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THE SYNTHESIS AND PROPERTIES OF 4,5-DIOXOVALERIC ACID, A POSSIBLE INTERMEDIATE IN THE BIOSYNTHESIS OF 5-AMINOLAEVULINIC ACID, AND ITS IN VIVO FORMATION IN *SCENEDESMUS OBLIQUUS*

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Summary

The formation of 5-aminolaevulinic acid for chlorophyll biosynthesis in the pigment mutant C-2A′ of the unicellular green alga *Scenedesmus obliquus* occurs via two pathways: the first and major pathway uses glycine and succinyl-CoA as precursors and the second, the C-5 pathway, uses the intact five C-atom skeleton of either glutamate or 2-oxoglutarate. The intermediates in this latter pathway of 5-aminolaevulinic acid biosynthesis are still a matter of controversy and discussion but, in this paper, we have demonstrated in this *Scenedesmus* mutant that 4,5-dioxovaleric acid occurs in the cells when illuminated in the presence of laevulinic acid and the amount of 4,5-dioxovaleric acid formed is dependent on the period of illumination. In a preliminary experiment we have shown, under similar conditions, that labelled 4,5-dioxovaleric acid can be obtained from both DL-[1-^{14}C]glutamate and [2-^{14}C]glycine. This suggests that 4,5-dioxovaleric acid is formed enzymically from the intact five C-atom skeleton of glutamate but may also arise from the deamination of ALA formed by the condensation of succinyl-CoA and glycine by the 5-aminolaevulinic acid synthase pathway.

To obtain positive identification the naturally occurring, and also the labelled, 4,5-dioxovaleric acid were isolated as the more stable benzoquinoxaline derivative by condensation with 2,3-diaminonaphthalene and identified by comparison with the benzoquinoxaline derivative of an authentic sample of 4,5-dioxovaleric acid prepared by the hydrogenation of the ozonide of benzyli-

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Abbreviations: PPO, 1,5-diphenyloxazole; POPOP, 1,4-bis-(5-phenyloxazolyl-2)-benzene.

dene laevulinic acid. The structures of the authentic sample of 4,5-dioxovaleric acid and its benzoquinoxaline derivative were confirmed by NMR and mass spectroscopy and both were further characterized by TLC and by infrared, ultraviolet and fluorescence spectroscopy.

Introduction

Tetrapyrroles, both cyclic and non-cyclic, are ubiquitous in nature and play important roles in the metabolism of animals, plants and microorganisms. The cyclic tetrapyrrole pigments include haemoproteins (haemoglobins, cytochromes, catalases and peroxidases), chlorophylls and the corrinoid, vitamin B-12. The non-cyclic tetrapyrroles, also formed via the porphyrin biosynthetic pathway, are represented by such bile pigments as bilirubin and biliverdin, and the accessory plant pigments, phycoerythrin, phycocyanin and phytochrome. The first specific steps in the biosynthesis of all tetrapyrroles, both cyclic and non-cyclic, is the formation of 5-aminolaevulinic acid which also appears to be rate-limiting and a key step in the regulation of tetrapyrrole formation.

In animal tissues and in some bacterial cells, 5-aminolaevulinic acid is known to be formed via the 'classical' pathway using succinyl-CoA and glycine as precursors which are condensed by the enzyme, 5-aminolaevulinic acid synthase (EC 2.3.1.37), which has been described in great detail (for reviews see Refs. 1—4).

In higher plants, Beale's [5—8], Harel's [9,10] and Kanangara's groups [11—13] as well as others [14] have shown that the five C-atom skeleton of glutamate or 2-oxoglutarate can be converted intact into 5-aminolaevulinic acid via the C-5 pathway; however, the intermediates in this plant pathway have not yet been clearly established. Different pathways have been considered [7,8,15] and are summarized by Beale et al. [8] and in Fig. 1. The most discussed pathways in higher plants are those involving glutamate-1-semialdehyde [12,13] or 4,5-dioxovaleric acid [6,14]: there is as yet no evidence to support yet another pathway involving 2-pyrrolidone-5-carboxyaldehyde as proposed by Beale et al. [8]. Because there have been very few demonstrations of the classical 5-aminolaevulinic acid synthase pathway claimed in higher plants [16,17], it is generally considered that the C-5 pathway is the preferred pathway of 5-aminolaevulinic acid formation in such organisms.

