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Alternative Routes for the Synthesis of 5-Aminolevulinic Acid in Maize Leaves[1]

II. FORMATION FROM GLUTAMATE

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ABSTRACT

Intact plastids from greening maize (*Zea mays* L.) leaves converted [^{14}C]glutamate and [^{14}C]2-ketoglutarate (KG) to [^{14}C]5-aminolevulinic acid (ALA). Glutamate appeared to be the immediate precursor of ALA, while KG was first converted to glutamate, as shown by the effect of various inhibitors of amino acid metabolism. Plastids from greening leaves contained markedly higher activity as compared with etioplasts or chloroplasts. The synthesis of ALA by intact plastids was light dependent. The enzyme system resides in the stroma of plastids or may be lightly bound to membranes. The solubilized system showed maximal activity around pH 7.9 and required Mg^{2+}, ATP, and NADPH although dependence on the latter was not clear-cut. A relatively high level of activity could be extracted from etioplasts. Maximal activity was obtained from plastids of leaves which had been illuminated for 90 minutes, after which activity declined sharply. The enzyme system solubilized from plastids also catalyzed the conversion of putative glutamate 1-semialdehyde to ALA in a reaction which was not dependent on the addition of an amino donor.

The system in maize greatly resembled the one which had been reported from barley. It is suggested that this system is the one responsible for the biosynthesis of ALA destined for chlorophyll formation.

Following the demonstration that ALA[2] is synthesized in greening leaves from a five-carbon atom precursor (2, 22), some uncertainties remained concerning the identity of the immediate precursor and the details of the pathway which apparently consisted of several steps (1, 8, 9, 19). Kannangara and Gough described in a series of papers (6, 7, 14–16, 19, 27) a system from barley plastids which was light induced and light driven, used glutamate as substrate, and required Mg^{2+}, ATP, and NADPH as cofactors. They suggested that glutamate was first converted to glutamate 1-P by a kinase-like enzyme, using Mg^{2+} and ATP. The activated glutamate was then reduced to Glu 1-SA by an NADPH-dependent dehydrogenase. Glu 1-SA was claimed to undergo an intramolecular transfer of the amino group catalyzed by an amino transferase. Thus, the conversion of glutamate to ALA should be independent of the addition of an external amino group donor.

The properties of this route—its location, dependence on light, ATP, and NADPH—strongly suggest that it is indeed the biosynthetic route of ALA destined for Chl formation. However, we have observed in studies with greening maize leaves that KG was superior to glutamate as precursor of ALA both *in vivo* and *in vitro* (9, 12, 22). Attempts to demonstrate the glutamate route in maize were unsuccessful (9) and observation with double labeling of ALA with ^{15}N and ^{14}C were also inconsistent with its synthesis by the glutamate route (11). Lohr and Friedman (21) suggested that the synthesis of ALA in maize proceeded from KG through DOVA. Their suggestion seemed to be supported by additional observations (9, 12). Ford and Friedmann (5) later reported that glutamate was the substrate in wheat leaves while Weinstein and Castelfranco concluded the same for cucumber cotyledons (28). However, in the two latter cases the activities reported were rather low and the exact nature of the reactions involved remained unclear.

This work demonstrates that the system described by Kannangara and Gough from barley operates also in maize plastids. Previous failure to demonstrate it was apparently due to difficulties in obtaining intact plastids from greening maize leaves and the marked sensitivity of the enzyme system to inactivation. The work lends further support to the conclusion of a preceding paper (13) that ALA is synthesized in greening maize leaves from glutamate and not from KG and DOVA, and describes some properties of the system. A preliminary report of some of its observations has been published as an abstract (23).

MATERIALS AND METHODS

Chemicals. ALA, LA, AOA, D-glutamate, *N*-acetyl L-glutamate, L-pyroglutamic acid, FCCP, L-albizziine, azaserine, Fd (type III from spinach), and MSX were purchased from Sigma. DL-β-Hydroxyglutamate was supplied by ICN Pharmaceuticals, Cleveland, OH. [5-^{14}C]KG and [U-^{14}C]glutamate were purchased from The Radiochemical Centre, Amersham. Glu 1-SA was synthesized and generously supplied by Dr. C. G. Kannangara, Carlsberg Laboratory, Copenhagen, Denmark. [U-^{14}C]Pyroglutamic acid and [U-^{14}C] *N*-acetyl L-glutamate were a gift from Dr. P. J. Lea, Rothamsted Experimental Station, Harpenden, England. A mixture of partially purified thioredoxin m and f from spinach were kindly donated by Professor Bob B. Buchanan, University of California, Berkeley. α-*N*-Ethyl-L-glutamine (25) was a gift from Professor R. Kulka, The Hebrew University of Jerusalem.

