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The biosynthesis of δ-aminolaevulinic acid in plants

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In animal and bacterial cells the first enzymic step unique to the tetrapyrrole biosynthetic pathway is the condensation of succinyl-CoA and glycine to yield δ-aminolaevulinic acid (ALA). The enzyme catalysing this reaction could not be detected in extracts from higher plants, or green or blue-green algae. Through the use of laevulinic acid, which competitively inhibits ALA dehydrase, and causes the accumulation of ALA *in vivo*, the ability of a number of specifically labelled ^{14}C radioactive compounds to label this ALA has been studied. Glycine and succinate are poor label donors, whereas α-ketoglutarate, glutamate and glutamine are able to donate ^{14}C to the ALA. Chemical degradation of the [^{14}C]ALA indicates that C_5 arises from C_1 of glutamate and the remaining four carbon atoms of the ALA arise from the remaining four carbon atoms of glutamate. This labelling pattern is incompatible with the succinyl-CoA–glycine condensation reaction and indicates a new pathway for ALA biosynthesis from the intact carbon skeleton of glutamate in greening plant tissues.

In this contribution I will consider the accumulating evidence indicating that δ-aminolaevulinic acid (ALA) is synthetized in plants from the intact carbon skeleton of glutamic acid, via an unexpected new pathway completely different from the succinyl transferase (ALA synthetase) route found in other organisms.

The tetrapyrrole pathway

ALA is the first identified intermediate unique to the tetrapyrrole pathway leading to haem, chlorophyll, vitamin B_{12} and the specialized linear tetrapyrroles found in plants (figure 1*a*). Although no single organism forms all of the end-products, many organisms can synthesize two or more of them. The regulation of such a branched pathway is obviously complex, especially in photosynthetic organisms which synthesize the various end-products in widely varying proportions, depending on physiological and environmental conditions.

Owing primarily to the classical isotope studies of Shemin and his co-workers (Shemin & Russell 1953), ALA was shown to be the precursor of all of the carbon and nitrogen atoms in haem. Later, ALA was also found to be the precursor of chlorophyll in photosynthetic bacteria (Burnham & Lascelles 1963) and in plants (Granick 1961).

ALA synthetase

In many organisms ALA is formed by the condensation of glycine and succinyl-CoA, mediated by the pyridoxal-requiring enzyme ALA synthetase (succinyl-CoA glycine succinyl transferase, EC 2.3.1.37, figure 1*b*). This enzymic activity was first demonstrated in photosynthetic bacteria (Kikuchi, Kumar, Talmage & Shemin 1958) and shortly thereafter in extracts of chicken erythrocytes (Gibson, Laver & Neuberger 1958). ALA synthetase has since

been reported in yeast (Porra, Barnes & Jones 1972), non-photosynthetic bacteria (Tait 1972), avian (Granick & Sassa 1971) and mammalian liver (Marver, Collins, Tschudy & Rechcigl 1966), and bone marrow (Bottomley & Smithee 1968).