The green alga, *Scenedesmus obliquus* (mutant C-2A'), which has been used in the present study has been shown [18—20] to possess both the classical and C-5 pathways: the former pathway has been shown to be the major pathway [18]. Our results demonstrate that 4,5-dioxovaleric acid is formed when dark-grown *Scenedesmus* cells are illuminated in the presence of laevulinic acid and it can also be shown to be formed in vivo from labelled glutamate. In these experiments 4,5-dioxovaleric acid was isolated as its more stable benzoquinoxaline derivative formed by condensation with 2,3-diaminonaphthalene [21] and identified by comparison with the same derivative of an authentic sample of 4,5-dioxovaleric acid prepared by the hydrogenation of the ozonide of benzylidene laevulinic acid [21].

These findings, together with previous results from this laboratory [18,19]

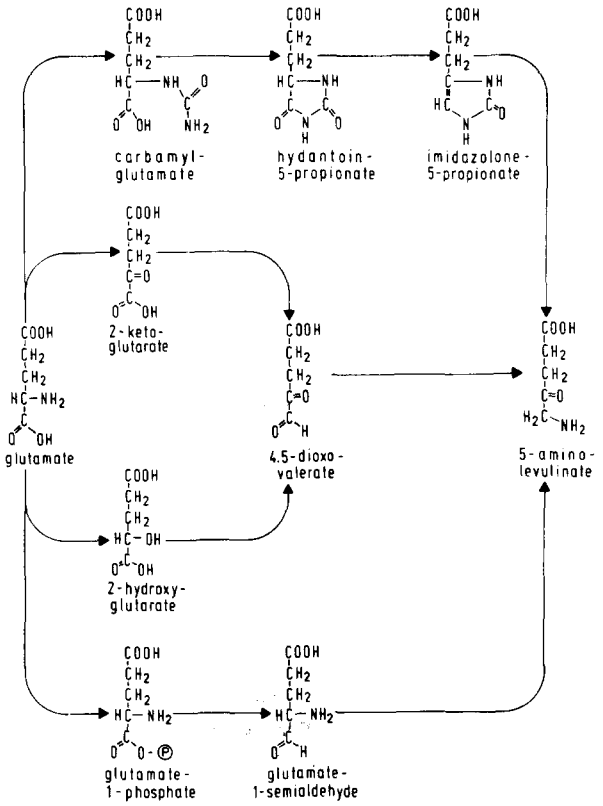


Fig. 1. Various postulated reaction sequences for the biosynthesis of 5-aminolaevulinic acid from glutamate via the C-5 pathway (see Ref. 8).

showing the conversion of the five C-atom skeleton of glutamate intact into 5-aminolaevulinic acid, suggest that 4,5-dioxovaleric acid may be an intermediate in the enzymic conversion of glutamate to 5-aminolaevulinic acid in these *Scenedesmus* cells.

Experimental

Chemicals

2,3-Diaminonaphthalene was obtained from Sigma Chemicals, Munich, and if too highly coloured was recrystallized from ethanol. DL-[1-^{14}C]Glutamate (spec. act. 57 Ci/mol) and [2-^{14}C]glycine (54 Ci/mol) were purchased from the Radiochemical Centre, Amersham, U.K. Lindlar catalyst (5% Pd/$CaCO_3$) was obtained from Fluka, AG, Basel.

Preparation of authentic samples of 4,5-dioxovaleric acid and its 2,3-diaminonaphthalene derivative

4,5-Dioxovaleric acid was synthesized by the hydrogenation of the ozonide of benzylidene laevulinic acid using a modified procedure of Kissel and Heilmeyer [21] (see Fig. 2).