Plant Material. Maize (*Zea mays* L., cv Neve Ya'ar 170) seeds were obtained and grown in the dark and leaves were treated and illuminated as previously described (13). Barley (*Hordeum vulgare*, cv Svalöfs Bonus) seeds were germinated and grown as previously described (11).

Isolation of Subcellular Fractions. Plastids were isolated after

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[2] Abbreviations: ALA, 5-aminolevulinic acid; Glu 1-SA, glutamate 1-semialdehyde; KG, 2-ketoglutaric acid; DOVA, 4,5-dioxovaleric acid; LA, levulinic acid; AOA, aminooxyacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone; MSX, L-methionine DL-sulfoximine; MV, methylviologen; DIT, sodium dithionite.

Table I. *Effect of Inhibitors of Amino Acid Metabolism on the Conversion of Glutamate and KG to ALA by Intact Maize Plastids*

Plastids were isolated from greening leaves. Incubation was carried out in the light at 25°C for 30 min, with 2.91 mg of plastid protein and 0.5 μCi of either [U-^{14}C]glutamate (285 mCi/mmol) or [5-^{14}C]KG (10.7 mCi/mmol) in 0.1 M Tricine buffer (pH 8.0), 0.6 M glycerol, 10 mM $MgCl_2$, 1 mM DTT, and 5 mM LA.

Inhibitor	Concn.	Radioactivity in ALA from	
		[^{14}C]Glutamate	[^{14}C]KG
	mM	*cpm*	
None		10,458[a]	8,909
MSX	5	7,340	8,006
L-Albizziine	10	11,631	5,390
Azaserine	2	14,225	2,832
D-Glutamate	5	8,236	8,226
β-OH glutamate	1	830	1,233
Aminooxyacetate	5	5,007	1,130

[a] Based on the specific radioactivity of the glutamate supplied and the content of cold glutamate in the plastid preparation, 1,000 cpm are equivalent to 0.38 nmol ALA.

Table II. *Synthesis of ALA from Glutamate by Isolated Plastids—The Effects of Light*

Plastids were isolated from either etiolated, greening (17 h low intensity red light followed by 3 h white light) or green (72 h white light) leaves. Incubation was carried out in the light, at 25°C for 30 min with 0.5 μCi [U-^{14}C]glutamate (285 mCi/mmol) as precursor. Other details as in Table I.

	Radioactivity in ALA per	
	Reaction mixture	mg protein h^{-1}
	cpm	
Etioplasts	2,200	880
Chloroplasts	562	67
Etiochloroplasts		
Complete	13,251[a]	5,303
No LA	4,210	
ATP, 1 mM	12,985	
DCMU, 1 μM	5,221	
DCMU, 10 μM	1,025	
FCCP, 5 μM	2,633	
FCCP, 20 μM	1,350	
Incubated in darkness	2,501	

[a] 1,000 cpm are equivalent to 0.32 nmol ALA.

grinding leaves in a modified Waring Blendor equipped with razor blades (13, 18), in 0.1 M Tricine buffer (pH 9.0), 0.6 M glycerol, 1 mM $MgCl_2$, and 1 mM DTT. The plastids (about 70% intact as judged by phase contrast microscopy and retention of the activity of NADP-glyceraldehyde-P dehydrogenase [13]) were either resuspended in the same buffer pH 8.0 for assays with intact organelles or broken in a Yeda Press in buffer with a lower concentration of glycerol (0.3 M) and the 'stroma' preparation obtained as already described (13). Gel filtration was performed by applying 1 ml of the stroma preparation to a mini-column (polypropylene Econo-Columns, Bio-Rad Laboratories) of Sephadex G-25 (Sigma, 50–150 μm particle size, 3 ml bed volume) and by collecting the first 1.3 ml of eluate which followed the void volume.

