0.236	6.2	0.240	5.6	0.313	10.9
Biopolymer	0.00943	0.25	0.0622	1.46	0.00184	0.064
Other volatile†	0.642	16.8	0.451	10.6	0.477	16.6

*Values represent the mean of three determinations.
†The 'other volatile' fraction includes all label unaccounted for in the experiment.

control leaves. The partitioning of [4-^{14}C]LA between the aqueous and organic fractions is 84% and 16%, respectively. This distribution is closer to that observed in such fractions derived from leaves treated with [^{14}C]LA in the absence of O_2 than from those treated aerobically (Table 2), an indication that most of the label in the former is probably in unreacted LA (see below). This suggests that an O_2-dependent step is required for LA metabolism.

Effect of a short-chain fatty acid. Valeric acid, an effective inhibitor of $^{14}CO_2$ evolution from [4-^{14}C]LA at 1.5 mM (Tables 1 and 3), had no effect upon the uptake of [^{14}C]LA or upon the amount of label found in the organic fraction (Table 3). However, it significantly reduced the proportion of label recovered in the basic, biopolymer, $^{14}CO_2$, and volatile fractions (Table 3). This reduction was offset by a corresponding increase in label appearing in the acidic and neutral fractions (Table 3). Furthermore, the acidic fraction obtained from tissue pretreated with this fatty acid was found to contain a significantly greater proportion of unreacted LA, relative to the amount of other acidic components, than control tissue (data not shown). Similar results were observed when caproic acid was substituted for valeric acid (data not shown), suggesting that these fatty acids may compete with LA at some biochemical step, possibly one early in LA metabolism.

Irradiation. The amount of label taken up and $^{14}CO_2$ evolved by irradiated tissue is greater than from tissue incubated in darkness (Table 4). Yet, the distribution of label among the various cellular fractions is similar under both conditions. Small differences are observed in the basic and biopolymer fractions: the former has a slightly lower proportion of label in irradiated tissue while the latter has somewhat more.

Analysis of the acidic fraction. Two-dimensional TLC of the acidic fraction derived from [4-^{14}C]LA-treated leaves indicated the presence of a number of labelled components (Fig. 3). In addition to LA, citric, malic and succinic acids could be identified on this chromatogram; this suggests that LA is metabolized to citric acid cycle intermediates. Of all the labelled compounds only one besides LA was significantly labelled in the absence of O_2: spot 16. LA was estimated to comprise from 27 to 84% of

Table 3. The incorporation of ^{14}C from [4-^{14}C]LA into various chemical constituents by etiolated barley shoots treated with valeric acid*

	Control		1.5 mM Valeric acid	
	Label incorporated		Label incorporated	
	dpm × 10^{-6}	%	dpm × 10^{-6}	%
Total incorporation	3.99	100	3.94	100
$^{14}CO_2$	0.0502	1.26	0.0116	0.29
Basic fraction	0.726	18.2	0.308	7.8
Acidic fraction	1.86	46.5	2.61	66.2
Neutral fraction	0.152	3.8	0.262	6.6
Organic fraction	0.332	8.3	0.333	8.4
Biopolymer	0.0445	1.1	0.0122	0.3
Other volatile	0.829	20.8	0.406	10.3

*See Table 2 for further details.

Table 4. The incorporation of ^{14}C from [4-^{14}C]LA into various chemical constituents by etiolated barley shoots in darkness or under illumination*

	Dark		Light	
	Label incorporated		Label incorporated	
	dpm × 10^{-6}	%	dpm × 10^{-6}	%
Total incorporation	3.40	100	4.06	100
$^{14}CO_2$	0.0458	1.35	0.0916	2.26
Basic fraction	0.628	18.5	0.670	16.5
Acidic fraction	1.52	44.7	1.82	44.8
Neutral fraction	0.00688	0.20	0.00859	0.21
Organic fraction	0.133	3.9	0.162	4.0
Biopolymer	0.0248	0.73	0.0656	1.62
Other volatile	1.04	30.7	1.25	30.8

*See Table 2 for further details.