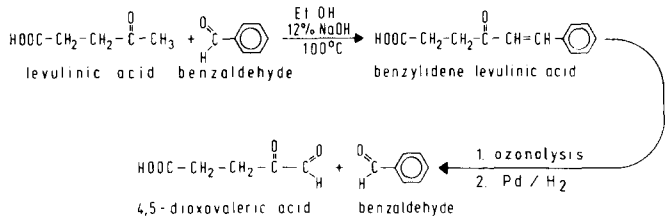


Fig. 2. The synthesis of 4,5-dioxovaleric acid from laevulinic acid and benzaldehyde via benzylidene laevulinic acid.

Formation of benzylidene laevulinic acid. The synthesis was carried out as described by Henke [24]. Benzaldehyde (20 ml; 0.2 mol) was dissolved in 200 ml 96% ethanol and laevulinic acid (22 ml; 0.2 mol) and 500 ml boiling water were added. Then 100 ml 12% NaOH was added with continuous shaking till the turbid solution became clear yellow. After 3—5 min the mixture was chilled to 0°C and the benzylidene laevulinic acid precipitated by the addition of 6 N HCl, first as an oil and later as a solid. The solid was filtered off and recrystallized three times from hot water. These crystals were then suspended in cold water, washed with diethyl ether and finally recrystallized from boiling water. The yield of crude material was 58% of theory but the yield of recrystallized material suitable for ozonolysis was 28% and had a melting point of 124°C (cf. Ref. 24). This material was further characterized by infrared, ultraviolet and NMR spectroscopy.

Infrared absorption (KBr); ν_{max}: 2800—3500 cm^{-1} (s, broad, —OH); 1710 (s, —COOH); 1660 (s, >C=O); 1620 (s, >C=O); 1580 (m); 1500 (m); 1400 (m); 1375 (m); 1110 (s).

Ultraviolet absorption (C_2H_5OH); λ_{max}: 287 nm.

^1H NMR spectrum (C^2HCl_3); ppm relative to external TMS: (δ = 2.6—3.3 (m, —CH_2—CH_2— group); δ = 7.3—7.5 (m, aromatic C—H); δ = 6.65—7.8 (AB system, J_{AB} = 16 Hz, olefinic C—H).

The formation of 4,5-dioxovaleric acid. The formation of the ozonide of benzylidene laevulinic acid and its subsequent hydrogenation [21,25] were carried our with care at 0°C to reduce risk of explosion. The acid (20.4 g) was dissolved in 200 ml absolute CH_3OH, chilled to 0°C and a stream of O_3/O_2 (4.75 g O_3/h), obtained by passing O_2 (20 l/h) through an ozonizer (Fischer Labor Technik, Bonn-Bad Godesberg), was passed through the chilled solution. The effluent gas from the reaction flask was passed through aqueous KI solution and when iodine was liberated the ozone supply was immediately terminated. The solution must also be maintained at 0°C for the hydrogenation which is carried out by vigorous shaking in the presence of Lindlar catalyst (5% Pd/$CaCO_3$; 250 mg) in a closed system so that H_2 uptake can be measured. To ensure that all the ozonide was reduced the reaction mixture was left under H_2 for 60 min after H_2 uptake ceased. The catalyst was then filtered off, the methanol removed by evaporation, the residue dissolved in 50 ml of H_2O, and the aqueous solution extracted three times with diethyl ether to remove the benzaldehyde formed. The water was then removed under vacuum over P_2O_5.

The product was a light-grey, glass-like substance with the following spectroscopic properties.

Infrared absorption (KBr); ν_{max}: 2800—3700 cm^{-1} (s, broad, —OH); 1775 (s, broad —COOH); 1725 (s, broad, >C=O); 1450 (m); 1410 (m); 1200 (m); 1145 (m); 1075 (m); 1010 (m).