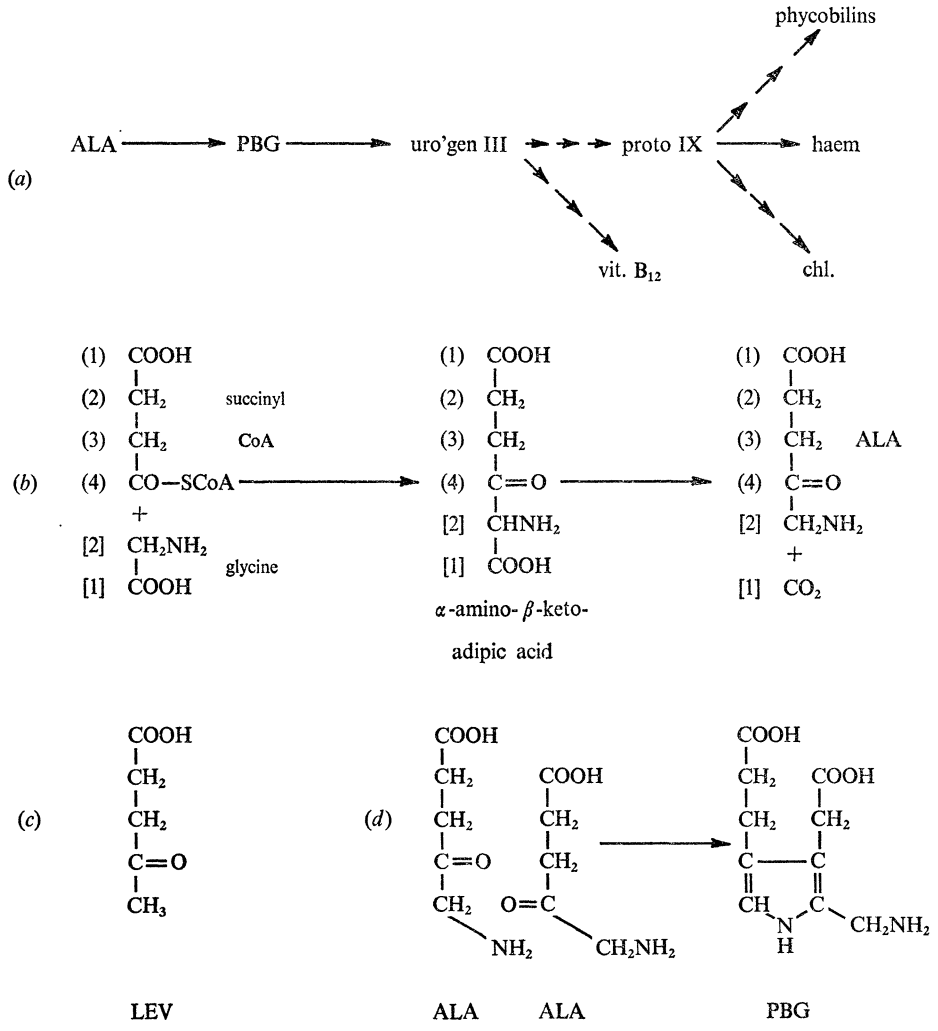

FIGURE 1. (*a*) Outline of the branched tetrapyrrole biosynthetic pathway from ALA – the first identified intermediate unique to the pathway – to the products haem, chlorophyll, vitamin B_{12}, and the plant phycobilins. (*b*) Illustration of the ALA synthetase reaction, which is a condensation of succinyl-CoA and glycine to form the unstable intermediate α-amino-β-ketoadipic acid, which then decarboxylates to yield ALA and CO_2, the CO_2 being derived from the C_1 carboxyl carbon of the glycine. (*c*) The structure of LEV, a competitive inhibitor of ALA dehydrase. (*d*) Illustration of the ALA dehydrase reaction, which is competitively inhibited by LEV, a structural analogue of ALA.

ALA SYNTHETASE IN PLANTS

Evidence for ALA synthetase in plant tissues is meagre and inconclusive. One early abstract claiming detection of the enzyme in spinach leaves was not detailed enough to allow for evaluation, and was never followed by a full report (Miller & Teng 1967). Another report, claiming isolation of the enzyme from cold-stored potato peels, did not adequately guard against the possibility of contaminating micro-organisms in the preparations (Ramaswamy & Nair 1973).

A report of the enzyme from a non-greening soybean callus culture likewise did not rule out possible contamination by micro-organisms (Wider de Xifra, del C. Battle & Tigier 1971). Moreover, since the tissue culture was not green, the relevance of the enzyme to chlorophyll biosynthesis is not apparent.

Because we, and others, have been unable to detect ALA synthetase in extracts from any green plants, including algae, we have considered other possible routes for ALA biosynthesis, and have sought to obtain indirect evidence bearing on ALA synthesis in these organisms.