Formation of ALA. The synthesis of [^{14}C]ALA by intact plastids was assayed in the presence of 0.5 μCi [U-^{14}C]L-glutamate (285 mCi/mmol) or [5-^{14}C]KG (10.7 mCi/mmol), 0.1 M Tricine buffer (pH 8.0), 0.6 M glycerol, 10 mM $MgCl_2$, 1 mM DTT, and 5 mM LA at 25°C for 30 min, while illuminating as previously described (13). Membranes and soluble fractions obtained from lysed plastids were assayed in a reaction mixture containing 0.1 M Tricine buffer (pH 8.0), 0.3 M glycerol, 25 mM $MgCl_2$, 5 mM LA, 1 mM ATP, 1 mM NADPH, and 0.5 μCi of [U-^{14}C]L-glutamate in a total volume of 2 ml, incubating at 25°C for 30 min. The reaction was terminated by the addition of 0.1 ml of 1 mg/ml ALA and 0.1 ml 70% (w/v) $HClO_4$. The isolation of [^{14}C]ALA by ion exchange chromatography and the preparation and purification of the pyrrole by differential extraction and TLC were performed as previ-

Table III. *Extraction and Fractionation of the ALA Synthesizing System from Maize Plastids*

Plastids isolated from greening maize leaves were either frozen in liquid N_2 and thawed, or broken twice in a 'Yeda Press'. Supernatants ('sup') and precipitates were obtained by centrifugation for 30 min, as specified. Gel filtration was performed in mini-columns of Sephadex G-25: 1 ml of the 120,000*g* supernatant was applied to the column and the enzyme fraction was collected in 1.2 ml. The low mol wt fractions were the 1st, 2nd, and 3rd ml of the following eluate. The reaction mixture consisted of 0.1 M Tricine buffer (pH 8.0), 0.3 M glycerol, 25 mM $MgCl_2$, 5 mM LA, 1 mM ATP, 1 mM NADPH, and 0.5 μCi of [U-^{14}C]L-glutamate in a total volume of 2 ml. Incubation was carried out at 25°C for 30 min.

	Radioactivity in ALA	
	mg^{-1} protein h^{-1}	Total
	cpm	
a. Freeze and thaw, 120,000*g* sup	8,261	211,865
b. Yeda Press, 20,000*g*		
sup	22,640	637,546
ppt	7,521	104,339
c. Yeda Press, 120,000*g*		
sup	28,348	397,332
ppt	866	38,210
d. c (sup) gel filtrated	107,168	
+ low mol wt fraction I	42,462	
+ low mol wt fraction II	71,706	
+ low mol wt fraction III	82,704	

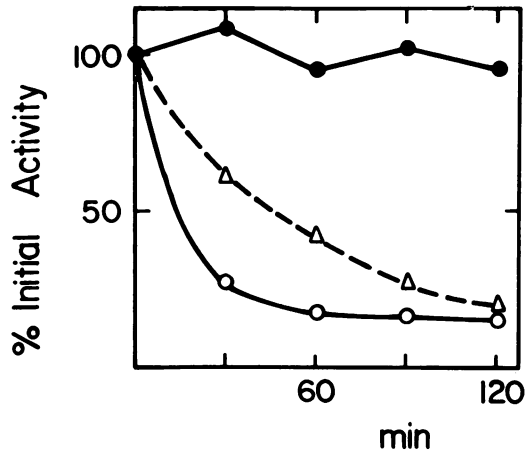

FIG. 1. Inactivation of the ALA synthesizing system at different pH values. Stroma preparations, after gel filtration through Sephadex G-25, were incubated in 0.1 M Tricine-KOH buffer, 0.3 M glycerol, 25 mM $MgCl_2$, and 1 mM DTT at 10°C and pH 9.0 (●), pH 7.9 (Δ), and pH 7.5 (○). Aliquots were withdrawn after various time intervals and the activity determined at pH 8.0, using [U-^{14}C]glutamate as substrate, as described for Table III. Reaction time, 20 min.