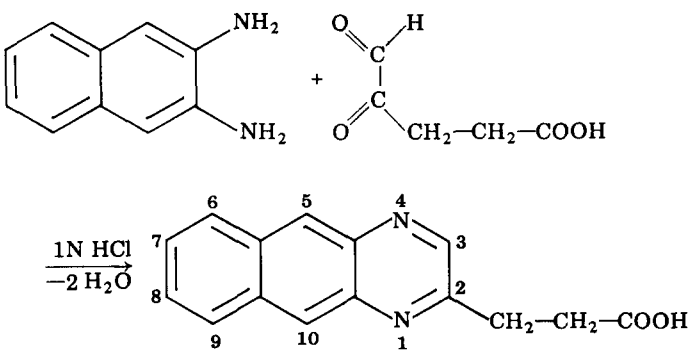
Ultraviolet absorption (H_2O); λ_{max}: 283 (shoulder) 256 (peak) nm.

^1H NMR spectrum (TFA); ppm relative to external TMS: δ = 1.8—2.6 (m, $—CH_2—CH_2—$ of ketone and $—CH_2—$ of enol); δ = 5.3 (=CH— of enol).

^{13}C NMR spectrum (TFA); ^{19}F-lock; ppm relative to external TMS: δ = 28—30 (C_2, C_3 keto form or C_2 enol form); δ = 101—103 (C_3 enol form); δ = 105—107 (C_4 enol form); δ = 115—117 (C_5 keto and enol form); δ = 180—182 (C_1 keto and enol form).

Mass spectrum (70 eV); *m/e*: 101 (100%, $M^+ - HCO^-$); 73 (24%, $C_3H_5O_2^+$); 55 (57%, $C_3H_4O^+$); 44 (11%, $CO_2^{+\cdot}$); 30 (13%, $CH_2O^{+\cdot}$); 28 (91%, $^{\cdot+}CO$); 26 (65%, $C_2H_2^{+\cdot}$).

The formation of the benzoquinoxaline derivative of 4,5-dioxovaleric acid. Because α-ketoaldehydes are highly reactive, 4,5-dioxovaleric acid was also characterized as its more stable benzoquinoxaline derivative which was formed by mixing equal volumes of concentrated equimolar ethanolic solutions of 4,5-dioxovaleric acid and 2,3-diaminonaphthalene as shown below:



The derivative, 3-(benzo[*g*]quinoxalinyl-2)propionic acid, was recrystallized from hot ethanol and had the following spectroscopic properties.

Infrared absorption (KBr); ν_{max}: 3100 cm^{-1} (m, CH aromatic); 2930 (m, CH aliphatic); 2650 (m, broad, —OH); 1752 (s, —COOH); 1590 (w, C=C, aromatic); 1435 (s); 1420 (s); 1498 (s); 1375 (s); 1332 (s); 1287 (m); 1208 (s); 1180 (s); 1163 (s); 1120 (s).

Ultraviolet absorption (1 N NaOH); λ_{max}: 365, 348, 268 nm.

^1H NMR spectrum (TFA); ppm relative to external TMS: δ = 2.9 (m, CH_2); δ = 3.3 (m, CH_2); δ = 7.2—7.9 (m, C—6—H to C—9—H); δ = 8.5 (d, ^4J = 3H, C—5—H to C—10—H); δ = 8.9 (s, C—3—H).

Mass spectrum (70 eV); *m/e*: 252 (46% M^+); 207 (100%, $M^+ - 45$; $CO^{+\cdot}$, $^{\cdot}OH$); 179 (5%, $M^+ - 73$; $CO^{+\cdot}$, $^{\cdot}OH$, $C_2H_4^{+\cdot}$); 152 (12%, $M^+ - 100$; $^{\cdot+}C_{11}H_6N$); 127 (6%, $M^+ - 125$; $C_{10}H_7^+$); 126 (3%, $M^+ - 126$; $C_{10}H_6$).