^{14}C incorporation into chlorophyll

In their isotope studies, Shemin and his co-workers were able to show that in duck erythrocytes, glycine and succinate supply all of the carbon and nitrogen atoms of haem, and that only the aminomethyl carbon of glycine contributes to the haem, while the carboxyl carbon is excluded as is predicted by the ALA synthetase reaction (figure 1*b*; Radin, Rittenberg, & Shemin 1950). Similar experiments were performed with plant tissues in order to determine whether chlorophyll is synthesized from the same precursors as is haem. Such experiments are fraught with difficulties, both technical and interpretive. Chlorophyll is difficult to purify and degrade. Also, certain parts of the chlorophyll molecule arise from sources other than the tetrapyrrole pathway. Chemical hydrolysis is necessary to free chlorophyll from the phytyl and methyl alcohol groups which are added to the molecule after the protoporphyrin IX stage. If these non-tetrapyrrole derived moieties are not removed, ^{14}C-labelling results cannot be unequivocally interpreted.

The first ^{14}C experiments with chlorophyll were performed on the green alga *Chlorella*. The authors reported the incorporation of both carbon atoms of glycine into the chlorin (tetrapyrrole) moiety of chlorophyll (Della Rosa, Altman & Salomon 1953). They also found that acetate is a much more effective label donor than is glycine. Both of these results were surprising, because the ALA synthetase reaction (figure 1*b*) excludes the carboxyl carbon of glycine from ALA, and because glycine would be expected to be a better label donor than acetate, being metabolically closer to the product. Other workers have also reported the incorporation of glycine into chlorophyll in greening plants (Brzeski & Rücker 1960; Wellburn & Wellburn 1971; Roberts & Perkins 1962, 1966), but these workers did not separate the chlorin moiety of chlorophyll from the non-tetrapyrrole derived groups on the molecule, so their results must be interpreted with caution.

In summary, the exclusive incorporation of the aminomethyl carbon of glycine into the tetrapyrrole portion of chlorophyll has not been demonstrated in plants, and therefore these experiments have not supported the ALA synthetase route for ALA formation on these tissues.

ALA accumulation in the presence of LEV

In the course of investigations of chlorophyll synthesis in *Chlorella*, we found that laevulinic acid (LEV), when added to growing cultures, caused the accumulation of ALA in the cells and in the culture medium (Beale 1970, 1971). The structure of LEV, which is identical to ALA except for the absence of the amino group, is shown in figure 1*c*. It is a competitive inhibitor of ALA dehydrase (EC 4.2.1.24, figure 1*d*) in *Rps. spheroides* extracts (Nandi & Shemin 1968) and in *Chlorella* (Beale 1970). When applied at optimal concentrations, LEV had very little effect on

growth rate, and inhibited chlorophyll synthesis by about 50 % (figure 2). However, the total amount of ALA formed (the amount appearing free in the cells and in the medium plus that required for the amount of chlorophyll formed, figure 2; Beale 1970, 1971) was not affected by the LEV. Thus, LEV appeared to function *in vivo* by partially blocking the metabolism of ALA, while not inhibiting the rate of synthesis of ALA or upsetting the biochemistry of the cells in any other noticeable way. This result also supplied direct evidence that the formation of ALA is the rate-controlling step in chlorophyll biosynthesis under the conditions employed. Through the use of [^{14}C]LEV, it was shown that the ALA accumulated in response to LEV, was not formed from the carbon atoms of the LEV, but rather was synthesized *de novo* (Beale 1971).