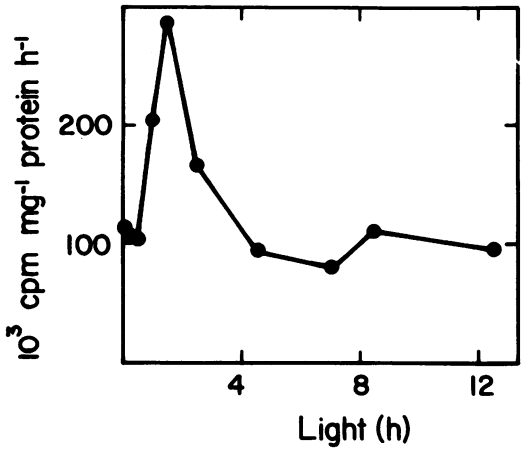

FIG. 2. Effect of the length of preillumination on the activity of ALA synthesizing system in maize plastids.

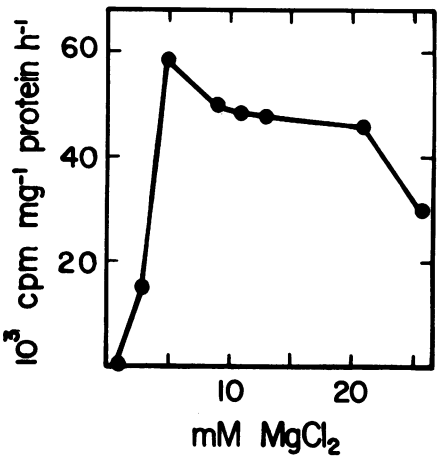

FIG. 3. Effect of Mg ion concentration on the synthesis of ALA from glutamate.

ously described (13). The ATP generating system consisted of 1 mM ATP, 8 mM P-creatine (Sigma), and 8 units of creatine phosphokinase (from rabbit muscle type I, Sigma). Colorimetric assays of ALA formation were carried out in a reaction mixture identical with that described for the radioactive assay for membrane and soluble fractions except that 1 mM L-glutamate was used as substrate. The determination of ALA too, was carried out as described (13) except that carrier ALA was not added upon stopping the reaction. Glu 1-SA aminotransferase was assayed according to Kannangara and Gough (16).

Glutamate. Glutamate was extracted from plastid preparations with boiling 80% ethanol and determined as previously described (11).

Protein. Protein was determined by the Coomassie Brilliant Blue G-250 assay according to Bradford (3), with BSA (Sigma, fraction V powder) as standard.

RESULTS AND DISCUSSION

In a preceding paper (13) we concluded that the conversion of KG to ALA by intact maize plastids was apparently due to their ability to readily convert KG to glutamate. This conversion can take place by several independent enzymes, all of which have been reported to occur in plastids from greening maize leaves (10, 26). Further support of this conclusion was lent by observations on the effect of various inhibitors of amino acid metabolism on the conversion of radioactive KG and glutamate to [^{14}C]ALA by

Table IV. *Effect of ATP and of Inhibitors and Activators of Glutamine Synthetase on the Synthesis of ALA by a Stroma Preparation from Maize Plastids*

	ALA[a]
	cpm mg^{-1} protein h^{-1}
No ATP	578
1 mM	36,788
4 mM	36,296
10 mM	16,634
ATP generating system[b]	44,587
Control (1 mM ATP)	42,208
$(NH_4)_2SO_4$, 10 mM	12,646
EDTA, 5 mM	27,954
Control	41,302
α-*N*-Ethyl-L-glutamine	
0.5 mM	43,270
1 mM	54,784
5 mM	37,920
MSX	
0.01 mM	45,934
0.1 mM	49,470
1.0 mM	53,070

[a] 1,000 cpm are equivalent to 10 pmol ALA formed in the reaction mixture. Assay conditions as in Table III.

[b] One mM ATP, 8 mM P-creatine, and 8 units of creatine phosphokinase.

Table V. *ALA Synthesizing System—The Nature of the Reductant*

Assay conditions as in Table III.

	ALA
	cpm mg^{-1} protein h^{-1}
Control, 120,000*g* sup	22,640
− NADPH	15,476
NADPH generating system[a]	30,768
After gel filtration of enzyme	
Complete	98,754[d]
− NADPH[b]	32,706
MV, DIT, and NADPH	48,797
MV, DIT[c]	48,570

[a] NADP (2 mM) and malic acid (4 mM).

[b] The following cofactors had no effect on the synthesis of ALA in the presence of NADPH, nor could they support ALA formation in its absence: Fd, 100 μg, in light; GSH, 5 mM; Na ascorbate, 5 mM; preincubation with thioredoxin—the enzyme was incubated with a mixture of thioredoxin m and f from spinach, in buffer with 10 μmol DTT for 10 min at 22°C before addition of the other reactants.