The formation of benzoquinoxaline derivatives of other α,β-dioxo compounds

Other naturally occurring compounds including pyruvate, 2-oxoglutarate,

TABLE I

SOME CHARACTERISTICS OF THE BENZOQUINOXALINE DERIVATIVES OF SOME NATURALLY OCCURRING $\alpha\beta$-DIOXO COMPOUNDS

Spectra were run in 1 N NaOH. The fluorescence excitation spectra were run with an excitation bandwidth of 3 nm and an emission bandwidth of 7.5 nm while the emission spectra were recorded with a 3 nm emission bandwidth and a 15 nm excitation bandwidth. The major peaks are in italics. The R_F values of the benzoquinoxaline derivatives were obtained with Kieselgel 60 analytical plates developed in toluene/acetic acid (3:1, v/v).

Benzoquinoxaline derivative of:	Fluorescence spectra (nm)				Absorption spectra (ultraviolet maxima (nm))	TLC chromatography (R_F values)
	Emission maxima		Excitation maxima			
4,5-Dioxovaleric acid	*558*	540	*366*	350	365,348,*268*	0.31
2-Oxoglutarate	*519*	505	*355*		353,338,*287,278,245*	0.38
Oxaloacetate	*502*	518	*338*	351	352,336,*287,278,244*	0.42
Pyruvate	*502*	517	352		352,336,*287,278,244*	0.42
Methylglyoxal	*540*	554	*365*	353	362,346,*265*	0.35

oxaloacetate and methylglyoxal condense with 2,3-diaminonaphthalene to form benzoquinoxaline derivatives which, like the 4,5-dioxovaleric acid derivative, are characterized by ultraviolet absorption which produces a green fluorescence. This fluorescence can be used for the qualitative and quantitative determination of benzoquinoxalines. The ultraviolet absorption and fluorescence emission and excitation spectra of all the above derivatives, including those of 4,5-dioxovaleric acid, are compared in Table I which also contains some relevant TLC data.

Organism and maintenance

The X-ray-induced mutant C-2A' of *S. obliquus* [22] was used: chlorophyll synthesis in algae normally occurs in the dark but is light-dependent in this mutant. Cultures were grown heterotrophically in the dark at 30°C in an inorganic nitrate-based medium [23] supplemented with 0.5% glucose and 0.25% yeast extract. Cultures were shaken at 80 rev./min and were maintained by subculturing every 3—4 days.

Illumination of cultures

For greening experiments cultures grown in the dark (see above), were taken at the end of logarithmic growth (3—4 days) and illuminated with a light bank containing five 40W/25-1 and four 40W/15-1 Osram lamps giving 20 000 lux ($1.7 \cdot 10^4$ erg/cm^2 per s, approx. 20 W/m^2). To produce 4,5-dioxovaleric acid the cultures were preilluminated for 4 h then 10 mM laevulinic acid added and illumination continued for periods up to 24 h.

Incubation conditions with DL-[1-^{14}C]glutamate and [2-^{14}C]glycine

To produce labelled 4,5-dioxovaleric acid, 250 ml of dark-grown culture was preilluminated for 4 h in the presence of approx. 16 μCi of labelled glutamate or glycine, diluted to 5 mM with unlabelled substrate, then treated with laevulinic acid as above for 20 h. The labelled 4,5-dioxovaleric acid was extracted as described below.

Extraction of unlabelled benzoquinoxaline derivative of 4,5-dioxovaleric acid from cells

4,5-Dioxovaleric acid in the cells and medium was quickly converted to the more stable benzoquinoxaline derivative as follows. Cultures were centrifuged (5 min at 15 000 × *g*). The cells were extracted with 5% perchloric acid and centrifuged at 20 000 × *g* for 15 min. Both the medium and perchlorate extract were adjusted to pH 3.1 and subjected to ion-exchange chromatography on Dowex 50W-X8 (Na^+ form) [8,18] to remove 5-aminolaevulinic acid which adheres to the column: it was subsequently found that this step was unnecessary. The eluate, containing 4,5-dioxovaleric acid, was made 1 N with respect to HCl, and 2,3-diaminonaphthalene (1 mg/10 ml of eluate) was added. After 18 h when the reaction was complete the pH was adjusted to 5.0 and the mixture extracted five times with small volumes of diethyl ether.