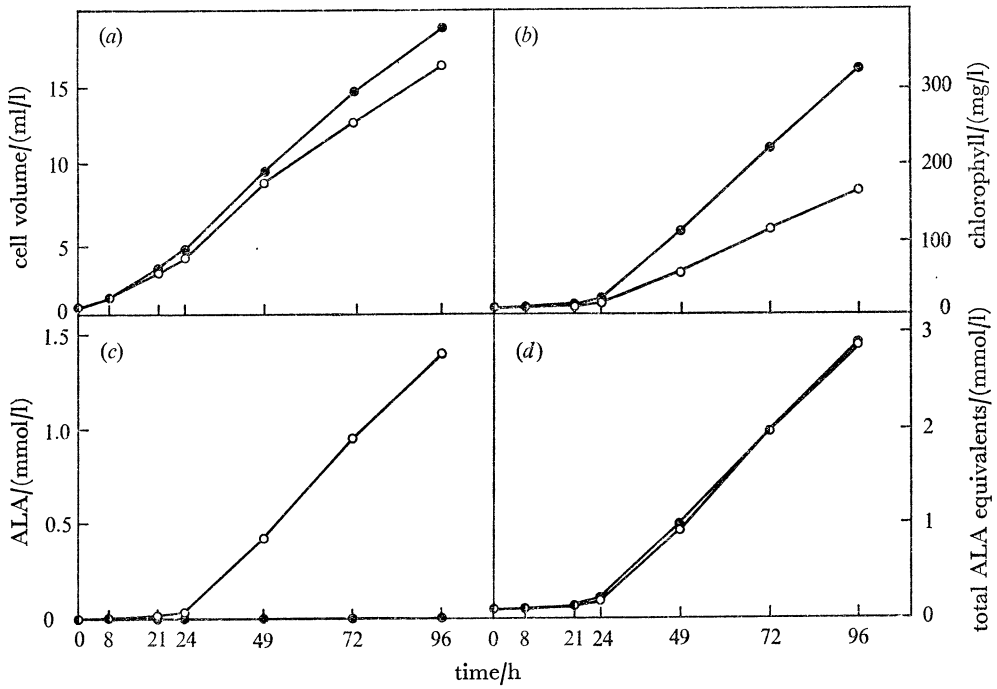

FIGURE 2. Effects of LEV on photoautotrophically growing *Chlorella*. Cultures were inoculated in mineral medium both with (●) and without (○) 10 mM LEV. Cell mass (*a*), chlorophyll (*b*), and ALA content (*c*) were determined at intervals during 96 h of growth in the light. Total ALA synthesized (*d*) was calculated as the molar sum of the ALA content (*c*) plus eight times the chlorophyll content, reflecting the biosynthesis of one molecule of chlorophyll from eight ALA molecules.

We have found that greening aetiolated tissues of higher plants also accumulate ALA in response to treatment with LEV. The tissues employed were bean and barley leaves and cucumber cotyledons (Beale & Castelfranco 1973, 1974*a*). Other workers have reported similar results with corn and bean leaves (Harel & Klein 1972), bacteria (Ho & Lascelles 1971) and *Euglena* (Richard & Nigon 1972).

^{14}C INCORPORATION INTO ALA

After it became possible to cause the accumulation of ALA in greening plant tissues by the administration of LEF, we attempted to label the ALA by incubating greening cucumber cotyledons with LEV plus ^{14}C-labelled presumed precursors of ALA: glycine or succinic acid (Beale & Castelfranco 1973, 1974*b*). Not only were these compounds unable to label the ALA to an appreciable extent, but both C_1 and C_2 of glycine contributed label equally (table 1),

TABLE 1. INCORPORATION OF ^{14}C FROM EXOGENOUS COMPOUNDS INTO ALA BY GREENING CUCUMBER COTYLEDONS IN THE PRESENCE OF 100 mM LEV PLUS 10 μCi OF THE INDICATED ^{14}C COMPOUNDS

(Pooled results from five separate experiments. All incubation times were 4 h.)