[c] 100 μg MV, 1 mg DIT, and 1 mg $NaHCO_3$.

[d] 1,000 cpm are equivalent to 10 pmol ALA formed in the reaction mixture.

isolated plastids (Table I). Inasmuch as there is a large difference in the content of glutamate and KG in etiochloroplasts (E. Harel, unpublished observations), the specific activities of the two precursors had been chosen to give similar rates of [^{14}C]ALA formation in the control assays. MSX, an efficient inhibitor of glutamine synthetase (24), inhibited the formation of ALA from glutamate by 30% and to a lesser extent than from KG. The difference can be explained by an increase in the endogenous pool of glutamate due to prevention of its conversion to glutamine. This would cause dilution of the [^{14}C]glutamate supplied, resulting in a lower level of incorporation into ALA. The difference cannot be due to a

Table VI. *Synthesis of ALA from Glutamate Using a NonRadioactive Assay*

Enzyme after gel filtration. The reaction mixture was identical to that of Table III, except that 1 mM L-glutamate was used as substrate.

	ALA Formation
	nmol mg^{-1} protein h^{-1}
Control	11.4
− NADPH	2.7
− ATP	0.6
− Glutamate	1.0
Boiled enzyme	0.2
0.1 mM glutamate	7.6
5 mM glutamate	8.0
NADPH generating system[a]	7.6

[a] NADP (2 mM) and malic acid (4 mM).

Table VII. *Glu 1-SA Aminotransferase Activity from Greening Maize Leaves*

The incubation mixture contained 0.1 M Tricine buffer (pH 8.0), 0.25 mM DTT, 1 mM Glu 1-SA, and 200–300 μg protein in a final volume of 1 ml. Incubation was carried out at 25°C for 20 min.

Enzyme Source and Reaction Mixture	ALA Formation
	nmol mg^{-1} protein h^{-1}
Roots	0.0
Etiolated leaves	68.7
Green leaves	43.3
Greening leaves (6 h light)	165.6
+ Glutamate, 5 mM	158.9
+ Aspartate, 5 mM	77.4
+ Aminooxyacetate, 2 mM	17.5

direct effect of MSX inasmuch as it does not inhibit ALA synthesis *in vitro* (*cf.* Table IV). L-Albizziine and azaserine, inhibitors of glutamate synthase (26), strongly inhibited the incorporation from KG, while the contribution from glutamate was increased. This increase could again be explained by an effect on the endogenous pool of glutamate. In this case, the pool was probably decreased, thereby increasing the *in situ* specific activity of the radioactive precursor. β-Hydroxyglutamate is an efficient inhibitor of the enzymic system which synthesizes ALA from glutamate in barley leaves (14). It strongly inhibited the formation of ALA from both glutamate and KG (Table I). On the other hand AOA, an antagonist of pyridoxal phosphate, strongly affected the incorporation from [^{14}C]KG to ALA but less so from [^{14}C]glutamate. This could be due to an effective inhibition of the transamination of KG to glutamate as compared to its meager effect on the conversion of glutamate to ALA (14). On the whole, the results shown in Table I strengthen the conclusion that isolated plastids convert KG to ALA through first transforming it to glutamate and not to DOVA (13).

The conversion of glutamate to ALA does not proceed through either *N*-acetylglutamate or pyroglutamic acid (2-pyrrolidone-5 carboxylic acid), which might be suggested to serve as intermediates in some hypothetical routes (2). Although the addition of cold Glu 1-SA (0.1 mM) inhibited the formation of [^{14}C]ALA from [^{14}C]glutamate in both intact plastids and stroma preparations by more than 90%, *N*-acetylglutamate had no effect and pyroglutamate was only slightly inhibitory at the same concentration. The above mentioned enzyme preparations were unable to convert [U-^{14}C]*N*-acetylglutamate (2 μCi/reaction mixture, 0.4 mmol/mCi) or [U-^{14}C]pyroglutamic acid (2 μCi/reaction mixture, 0.86 mCi/mmol) to [^{14}C]ALA.