Pyruvate, 2-oxoglutarate, oxaloacetate and methylglyoxal, like 4,5-dioxovaleric acid, are present in the column eluate and form diethyl ether-soluble benzoquinoxalines under the conditions described above but they may be separated by TLC as follows.

The pooled diethyl ether extracts were evaporated to dryness and the residue, redissolved in methanol, was applied to preparative TLC plates (20 cm × 20 cm × 2 mm; Kieselgel G; Merck) and developed in toluene/acetic acid (3 : 1, v/v). The benzoquinoxaline zones, identified by their green fluorescence, were scraped off, pooled and eluted with methanol. Further separation of unlabelled 4,5-dioxovaleric acid samples were then carried out on analytical TLC plates (20 cm × 20 cm × 0.2 mm; Kieselgel 60; Merck) by two successive developments in toluene/acetic acid (3 : 1, v/v) against authentic markers of 2,3-diaminonaphthalene derivatives of pyruvate, 2-oxoglutarate, oxaloacetate, methylglyoxal and 4,5-dioxovaleric acid: the R_F values are given in Table I. The 4,5-dioxovaleric acid derivative zone was scraped from the plate, extracted with 1 N NaOH and determined quantitatively by fluorescence spectroscopy by comparison with a calibration curve obtained with 0—10 μg (0—40 nmol) of the authentic benzoquinoxaline derivative of 4,5-dioxovaleric acid in 1 N NaOH. The calibration was linear to 20 nmol and deviated only slightly from linear between 20 and 40 nmol.

Extraction of labelled benzoquinoxaline derivative of 4,5-dioxovaleric acid from cells and culture medium

Cell extracts, prepared with perchlorate as above, and medium were each made 1 N with respect to HCl and treated with 2,3-diaminonaphthalene for 18 h. The benzoquinoxaline derivatives were extracted into diethyl ether at pH 5.0 and subjected to TLC on analytical plates (as above) using toluene/acetic acid (3 : 1, v/v). The 4,5-dioxovaleric acid band was eluted with methanol and subjected twice more to TLC separations firstly in toluene/CH_3OH (2 : 1, v/v) and finally in toluene/acetic acid (3 : 1, v/v). The radioactive bands were located by a Berthold Scanner (model LB2723). The labelled band was eluted with methanol (1.0 ml) and an aliquot (0.1 ml) was mixed in a scintillation mixture of PPO (0.7%, w/v) and POPOP (0.01%, w/v) in sulphur-free toluene and radioactivity determined in a Beckman Scintillation Counter (model LS3133T). The remainder of the methanol solution (0.9 ml) was added

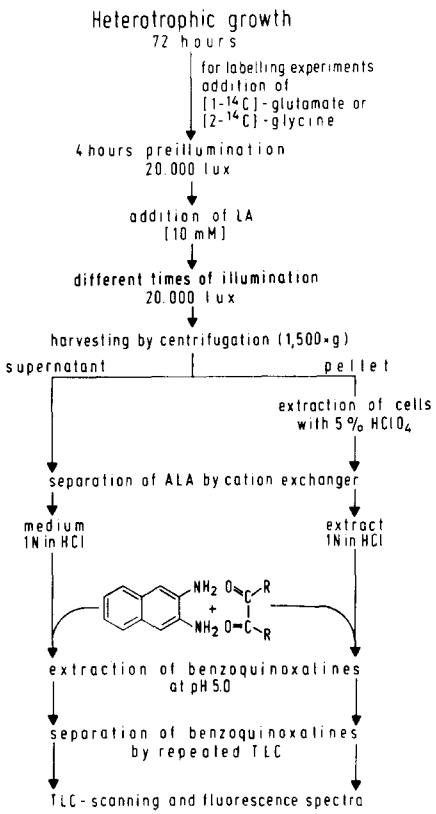


Fig. 3.Recommended procedure for the in vivo labelling of 4,5-dioxovaleric acid, its isolation and determination of specific activity.

to 1 N NaOH (3 ml) and the concentration of 4,5-dioxovaleric acid determined by fluorescence emission spectroscopy as described above. The recommended scheme of procedures to be used is shown in Fig. 3.