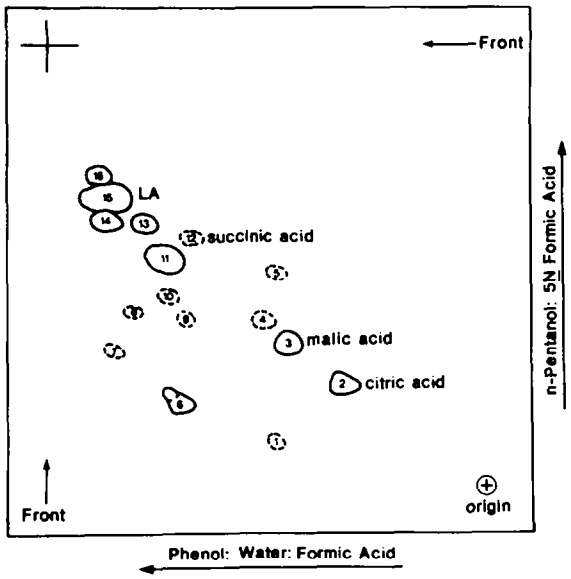

Fig. 3. Diagrammatic representation of an autoradiogram prepared after two-dimensional TLC of the acidic fraction obtained from etiolated barley shoot segments incubated with [4-^{14}C]LA. Solid circles represent spots with high activity, dashed circles represent spots with low activity (less than 5 × background).

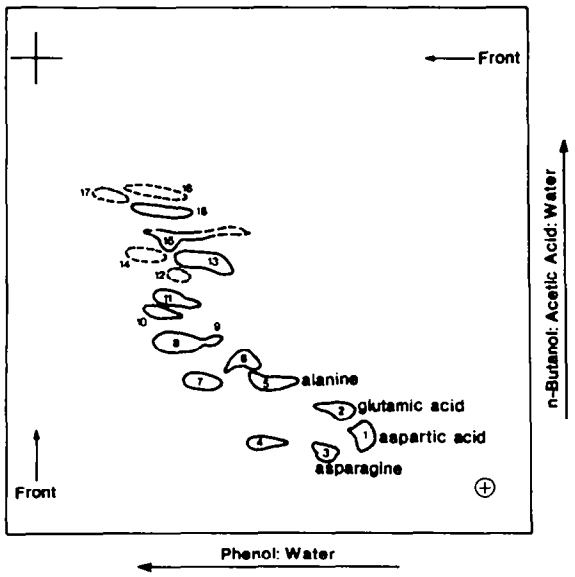

Fig. 4. Diagrammatic representation of an autoradiogram prepared after two-dimensional TLC of the basic fraction obtained from etiolated barley shoot segments incubated with [4-^{14}C]LA. Solid circles represent spots with high activity, dashed circles represent spots with low activity (less than 5 × background).

the acidic fraction, depending upon treatment (data not shown). In each instance, LA contained more label than any other component.

Analysis of the basic fraction. Two-dimensional TLC of the basic fraction also indicated the presence of a number of labelled components (Fig. 4). Aspartic and glutamic acids, asparagine and possibly alanine could be identified; the presence of aspartic and glutamic acids was confirmed by TLE (thin-layer electrophoresis) in one dimension followed by TLC in the second dimension (data not shown). All basic fractions which were examined exhibited the same two-dimensional chromatographic distribution as observed in Fig. 4.

ALA formation from LA

The finding that [4-^{14}C]LA is metabolized to citric acid cycle intermediates (see above), and that the citric acid cycle provides precursors for ALA biosynthesis [1, 8], suggested that LA metabolism could be a significant factor in tracer experiments in which LA is used to study ALA biosynthesis. To determine the contribution of LA metabolism to ALA formation, leaf tissue was incubated under illumination for 4 hr with 4 μCi of [4-^{14}C]LA (20 mCi mmol^{-1}, 0.2 mM) in the presence of 5 mM DA or with 11 μCi of [4-^{14}C]LA (0.73 mCi mmol^{-1}, 15 mM). The results demonstrate that less than 0.1% of the label

Table 5. The incorporation of the C-4 of LA into ALA

Treatment	Radioactivity in extract dpm × 10^{-6}	Radioactivity in ALA dpm × 10^{-3}	Radioactivity in ALA % of Total
[4-^{14}C]LA + DA	5.33	4.21	0.08
15 mM [4-^{14}C]LA	2.82	1.12	0.04

incorporated from [4-^{14}C]LA is recovered in ALA (Table 5). It is concluded that LA metabolism does not contribute significantly to ALA biosynthesis.

DISCUSSION

The kinetics of levulinic acid (LA) uptake by etiolated barley leaves is quite different from that of its oxidation to $^{14}CO_2$ (Fig. 1). Furthermore, less than 15% of the LA absorbed during a 2- or 4-hr incubation was estimated to be apoplastic. Hence, it is not clear why the onset of rapid $^{14}CO_2$ evolution occurs after a long lag period, a lag period which is not observed for LA uptake. A similar lag period was observed when these leaves were fed [1-^{14}C]LA [17]. These data suggest that the rate of LA metabolism to $^{14}CO_2$ is not limited by its absorption into the tissue.