The activity of the ALA synthesizing system was high in greening plastids and relatively low in etioplasts and in plastids from fully greened leaves (Table II). Omission of LA from the reaction mixture apparently enabled the utilization of ALA for Chl formation and markedly reduced the amount of label found in ALA (Table II). The addition of ATP had no effect on the intact plastid preparation apparently due to its inability to cross the plastid envelope. DCMU, an inhibitor of photosynthetic electron transfer and FCCP, an efficient uncoupler, strongly inhibited ALA formation. The amount of ALA formed by plastids incubated in darkness was markedly lower than the amount synthesized by illuminated ones. The observations reported in Table II suggest that the conversion of glutamate to ALA by isolated maize plastids utilized photosynthetically produced ATP and reducing power and confirm our claim (13) that the plastid preparations used had a high percentage of intact organelles.

Gough and Kannangara (7) reported that the ALA synthesizing system in barley is located in the plastid stroma. However, when maize plastids were broken by freezing and thawing, only 30% of the activity was released to the high-speed supernatant (Table III). A greater part of the activity was solubilized by passing the suspension twice in the Yeda Press. It appeared that the presence of some membrane-bound component inhibited the conversion of glutamate to ALA or acted on the ALA formed since centrifugation at 120,000*g* paradoxically increased the activity measured in the supernatant and decreased that assayed with the precipitate, as compared to centrifugation at 20,000*g* (Table III). Gel filtration of the 120,000*g* supernatant through Sephadex G-25 increased the activity of the preparation, probably by removing an inhibitor, cold glutamate, or both. Addition of the low mol wt fractions obtained from the Sephadex column back to the reaction mixture inhibited ALA formation (Table III). This inhibition could not be explained only by isotopic dilution of the [^{14}C]glutamate inasmuch the increase was observed also in a nonradioactive assay—from 13.6 before to 23.2 nmol ALA mg^{-1} protein h^{-1} after gel filtration.

The rate of synthesis of [^{14}C]ALA from [^{14}C]glutamate by stroma preparations was linearly dependent on protein concentration of up to 2 mg/reaction mixture. ALA accumulated at a constant rate only during the first 20 min of the reaction, after which the rate was markedly slowed down. Maximal activity was observed around pH 7.9. As in many of its other properties, the ALA synthesizing system from maize plastids resembled the system from barley also in the dependence of its stability on pH (17). The enzyme system was stable at pH 9.0 in the presence of Mg^{2+} and DTT but underwent rapid inactivation at pH 7.9 and below (Fig. 1).

The level of solubilized ALA synthesizing activity decreased with the age of the seedlings, following the emergence of leaves from the coleoptiles. Although activity was highest in 8-d-old seedlings, we went on working with 9- to 10-d-old ones because younger leaves are too difficult to harvest. The activity assayed with leaves of identical age and stage of greening was fairly constant. Thus, the mean rate of [^{14}C] ALA formation with 9-d-old leaves in six independent experiments was 37,311 ± 1,992 (SE) cpm mg^{-1} protein h^{-1}. This value was similar to the one obtained with 6-d-old barley leaves, under identical assay conditions—29,160 ± 2,650 (SE, seven experiments). Activity increased sharply during the first 90 min of greening and decreased thereafter (Fig. 2). Surprisingly, etiolated leaves contained a relatively high level of activity, similar to that found after 5 h illumination or longer.

The cell-free system (stroma supernatant after gel filtration) showed an absolute dependence on Mg ions (Fig. 3) and ATP (Table IV). Activity decreased at high concentrations of either cofactor. Maize plastids contain a relatively high level of glutamine synthetase activity (10) which utilizes glutamate as substrate, while requiring Mg^{2+} and ATP as cofactors. It seemed likely that low concentrations of NH_4^+ in the preparation could enable glutamine

synthetase to act, thereby lowering the rate of ALA synthesis. Addition of $(NH_4)_2SO_4$ to the reaction mixture indeed inhibited ALA formation (Table IV). So did EDTA, which causes activation of glutamine synthetase (24). α-*N*-ethyl-L-glutamine (25) and MSX, both inhibitors of the enzyme, enhanced the formation of ALA from glutamate (Table IV).