Spectrometers

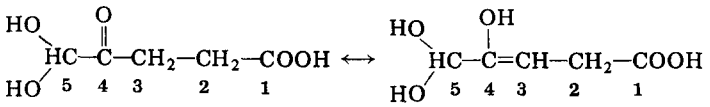
Spectrometers used in these experiments were : infrared absorption, Perkin-Elmer model 457; ultraviolet absorption, Shimadzu model MPS 5000; ^{1}H NMR spectra, Varian model T60; ^{13}C NMR spectra, Varian model XL100; mass spectra, Varian model MAT7; fluorescence spectra, Shimadzu corrected spectrofluorimeter model RF502.

Results and Discussion

Interpretation of the spectroscopic data and the structure of 4,5-dioxovaleric acid

4,5-Dioxovaleric acid has been examined spectroscopically [21,25] but the present work not only confirms but extends previous findings. As shown (see Experimental) the infrared spectrum revealed peaks at 1775 and 1725 cm^{-1} for carboxyl and carbonyl groups, respectively. The ^{1}H NMR data showed a broad

signal, δ = 1.8—2.6 ppm associated with the protons of C_2 and C_3 (see structures below) in the keto and enol form and a multiplet at δ = 5.3 ppm due to the proton of C_3 in the enol form. ^{1}H NMR data, however, do not provide any information about the functional group at C_5 and so a ^{13}C NMR spectrum was undertaken. Signals above δ = 190 ppm, characteristic for aldehyde groups, were not present but rather a broad band at δ = 115—117 ppm. This indicates that the aldehyde is hydrated thus confirming the ^{1}H NMR data suggesting that 4,5-dioxovaleric acid exists in the following two tautomeric (keto and enol) forms:



Because of instability and the probable loss of an HCO radical, the mass spectrum of 4,5-dioxovaleric acid at 70 eV showed no M^+ peak at 130 but rather a peak at 101 thus giving no conclusive proof of the structure of 4,5-dioxovaleric acid. Nonetheless, confirmation of this structure was obtained when the mass spectrum of the benzoquinoxaline derivative of 4,5-dioxovaleric acid showed a M^+ peak at 252; further, the cleavage of the carboxyl and the ethylene groups and further degradation to the naphthalene skeleton could be clearly demonstrated, thus conclusively proving the structure of the benzoquinoxaline derivative of 4,5-dioxovaleric acid.

The isolation of 4,5-dioxovaleric acid from S. obliquus cultures

To isolate 4,5-dioxovaleric acid, synthesized in vivo by *Scenedesmus*, a cell suspension was preilluminated and incubated in the light with laevulinic acid. The addition of laevulinic acid, an inhibitor of 5-aminolaevulinic acid dehydratase [26], caused not only the accumulation of 5-aminolaevulinic acid [20] but also of 4,5-dioxovaleric acid and both were secreted into the culture medium. After the conversion of all the dioxo compounds in the cells and medium to their benzoquinoxaline derivatives (see Experimental), the 4,5-dioxovaleric acid derivative, 3-(benzo[*g*]quinoxalinyl-2)propionic acid, was separated from other benzoquinoxalines by TLC procedures (see Experimental) and identified by comparison with an authentic marker. The identity of the derivative of this naturally occurring 4,5-dioxovaleric acid was further confirmed by comparison of the ultraviolet absorption, fluorescence emission and excitation spectra with those of the authentic compound. Since under these conditions by far the greater part of the 4,5-dioxovaleric acid formed was excreted into the medium, the time course of 4,5-dioxovaleric acid formation was obtained (see Fig. 4) by assaying only the medium. That illumination was required for 4,5-dioxovaleric acid formation by *Scenedesmus* cells incubated with laevulinic acid again confirms the light dependence of this C-5 pathway as suggested by the experiments of Klein et al. [20].