The catabolism of LA to CO_2 appears to include the citric acid cycle. This conclusion is drawn from the following observations: (a) the acidic fraction of [4-^{14}C]LA-treated tissue contains labelled citric, malic and succinic acids (Fig. 3); (b) the basic fraction contains labelled asparagine, aspartic and glutamic acids (Fig. 4); (c) when etiolated barley tissue was incubated with [4-^{14}C]LA in the presence of respiratory inhibitors or intermediates of the citric acid cycle, $^{14}CO_2$ production was reduced (Table 1). Furthermore, the acidic fraction of leaves fed [4-^{14}C]LA under anaerobic conditions did not contain detectable amounts of labelled citric, malic or succinic acids (data not shown).

Incubation of etiolated barley leaves with short-chain fatty acids effectively reduced the evolution of $^{14}CO_2$ from [4-^{14}C]LA (Table 1). Since these compounds are structural analogs of LA, this result suggests that, in addition to involving the citric acid cycle, LA metabolism may include reactions similar to those found in fatty acid oxidation.

It is unlikely that these fatty acids exert their effect on LA metabolism solely through precursor dilution of the citric acid cycle pools for two reasons: (i) they are more effective inhibitors of CO_2 production from LA than acetic or pyruvic acid or any of the citric acid cycle intermediates tested (Table 1), and (ii) even though etiolated barley shoots take up more caproic acid than succinic acid, a smaller proportion of the former is metabolized to CO_2 [unpublished observations]; this suggests that exogenous succinic acid can dilute the pools of citric acid cycle intermediates more than caproic acid.

The acidic fraction obtained from tissue incubated with [4-^{14}C]LA in the presence of valeric (or caproic) acid contained a greater proportion of the incorporated label than in control tissue (Table 3). Within this fraction, less label was found in citric and malic acids, and more in LA, than in control tissue (data not shown). The greater proportion of LA in the acidic fraction suggests that fatty acids retard reactions early in LA metabolism. Thus, they may compete with LA for one or more enzymes.

The stimulation of $^{14}CO_2$ production *in vivo* from [4-^{14}C]LA by ALA and by illumination (Table 1) is of particular interest because ALA accumulates in irradiated tissue in the presence of excess [^{12}C]LA. This suggests that, when LA is used as an *in vivo* inhibitor of ALA dehydratase, illumination and/or the resultant *in vivo* accumulation of ALA might accelerate LA metabolism, resulting in more acyl-CoA compounds entering the citric acid cycle and increasing the pool size of ALA precursors, i.e. of α-ketoglutaric and glutamic acid [20]. However, the possibility that LA metabolism might significantly alter the ALA pool is quite remote since only a small amount of label was detected in ALA produced by irradiated barley tissue incubated with [4-^{14}C]LA (Table 5). These results agree with two earlier reports [17, 21] which indicated that LA was not a direct precursor of ALA.

At a high concentration, 15 mM, one which effectively inhibits greening in barley [9], LA metabolism was found to be qualitatively similar to that at a low concentration, 0.2 mM, although to a proportionately lower extent (data not shown). It is conceivable, therefore, that at concentrations which inhibit ALA metabolism, LA catabolism could make a substantial contribution to the pool-size of citric acid cycle intermediates and, consequently, influence the distribution of label within intermediates derived from precursors being evaluated in tracer experiments. However, since this ketoacid is a relatively poor precursor of ALA (Table 5), the contribution of LA to the pool sizes of citric acid cycle intermediates is probably not significant in such tracer experiments.

Although the metabolic fates of the C-1 and C-4 of LA have elements in common, there are important differences: (a) the length of the lag period in CO_2 evolution from the C-1 is shorter than that observed for the C-4 (*cf.* [17] with Fig. 1); (b) the proportion of CO_2 produced from the C-1 is greater than from the C-4 (*cf.* [17] with Fig. 1); (c) major differences in the distribution of label among the various fractions were observed between etiolated and greening barley tissue for the C-1 but not the C-4 (*cf.* [17] with Table 4); (d) ALA and illumination stimulated $^{14}CO_2$ production from [1-^{14}C]LA to a greater extent than for [4-^{14}C]LA (*cf.* [17] with Table 1); (e) the distribution of label within the acidic fraction differed between [1-^{14}C]LA-fed and [4-^{14}C]LA-fed leaves (*cf.* [17] and [22] with [23]).