radioactive compound applied	molar radioactivity μCi/μmol	accumulated ALA nmol/g fresh tissue	accumulated ALA (counts/min)/(10^6 counts/min applied × g fresh tissue)
[1-^{14}C]glycine	46.5	289	12
[2-^{14}C]glycine	36.7	188	13
[2-^{14}C]glycine	36.7	277	16
[1,4-^{14}C]succinate	20.4	253	48
[U-^{14}C]L-proline	200	272	56
[^{14}C]formate	52.5	320	35
[1-^{14}C]glyoxylate	7.4	219	49
[2-^{14}C]glyoxylate	7.4	232	140
[U-^{14}C]acetate	47.5	302	166
[U-^{14}C]L-glutamine	45	258	405
[U-^{14}C]α-ketoglutarate	200	198	229
[U-^{14}C]α-ketoglutarate	200	239	277
[1-^{14}C]DL-glutamate	25	229	232
[1-^{14}C]DL-glutamate	25	274	436
[1-^{14}C]DL-glutamate	5.3	234	231
[3,4-^{14}C]DL-glutamate	55.5	251	262
[3,4-^{14}C]DL-glutamate	55.5	277	308
[3,4-^{14}C]DL-glutamate	55.5	245	491
[3,4-^{14}C]DL-glutamate	14.2	253	392

clearly not as would be predicted by the ALA synthetase reaction (figure 1*b*). When other ^{14}C compounds were used instead of glycine or succinic acid, only the metabolically closely related five-carbon compounds – glutamate, α-ketoglutarate and glutamine – were found to donate appreciable label to the ALA – as much as 30 times more than glycine (table 1). Moreover, both C_1 and C_3–C_4-labelled glutamate were equally able to label ALA (table 1). Glutamate might enter ALA via conversion to α-ketoglutarate, thence to succinyl-CoA by the tricarboxylic acid enzyme complex α-ketoglutarate dehydrogenase, and finally condensation with glycine by ALA synthetase. But in this route, C_1 labelled glutamate would not be expected to label the ALA, because the C_1 is lost at the α-ketoglutarate dehydrogenase step. This result, along with the equal but poor ability of either C_1 or C_2 labelled glycine to label the ALA, suggested that the ALA was being synthesized from the five carbon skeleton of glutamate via a route other than ALA synthetase.

Two other greening plant tissues were studied – bean and barley leaves – and essentially similar results were found as with cucumber cotyledons: glycine labelled in either C_1 or C_2 was a relatively poor label donor to ALA, whereas glutamate, whether labelled in C_1 or C_3 and C_4, was a relatively good label donor to ALA (table 2).

As a control experiment, avian blood, a tissue known to form ALA via ALA synthetase, was incubated with LEV plus [^{14}C]glycine or [^{14}C]glutamate and the accumulated ALA was purified and assayed for radioactivity exactly like the plant extracts. In this case the C_2 of glycine was a much better label donor to ALA than was the C_1 of glycine. Also, only C_3–C_4 labelled glutamate, and not C_1 labelled glutamate, was able to label the ALA, presumably

through conversion to α-ketoglutarate and thence to succinyl CoA (table 2). Thus, avian blood was clearly different from the three tested plant tissues, in its relative ability to utilize the four ^{14}C specifically labelled compounds of table 2 for labelling ALA.

TABLE 2. COMPARISON OF RELATIVE ABILITY OF SPECIFICALLY LABELLED ^{14}C COMPOUNDS TO LABEL ALA ACCUMULATED IN THE PRESENCE OF LEV BY GREENING PLANT TISSUES AND AVIAN BLOOD

(Results for each tissue are compared to the ability of [1-^{14}C]glycine to label ALA.)

radioactive compound applied	cucumber cotyledons	bean leaves	barley leaves	turkey blood
[1-^{14}C]glycine	1.0	1.0	1.0	1.0
[2-^{14}C]glycine	1.1	2.4	2.4	8.1
[1-^{14}C]DL-glutamate	27	6.5	10	0
[3,4-^{14}C]DL-glutamate	27	23	12	1.8

TABLE 3. COMPARISON OF RELATIVE RATES OF $^{14}CO_2$ AND [^{14}C]ALA FORMATION BY GREENING PLANT TISSUES INCUBATED WITH LEV PLUS THE SPECIFICALLY LABELLED ^{14}C COMPOUNDS INDICATED

(The data in each column are normalized with respect to the relative result with [1-^{14}C]glycine.)