In subsequent experiments using an incubation period of 20 h, 4,5-dioxovaleric acid production rates 10-fold higher than those reported in Fig. 4 have been achieved.

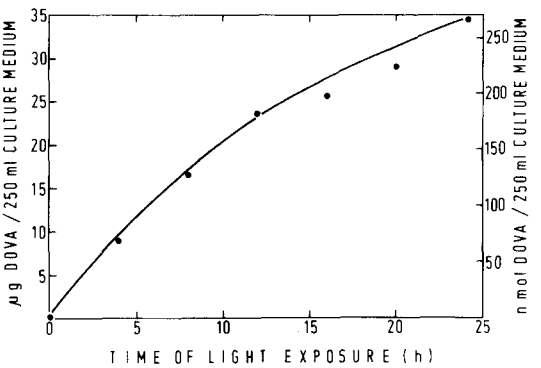


Fig. 4. Time course and light dependency of 4,5-dioxovaleric acid (DOVA) accumulation in the culture medium of *S. obliquus* C-2A' after addition of laevulinic acid.

The formation of labelled 4,5-dioxovaleric acid from [1-^{14}C]glutamate or [2-^{14}C]glycine

Labelling experiments were performed to establish the role of 4,5-dioxovaleric acid as an intermediate in the biosynthesis of 5-aminolaevulinic acid by the C-5 pathway. Cells were incubated with labelled glutamate or glycine as described in Experimental.

Labelling of 4,5-dioxovaleric acid by labelled glutamate via the C-5 pathway was observed. However, only a very small proportion of the administered label was incorporated; nonetheless, the isolated benzoquinoxaline derivative was purified by TLC to constant specific activity. The labelling observed accounted for the formation of approximately 44 nmol of 4,5-dioxovaleric acid by 250 ml of culture.

Because the glutamate was labelled in the C_1 position there was no possibility that it could have given rise to labelled succinyl-CoA from which labelled 5-aminolaevulinic acid could be derived to permit labelled 4,5-dioxovaleric acid formation by reversal of 4,5-dioxovalerate transaminase activity. However, the administration of [2-^{14}C]glycine gave rise to an almost identical proportion of label entering 4,5-dioxovaleric acid and such incorporation presumably does occur by this 5-aminolaevulinic acid deamination route. In this experiment with labelled glycine the 4,5-dioxovaleric acid was again purified to constant specific activity as its benzoquinoxaline derivative.

The incorporation of so little labelled glutamate is, perhaps, to be expected since Neuberger's group [27] has reported that the equilibrium of the dioxovalerate transaminase very strongly favours 5-aminolaevulinic acid formation. The incorporation of so little label also suggests that the algal cells may not be freely permeable to glutamate. To check this latter possibility cells were incubated in the presence of 5 mM unlabelled glutamate to see if there was any enhancement of 4,5-dioxovaleric acid formation. The results of this experiment were inconclusive since 4,5-dioxovaleric acid formation in the presence of glutamate was only two-thirds of that observed in its absence.

In conclusion, these findings of the occurrence of 4,5-dioxovaleric acid in cultures of *Scenedesmus* cells and also of the incorporation of [1-^{14}C]glutamate into 4,5-dioxovaleric acid, along with previous results from this laboratory

showing the conversion of the intact five C-atom skeleton of glutamate into 4,5-dioxovaleric acid [18,19], suggest that 4,5-dioxovaleric acid may be an intermediate in the conversion of glutamate to 5-aminolaevulinic acid by the C-5 pathway in these algal cells. Because of the very low rate of incorporation of label, however, final proof of this suggestion may have to await the outcome of experiments with cell-free preparations which are already in progress.

Acknowledgements

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