In conclusion, it has been shown that etiolated and greening barley tissue can incorporate the C-4 of LA into CO_2 and a number of cellular constituents, and that this metabolism may involve fatty acid oxidation and the operation of the citric acid cycle.

EXPERIMENTAL

Purification and isolation of LA. Radiolabelled LA was diluted in MeOH and purified by TLC on silica gel (Merck, 0.1 mm) with $CHCl_3$–Me_2CO–HCO_2H (80:20:1). The plates were scanned and the radiolabelled peak corresponding to [^{14}C]LA was identified by reference to [^{12}C]LA, $R_f = 0.47$. The latter was visualized with 0.1 N $AgNO_3$–0.1 N NH_4OH (1:1).

Purified [^{14}C]LA was stored in the dry state on the chromatograms. When needed, it was eluted from the labelled zone with Me_2CO–MeOH (4:1), and the silica slurry was suction-filtered through an asbestos pad. The filtrate was taken to dryness and then brought to volume with H_2O for radioassay. Purity was

ascertained by TLC and found to be greater than 95%. Periodic reassays indicated that [^{14}C]LA remained stable on the dry chromatogram.

Growth and preparation of plant tissue. Seeds of *Hordeum vulgare* L. var. 'larker' (Field Seed Farm; Byron, MN) were germinated and grown in darkness for 7 days, and then the apical 5 cm of the shoots was harvested according to the method of Duggan *et al.* [17, 24]. All manipulations involving living material were performed under a dim green safelight unless otherwise stated.

One gram samples of tissue were placed into 125-ml Erlenmeyer flasks containing 0.1 M Pi buffer (pH 3.0) plus the indicated chemical. The flasks were stoppered, and the samples were preincubated for 30 min at 22 ± 1° in darkness. At zero time, 4 μCi of [4-^{14}C]LA (20 mCi mmol^{-1}) was added, bringing the final volume to 1.0 ml. Incubations were carried out at 22 ± 1° in darkness for 4 hr unless otherwise stated. Illumination, when provided, was at 12 W m^{-2} [17].

Measurement of $^{14}CO_2$ evolution in vivo. Respired $^{14}CO_2$ was captured on hanging filter paper discs satd with 2 N KOH. The discs were removed periodically and replaced as previously described [25]. The amount of captured radiolabel was estimated using a Packard PRIAS Liquid Scintillation Counter (Model 240 CL/D) in the dpm mode.

At the end of the incubation, the incubation medium was diluted, and the tissue rinsed with an excess of H_2O. Aliquots of the diluted medium were analysed for radioactivity to estimate the amount of [^{14}C]LA which was not absorbed by the tissue [17].

Determination of apoplastic and symplastic LA. Etiolated barley shoot segments were incubated with [^{14}C]LA in darkness for 2 or 4 hr. The samples were rinsed with H_2O, strained through cheesecloth under reduced light, and the amount of unabsorbed [^{14}C]LA in the rinse soln determined. The tissue segments were blotted with absorbent paper and transferred to glass cylinders, one aperture of which was covered with a layer of cheesecloth. These 'straining' tubes were inserted into larger tubes containing 10 ml of 2.5 mM [^{12}C]LA. The nested tubes were then placed in a shaking water-bath at 30°, and, at 20-min intervals, the straining tubes were drained and transferred to fresh solns of 2.5 mM [^{12}C]LA [26]. Aliquots of the washes were taken for radioassay.

The first 20-min wash was taken to dryness by rotary evaporation and the residue resuspended in 1.0 ml of H_2O. The washes taken between 60 and 180 min were pooled, dried, and the residue resuspended in 1.0 ml of H_2O. Aliquots from each of these two samples were analysed by TLC as described above for LA.