	cucumber cotyledons		bean leaves	
radioactive compound applied	ALA	CO_2	ALA	CO_2
[1-^{14}C]glycine	1.0	1.0	1.0	1.0
[2-^{14}C]glycine	1.1	0.9	2.4	0.23
[1-^{14}C]DL-glutamate	27	0.9	6.5	1.25
[3,4-^{14}C]DL-glutamate	27	0.13	23	0.20

We next examined the possibility that our labelling results were due to differential permeability of the plant tissues to the ^{14}C compounds. Greening cucumber cotyledons and bean leaves were incubated with specifically labelled [^{14}C]glycine or [^{14}C]glutamate, and the respired CO_2 was trapped and analysed for radioactivity (Beale & Castelfranco 1974*b*). These compounds were all respired at comparable rates by both tissues (table 3). The relative amounts of ^{14}C incorporation into CO_2 were very different from the pattern of incorporation into ALA, demonstrating that the relative ability of the tissues to use these compounds as ALA precursors was not a reflection of their ability to enter the cells. This result also indicates that the ALA was not labelled by re-fixation of the respired $^{14}CO_2$, because the amount of label appearing in the ALA was not related to the rate of $^{14}CO_2$ formation from the labelled precursors.

In summary, [^{14}C]glycine and [^{14}C]succinate were relatively poor label donors to ALA, whereas glutamate, α-ketoglutarate and glutamine were relatively good label donors to ALA, in greening plant tissues. The relative abilities of these compounds to donate label to ALA was unrelated to their ability to be respired by the tissues. Both C_1 and C_3–C_4-labelled glutamate could donate label to the ALA. Neither C_1 nor C_2 labelled glycine could label the ALA to an appreciable extent. This labelling pattern was quite different from that found in avian blood, a tissue known to contain ALA synthetase. These results indicate that ALA is synthesized in these plant tissues from the five carbon skeleton of glutamate, rather than from glycine and succinate.

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These results have recently been confirmed (Castelfranco & Jones 1975). These authors reported that both chlorophyll and haem that were formed in greening barley were labelled effectively by [1-^{14}C]glutamate and [U-^{14}C]glutamate, while neither [1-^{14}C]glycine nor [2-^{14}C]-glycine were able to donate appreciable label to either chlorophyll or haem.

However, in a report by Porra & Grimme, the ALA of regreening, bleached *Chlorella fusca* was found to be labelled by [1,4-^{14}C]succinate and [2-^{14}C]glycine, but not by [1-^{14}C]glycine, [1-^{14}C]α-ketoglutarate, [5-^{14}C]α-ketoglutarate, [1-^{14}C]glutamate or [5-^{14}C]glutamate (Porra & Grimme 1974). These authors did not examine the relative ability of these compounds to enter the cells. Perhaps different ALA biosynthetic pathways may exist in different plant species, or even in a single species under differing physiological conditions.

Chemical degradation of [^{14}C]ALA from greening plants

In our most recent experiments we have determined the location of the ^{14}C within the ALA which accumulates in greening barley leaves in the presence of LEV plus specifically labelled [^{14}C]glutamate (Beale, Gough & Granick 1975). Five gram samples of etiolated leaves from 7-day-old barley seedlings were exposed to light for 4 h, then, still in the light, they were incubated for 2 h with 6.7 μCi of DL-glutamate labelled with ^{14}C either in C_1 or C_3 and C_4, and 25 mM LEV. The accumulated ALA was purified, then cleaved between C_4 and C_5 with alkaline periodate (Shemin, Russell & Abramsky 1955). The C_5 fragment was isolated as the formaldehyde dimedon derivative, while the C_1–C_4 fragment, as succinic acid, was extracted into ether at acid pH (figure 3*a*).

We found that [1-^{14}C]glutamate labelled the C_5 of ALA predominately, while [3,4-^{14}C]-glutamate labelled the C_1–C_4 portion of ALA almost exclusively (table 4). This result is schematically summarized in figure 3*b*.