The conversion of glutamate to ALA required Mg^{2+}, ATP, and NADPH. However, while requirement for the first two was absolute, the dependence on exogenous NADPH was not. Omission of NADPH from a reaction mixture with the 120,000*g* supernatant as enzyme inhibited the reaction by only 30% (Table V). The effect was more pronounced after gel filtration of the enzyme, but even then inhibition was not complete (67%). NADPH could be produced in the reaction mixture from NADP and malate, which together with the NADP-malic enzyme in the preparation (13.1 μmol NADP reduced mg^{-1} protein h^{-1}) could constitute a 'regenerating' system (Table V). It seemed possible that NADPH might contribute to the reaction indirectly and not through being its 'native' reductant. However, ascorbate, reduced MV, GSH, Fd, or a mixture of thioredoxin m and f did not improve the performance of NADPH, nor could they replace it in the reaction (Table V).

ALA formation could be followed also with a nonradioactive assay although the amounts formed were low compared with those expected from the rate of Chl synthesized by the tissue of origin (20). The response of the system to glutamate, ATP, and NADPH conformed with the data obtained by the radioactive assay (Table VI). The reaction continued at a considerable, though progressively diminishing rate, for at least 2 h.

The soluble stroma preparation which converted glutamate to ALA also catalyzed the enzymic conversion of a substance presumed to be Glu 1-SA to ALA, in a reaction which did not require the addition of an amino group donor (Table VII). Glutamate, at 5 mM, did not affect the rate of the reaction while aspartate, at an identical concentration, and AOA (2 mM), were strongly inhibitory. The strong inhibition by aspartate and AOA are in contrast to the properties of the barley enzyme. The pH optima of the two enzymes were similar—pH 8.0 to 8.5. The apparent K_m of the maize enzyme for Glu 1-SA was 1.8 mM (0.4 mM in barley), but both values should be regarded as rough estimates due to uncertainties regarding the purity and stability of the substrate. It is interesting to note that contrary to DOVA transaminase (13), the 'Glu 1-SA aminotransferase' was observed only in leaves and was completely absent from roots (Table VII). Activity in greening leaves was markedly higher than in etiolated or fully greened ones.

It thus appears that the synthesis of ALA destined for the formation of Chl and other porphyrins in plastids, takes place from glutamate, utilizing ATP and probably NADPH as a reducing agent. The suggestion of Kannangara and Gough (15) on the nature of the reactions involved seems reasonable and is in agreement with most of the observations obtained in both barley and maize. However, the suggested route has not been rigorously established with regard to reactions and intermediates. Although the reaction shows an absolute dependence on Mg^{2+} and ATP (Fig. 3; Table IV), it is by no means clear that glutamate is indeed phosphorylated at carbon 1 by a kinase-like enzyme. The fact that inhibitors of glutamine synthetase do not inhibit ALA formation effectively suggests perhaps that glutamate 5-P is not an intermediate. Nevertheless, glutamate could be activated by adenylation of the carboxyl at position 1. Conclusion as to how ATP is involved in the activation of glutamate is dependent on isolation of the enzyme responsible and characterization of the intermediate, whether free or enzyme bound.

Attempts at isolation of the 'glutamate kinase' or the glutamate activating enzyme and the characterization of the reaction should take into account interference by glutamine synthetase and other enzymes, *e.g.* aminoacyl synthetases. There is yet no adequate characterization of the Glu 1-SA or data on its stability. The possibility that it might be contaminated by glutamate or oxidized to it under the conditions of the assay has not been ruled out. This, along with the possible release of amino acids by proteolysis during the reaction require further substantiation of the claim that Glu 1-SA is converted to ALA by an intramolecular transfer of the amino group of the former (15). The exact identity of the cofactor involved in the reduction of the activated glutamate should also be established. In any event, complete elucidation of the reactions taking part in ALA biosynthesis is dependent on isolation of the enzymes and identification of the intermediates involved.

Although the ALA synthesizing system is easily released to a soluble form, it appears that at least some of the activity is lightly bound to a membrane fraction and is not released merely by rupture of the plastid envelope (Table III). Etiolated leaves synthesize only insignificant amounts of ALA and the ability for massive synthesis is light induced and develops after a lag period (4, 20). The 'induction' could be abolished by inhibitors of protein synthesis (20). Such observations were the basis for several models for the control of ALA and Chl synthesis (1, 8). However, the significant levels of ALA synthesizing activity found in etiolated barley (16) and maize (Fig. 2) suggest that such models should be revised, at least with regard to the explanation of the lag phase. In addition, the source of ATP and NADPH for ALA formation during the early hours of greening should be accounted for.

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