Tissue fractionation. Tissue was fractionated into various fractions according to the method of Duggan *et al.* [17, 24]. Under reduced illumination, leaf segments were separated from the rinse soln by straining through cheesecloth, then blotted and pulverized under liquid N_2 with a mortar and pestle. The ground tissue was extracted with MeOH–$CHCl_3$ (2:1) followed by MeOH–$CHCl_3$–H_2O (2:1:0.8) as previously described [17, 24]. The washed pellet, referred to as the 'biopolymer', was dried *in vacuo* and radioassayed [17, 24]. The soluble fraction was further separated into the 'aqueous' and 'organic' fractions by the addition of a 0.26 vol each of H_2O and $CHCl_3$ [17, 24]. The aqueous fraction was applied to a Dowex-50 × 8 resin column, and the column was washed with 50% MeOH. Basic compounds held by the resin were eluted with 0.5 M NH_4OAc. This 'basic fraction' was taken to dryness and the residue dissolved in 50% MeOH. The methanolic effluent was adjusted to pH > 7.0, applied to a column of Dowex-1 × 8 resin, and the column washed with 50% MeOH. The unbound material is referred to as the 'neutral fraction'. The bound acidic compounds, comprising the 'acidic fraction', were eluted with MeOH–HOAc–H_2O (1:1:1), the effluent taken to dryness and the residue dissolved in H_2O. An aliquot of each fraction was taken for radioassay.

TLC and TLE. Components of the acidic fraction were separated by two-dimensional TLC on cellulose (Merck, 0.1 mm). The chromatogram was developed in the first dimension with PhOH–H_2O–HCO_2H (75:25:1), dried, and then developed in the second dimension with *n*-pentanol–5 N HCO_2H (1:1). Organic acid standards were chromatographed in parallel and visualized with 0.1 N $AgNO_3$–0.1 N NH_4OH (1:1).

Components of the basic fraction were also separated by two-dimensional TLC on cellulose. The chromatogram was developed in the first dimension with PhOH–H_2O (72:28), dried, and then developed in the second dimension with *n*-BuOH–HOAc–H_2O (4:1:1).

The basic fraction was also subjected to TLE (thin-layer electrophoresis) [17]. Samples were applied to 20 × 20-cm glass-supported cellulose TLC plates (Merck, 0.1 mm) 1 cm from one edge and midway between two opposite edges. The cellulose layer was satd with 50 mM sodium 2-[N-morpholino]ethanesulphonate buffer (pH 6.0) and subjected to electrophoresis for 45 min at 32 V cm^{-1} in a Brinkmann Desaga TLE chamber cooled to 4°. The plate was allowed to dry and then chromatographed in the second dimension with *n*-BuOH–HOAc–H_2O at 22 ± 1°. Amino acid standards were run in parallel to both two-dimensional chromatographic techniques and visualized with ninhydrin.

Autoradiography. Chromatograms were overlaid with a sheet of Kodak X-Omat RP X-ray film and held at −80° for 1 month. The resulting autoradiograms were developed according to the manufacturer's specifications.

Isolation of ALA. The incorporation of [4-^{14}C]LA into ALA was determined under two sets of conditions. In the first, etiolated barley shoots were incubated with 4 μCi of [4-^{14}C]LA (20 mCi mmol^{-1}, LA = 0.2 mM) in the presence of 5 mM 4,6-dioxoheptanoic acid (DA). In the second condition, the etiolated shoots were incubated with 11 μCi of [4-^{14}C]LA (0.73 mCi mmol^{-1}, LA = 15 mM). Both incubations were carried out under illumination for 4 hr. The tissue was then rinsed and collected, and the amount of [^{14}C]LA which was not absorbed was determined.

Treated tissue was frozen in liquid N_2 and then ground to a powder with a mortar and pestle. The DA-treated tissue was extracted with 5% TCA according to the method of Harel and Klein [6]. The tissue incubated with 15 mM LA was fractionated as described above to obtain the 'aqueous fraction'. In each case, the pH of the extract was adjusted to 4.25 with 0.2 N sodium citrate buffer. Carrier [^{12}C]ALA was added, and the extracts were applied to Dowex-50 × 8 resin columns. The columns were eluted with 0.2 N sodium citrate buffer (pH 4.25). Collected fractions were assayed for ALA according to the method of Mauzerall and Granick [27].

The fractions which gave a positive colorimetric test for ALA were pooled, acetylacetone was added, and the mixture was heated to form the ALA-pyrrole. After cooling, the soln was extracted × 4 with equal vols of EtOAc, and the radioactivity in the pooled EtOAc washes containing the pyrrole was determined.

Biochemicals. [4-^{14}C]LA (20 mCi mmol^{-1}) was purchased from Amersham Corp., Arlington Hts., IL. ALA (grade I), *n*-caproic acid, [^{12}C]LA (grade I, 98%), sodium 2-[N-morpholino]ethanesulphonate, valeric acid, and amino acids were obtained from Sigma Chemical Co., Saint Louis, MO. DA was obtained from Calbiochem-Behring Corp., La Jolla, CA.

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