From the results of this experiment, and the others discussed earlier, we conclude that in greening aetiolated plant tissues, ALA is formed from the intact carbon skeleton of glutamate, via a new pathway, which does not involve the succinyl transferase reaction of ALA synthetase.

Possible routes of ALA biosynthesis in greening plants

The rather direct involvement of glutamate (or a closely related metabolite) in ALA biosynthesis, indicated by our ^{14}C results, requires that we consider pathways such as those illustrated in figure 4. Some of these have been previously discussed (Tait 1968; Beale & Castelfranco 1974*b*).

The most direct route involves reduction of the C_1 of glutamate, to form the 1-semialdehyde, followed by internal transamination to ALA. There is no evidence for the existence of either of these reactions, and glutamate-1-semialdehyde is not a known metabolite.

Glutamate might condense with carbamyl phosphate to form hydantoin propionic acid. Conversion to imidazole propionic acid by reactions analogous to the reversal of histidine catabolism, followed by opening of the ring, would yield CO_2, NH_3 and ALA. In this pathway the nitrogen of ALA is derived from carbamyl phosphate, but all of the carbons are from glutamate.

Another interesting hypothetical pathway involves cyclization of glutamate-5-semialdehyde to pyrroline carboxylate, the addition of oxygen to form the pyrrolidone ring, then cleavage of

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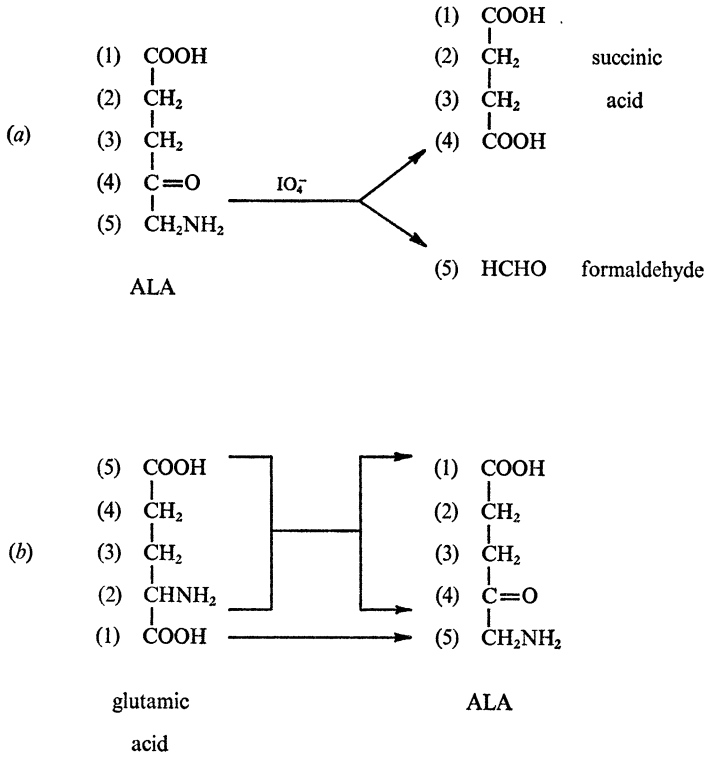

FIGURE 3. (*a*) Illustration of the alkaline periodate oxidation of ALA. The C_4–C_5 bond is broken, and the C_5 fragment is isolated as the dimedon derivative of formaldehyde, while the C_1–C_4 fragment is isolated as succinic acid. (*b*) Summary of experimental results. [^{14}C]glutamate is incorporated into greening plant tissue in the presence of LEV. The C_1 of glutamate is incorporated predominantly into the C_5 of ALA, and the remainder of the glutamate molecule into the remainder of the ALA.

TABLE 4. PERIODATE CLEAVAGE OF ACCUMULATED [^{14}C]ALA

(Greening aetiolated barley leaves were incubated for 2 h with specifically labelled [^{14}C]glutamate plus 25 mM LEV. The accumulated ALA was purified by ion exchange chromatography, then subjected to alkaline periodate oxidation. The C_5 fragment was isolated as the dimedon derivative of formaldehyde, and the C_1–C_4 fragment as succinic acid.)

		total ALA accumulated			periodate cleavage products: C_5 fragment		C_1–C_4 fragment	
source of radioactivity	radioactivity applied count/min	nmol	count/min	% of applied label	count/min	% of label in ALA	count/min	% of label in ALA
[1-^{14}C]DL-glutamate	8300000	723	54400	0.65	41300	76	4100	7.5
	8300000	656	47200	0.57	37400	79	3900	8.3
[3,4-^{14}C]DL-glutamate	7900000	665	65100	0.82	1600	2.4	38600	59
	7900000	706	75300	0.95	1700	2.3	45500	60

the ring by a reaction analogous to the D-proline reductase of *Clostridium* (Hodgins & Ables 1969). However, in this route C_5 of ALA would arise from C_5 of glutamate, and C_1 from C_1. This predicted labelling pattern is inconsistent with that actually found (Beale *et al.* 1975; table 4, figure 3*b*). Therefore this route is ruled out.

Finally, glutamate can be transaminated to α-ketoglutarate, or hydrolytically deaminated to

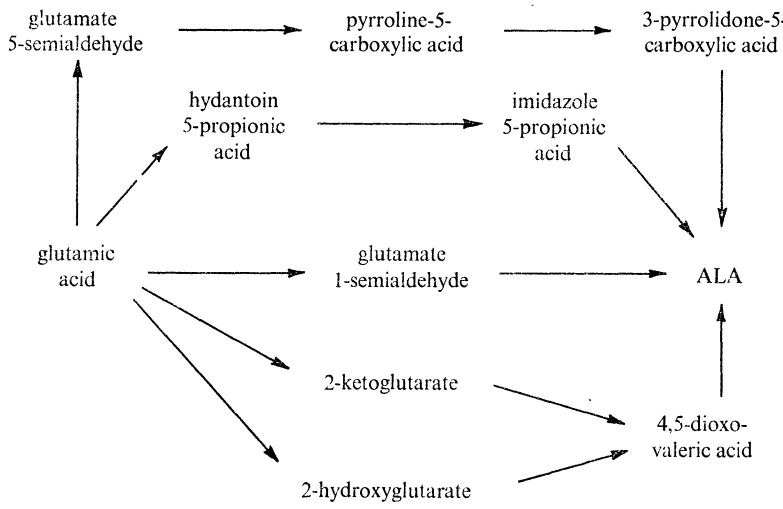

FIGURE 4. Hypothetical pathways for incorporation of the intact carbon skeleton of glutamic acid into ALA.

α-hydroxyglutarate, then converted to 4,5-dioxovaleric acid. The transformation from hydroxyglutarate is analogous to the reversal of the glyoxylase reaction, which transforms methylglyoxal to lactic acid, a reaction involving two enzymes and requiring glutathione as a co-factor (Racker 1951). Transamination of 4,5-dioxovaleric acid to ALA has been reported in extracts of *Chlorella* (Gassman, Plusčec & Bogorad 1968) and photosynthetic bacteria (Neuberger & Turner 1963). The formation of dioxovaleric acid from glutamate, however, has not been reported.

SUMMARY AND CONCLUSION

Evidence has been presented indicating that in greening higher plant tissues, ALA is synthesized from the intact carbon skeleton of glutamic acid, by a new pathway not involving the succinyl transferase reaction of ALA synthetase found in bacteria and vertebrates.

A number of hypothetical routes for the conversion of glutamic acid to ALA in a manner consistent with the observed ^{14}C labelling pattern have been suggested. While none of these pathways have yet been shown to occur, many of the individual reactions are known, and others are analogous to known reactions.

Our present search for an ALA synthesizing system in greening plants will hopefully lead us to an understanding of how ALA is formed, and how the tetrapyrrole pathway of chlorophyll synthesis is regulated, in these organisms.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES