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CHEMICAL SYNTHESIS OF 4,5-DIOXOVALERIC ACID AND ITS NONENZYMATIC TRANSAMINATION TO 5-AMINOLEVULINIC ACID

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Key Word Index—4,5-Dioxovaleric acid; 5-aminolevulinic acid; transamination; glycine; glyoxalate; porphyrin synthesis.

Abstract—4,5-Dioxovaleric acid (DOVA) was synthesized from 5-bromolevulinic acid via formation of the pyridinium bromide of 5-bromolevulinic acid, followed by nitrone formation with *p*-nitrosodimethylaniline, and hydrolysis of the nitrone to yield DOVA. Partial purification of DOVA was obtained by passage of the reaction mixture through a cation exchange column. DOVA was identified by paper electrophoresis and by a specific fluorometric assay. DOVA was nonenzymatically transaminated to 5-aminolevulinic acid (ALA) with glycine serving as the amino donor. Other compounds tested were less effective amino donors. Glyoxylic acid was identified as a reaction product by paper electrophoresis and a specific colorimetric test. ALA was identified by paper electrophoresis, paper chromatography of a pyrrole derivative, reaction with Ehrlich reagent, and by its enzymatic conversion by a barley extract to porphobilinogen and uroporphyrin. The nonenzymatic transamination was inhibited by Tris and was stimulated by high pH. The existence of this nonenzymatic activity is discussed in relation to previous reports of DOVA transaminase activity in cell extracts.

INTRODUCTION‡

The formation of ALA is the first known step of the tetrapyrrole biosynthetic pathway, leading to hemes, corrins, bilins and chlorophylls [1]. ALA formation in animals and bacteria (including photosynthetic bacteria) is catalysed by ALA synthetase [succinyl CoA:glycine *C*-succinyl transferase (decarboxylating) EC 2.3.1.37] [2]. In animals, ALA synthetase is found within the mitochondria, where it exists in close proximity to a source of the substrate succinyl CoA and to heme, the end product of the tetrapyrrole pathway in these organisms [3].

ALA (1) has recently been found to be formed in greening plants from glutamate (2) or 2-ketoglutarate (3), via a path that does not involve the ALA synthetase reaction [4]. In plants, the intact carbon skeleton of glutamate is incorporated into ALA, C_1 of glutamate becoming C_5 of ALA [5, 6]. DOVA (4) has been proposed as an intermediate between glutamate or 2-ketoglutarate and ALA in the plant pathway. ALA could be formed by reduction of the C_1 carboxyl group of 2-ketoglutarate to the aldehyde, followed by a transamination reaction on the aldehyde group. This pathway would yield ALA with the carbon to carbon correspondence that has been found experimentally [5, 6].

There have been reports of DOVA transaminase activity in animal tissues [7, 8], bacteria, both photosynthetic [9–11] and non-photosynthetic [12], algae [13, 14] and higher plant tissues [13]. Because DOVA was not detected in any of these organisms, and because no DOVA-forming activity was found, the possibility could not be excluded that the observed transaminase activity was either the reversal of a reaction that functions *in vivo* to degrade ALA [15], or that the activity was due to an anomolous reaction, of no physiological significance, catalysed by a transaminase enzyme that normally functions *in vivo* with other substrates.

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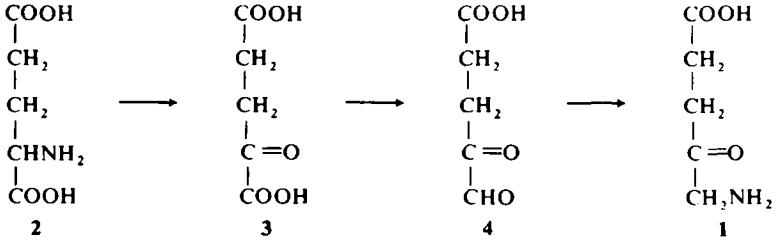
‡Abbreviations used: ALA, 5-aminolevulinic acid; CAPS, cyclohexylaminopropane sulfonic acid; DOVA, 4,5-dioxovaleric acid; EPPS, 4-(2-hydroxyethyl)-1-piperazine propane sulfonic acid; HEPES, *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid; MOPS, morpholinopropane sulfonic acid.



Scheme 1. Formation of 5-aminolevulinic acid from glutamic acid.

Table 1. Nonenzymatic transamination of DOVA to ALA

Amino donor	nmol ALA
Glycine	14.9
Phenylalanine	3.2
Glutamate	1.95
Alanine	1.39
Valine	1.25
Proline	0.28
None	0.00
Glycine (50 mM)	33.3
Glycine (50 mM), minus DOVA	0.97
Glycine (50 mM), zero time	2.09

Incubation of 10-fold diluted DOVA with 25 mM amino donor (except where indicated) in 0.1 M EPPS buffer at pH 8.4, for 2 hr at 37°. ALA was measured by reaction with ethyl acetoacetate, followed by addition of Ehrlich – Hg reagent (see text).

In this paper we present improved methods for the chemical synthesis and purification of DOVA, and describe an interesting nonenzymatic transamination reac- yielding net ALA from DOVA under mild conditions.

RESULTS

Synthesis of DOVA from 5-bromolevulinic acid

The procedure described in the Experimental yielded a pale yellow liquid solution of DOVA. The yield of product was not determined directly, but upon further reaction to form ALA (see below) the overall yield of ALA was 60%, based on the starting material, 5-bromolevulinic acid. High-voltage paper electrophoresis of the DOVA followed by visualization with $AgNO_3$, yielded a single spot, which migrated 4.0 cm toward the anode under our electrophoretic conditions (see Experimental). Treatment with 2,3-diaminonaphthalene yielded a derivative with intense green fluorescence, having an excitation maximum at 368 nm and an emission maximum at 512 nm [8].

Table 2. Effects of varying buffering agent and pH on the formation of ALA

Buffer	pH	nmol ALA
EPPS	8	20.3
	8.5	30.7
	9.0	35.7
CAPS	9.0	18.1
	9.5	26.8
	10.0	35.4
	10.5	39.9
HEPES	8.1	14.5
MOPS	8.4	10.0
Imidazole	8.4	3.8
Phosphate	8.6	2.1
Pyridine	8.5	6.8
Bicarbonate	9.0	8.8
	9.5	20.0
None	8.5	9.0
	9.5	36.8

Conditions are as for Table 1, except for choice of buffer and pH. Glycine concentration was 50 mM.

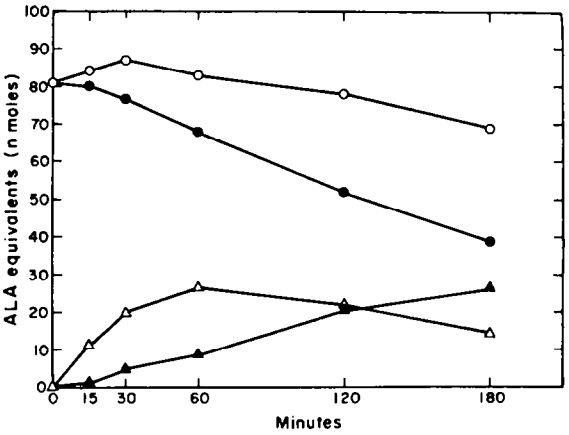


Fig. 1. Conversion of the nonenzymatic DOVA transamination product (●) into porphobilinogen (Δ) and uroporphyrin (▲) by a barley extract. Porphobilinogen concentration was multiplied by 2, and urophophyrin concentration was multiplied by 8, to reflect the stoicheometry of the synthesis of these from 2 and 8 molecules of ALA, respectively. The sum of the molar equivalents of ALA in all of these compounds is also shown (○). See text for details of incubation and analysis.

Nonenzymatic transamination of DOVA to ALA

Incubation of DOVA with glycine resulted in the formation of ALA and glyoxylate. The requirements for both DOVA and glycine are shown in Table 1. Glycine was by far the most effective amino donor found, but other α-amino acids were slightly effective (Table 1). The reaction was stimulated by, but was not dependent upon, the addition of various buffering agents (Table 2). The pH optimum of the reaction varied somewhat with the different buffers employed. Maximum yield of ALA was obtained at pH 9 in EPPS buffer, pH 10.5 in CAPS buffer, and pH 9.5–10 when buffer was omitted and the pH was carefully adjusted with NaOH.

The reaction exhibited a linear temperature dependence between 37° and 100°, and at 60° the maximum yield of ALA was obtained after 60 min of incubation. Longer incubation times resulted in net loss of ALA. Starting with glyoxylate and ALA, the reverse reaction, yielding glycine and DOVA, could not be detected.

Identification of the reaction product ALA

The reaction yielded a single Ehrlich-positive product after reaction with ethyl acetoacetate, having an electrophoretic mobility identical to that of an authentic ALA standard. After reaction with ethyl acetoacetate, chromatography in butanol–propanol–ammonia yielded a single Ehrlich-positive spot, with R_f 0.49, identical to that of the pyrrole formed with authentic ALA. The visible absorption spectrum of the Ehrlich-positive pyrrole formed by reaction of the product with ethyl acetoacetate was identical to that obtained with authentic ALA with an absorption maximum at 552 nm and a shoulder at 520 nm. Incubation of the reaction product with a cell extract obtained from barley leaves (see Experimental) resulted in enzymatic conversion to prophobilinogen, and the further enzymatic conversion of the prophobilinogen to uroporphyrin (Fig. 1), as determined by spectrofluorometry [16]. The transamination was strongly inhibited by Tris, 50 mM causing a 90% decrease in the yield of ALA.

Identification of the reaction product glyoxylate

The reaction also yielded a product with an electrophoretic mobility identical to authentic glyoxylic acid, and which, like glyoxylic acid, could be visualized with $AgNO_3$, indicating the presence of a reducing group. Upon reaction with 2,3,4-trihydroxybenzoic acid, a blue pigment was formed which has a visible spectrum identical to that of the derivative of authentic glyoxylic acid, with a maximum at 592 nm. This reaction with 2,3,4-trihydroxybenzoic acid is reported to be absolutely specific for glyoxylate [17, 18].

DISCUSSION

Older methods for DOVA synthesis have many drawbacks [19, 20]. The attack by bromine on levulinic acid is nonspecific, and the harsh reaction conditions and lengthy refluxing times allow DOVA to polymerize. Purification of the dark-brown tarry product is very difficult. Under the conditions employed in our procedure, extremes of pH are avoided and the temperature is kept at 0° during the formation of DOVA from the pyridinium salt of 5-bromolevulinate, thus preventing the consequences of high temperature and pH on DOVA. Passage of the crude reaction product through the cation exchange column removes such cationic materials as pyridine, 5-pyridinium levulinate and dimethylanilines; the pale yellow effluent is of a purity sufficient for use in biological experiments. Synthesis of derivatives of DOVA by the Kröhnke method has been described earlier [21], but free DOVA was apparently not isolated.

The interesting nonenzymatic transamination of glycine and DOVA to ALA and glyoxylate can occur under conditions similar to those employed in enzyme assays. It thus becomes necessary to re-examine earlier reports of DOVA transaminase activity in cell extracts based upon the formation of ALA from DOVA [8–11, 13, 14]. Especially in nondialysed preparations, the actual enzymatic activity could be the formation of glycine, which then could react spontaneously with added DOVA to yield ALA. The ability of cell extracts to stimulate ALA formation from DOVA thus might not indicate the presence of a DOVA transaminase activity.

The large difference between the effectiveness of glycine and the other α-amino acids in this nonenzymatic reaction is somewhat puzzling. Perhaps the explanation lies in the fact that only glycine, because of the lack of substituents at the α carbon atom, yields an aldehyde rather than a ketone upon transamination. The inhibition of the nonenzymatic activity by Tris, as well as the reported inhibition of enzymatic DOVA transaminase by Tris [14], could be due to reaction of the aldehyde group of DOVA with the amino group of Tris to form the imino complex, as was reported for other aldehydes [22].

Exchange of the ALA amino group among molecules of ALA and DOVA has been reported [23], but in that reaction no net synthesis of ALA can occur. The presence of such an exchange reaction could, however, cause anomalous apparent transfer of ^{14}C between pools of DOVA and ALA, and thus complicate the interpretation of results in experiments designed to study the metabolism of ^{14}C-DOVA or ^{14}C-ALA [24]. The high degree of reactivity of the α-ketoaldehyde group of DOVA toward primary amines such as ALA, glycine, and Tris indicates that caution should be observed both in experimental studies with this compound and in the interpretation of results of these studies. The nonenzymatic formation of ALA reported here is not likely to be involved in tetrapyrrole biosynthesis in living organisms. It is possible, however, that reactions of this type are important in nonbiological or prebiological porphyrin formation [25, 26].

EXPERIMENTAL

Synthesis of DOVA. The method of Kröhnke and Borner [27, 28] for the synthesis of other 2-ketoaldehydes was unsuitable for DOVA because of instability and lack of ether solubility of the product. Accordingly, the method was modified to carefully control reaction pH, and purification was carried out by ion exchange chromatography. One millimole (195 mg) 5-bromolevulinic acid was dissolved in 10 ml anhydrous Py. The soln was heated at 60° for 60 min, then the Py was removed by evapn *in vacuo* at 60°. The pale yellow syrup remaining was dissolved in 0.5 ml H_2O. After cooling to 0°, a soln of 150 mg *p*-nitrosodimethylaniline in 10 ml 95% ice cold EtOH was added. One ml 2N ice-cold NaOH was slowly added while swirling to mix. This caused the pH to rise to 10.75 and the color to change from green to deep red. After allowing the reaction mixture to stand for 15 min at 0°, 6 ml ice-cold 1N HCl were added, lowering the pH to 2.0 and causing the color to lighten noticeably. After the soln was allowed to stand for 15 min at 0°, it was passed through a cation exchange column (5 ml bed volume of Dowex 50W-X8, 200–400 mesh, in the (H^+) form) followed by 5 ml H_2O. The effluent from the column was evapd to a small vol. *in vacuo* at 30° to remove EtOH, then diluted to 10 ml with H_2O and stored at −20°.

DOVA was detected by its ability to reduce $AgNO_3$ after high voltage electrophoresis, and by the fluorescence characteristics of its derivative with 2,3-diaminonaphthalene [8].

Paper electrophoresis. Electrophoretic separations were carried out on Whatman 3MM paper. The solvent was 50 ml HOAc diluted to 2 l. with H_2O and adjusted to pH 4.3 with Py. A potential of 3000 V was applied across 80 cm (37.5 V/cm), and the temp. was maintained at 15° by thermal contact with a water-cooled aluminium block. Electrophoresis was normally carried out for 60 min. Under our conditions, DOVA, glutamate, glyoxylate and 2-ketoglutarate migrated 4, 7, 10, and 18 cm toward the anode, while glycine and ALA migrated 2 and 8 cm toward the cathode, respectively. All migration distances are corrected for neutral spot (glucose) migration due to capillarity and/or electro-osmosis. A previously published electrophoretic procedure [24] was unsuccessful in our hands.

Detection of compounds after electrophoresis. The $AgNO_3$ method of ref. [29] was used for detection of DOVA, glucose, glyoxylate and ALA. ALA was also detected by the Ehrlich reaction with the pyrrole formed on the paper (see below). Amino acids were detected with ninhydrin.

Spectrophotometric identification of glyoxylic acid. Glyoxylic acid was allowed to react with 2,3,4-trihydroxybenzoic acid in conc H_2SO_4 as described by Ecgriwe [17, 18]. One mg trihydroxybenzoic acid was placed in a small test-tube, 25 μl of test solution was added, followed by 0.5 ml conc H_2SO_4. The tube was capped and allowed to stand at room temp. for 24 hr, after which the visible spectrum was recorded.

Determination of ALA. ALA was allowed to react with ethyl acetoacetate at pH 6.8 [30]. The pyrrole formed from this reaction was determined with Ehrlich Hg reagent modified according to ref. [31]: 4 g *p*-dimethylaminobenzaldehyde and 0.7 g $HgCl_2$ are dissolved in 168 ml HOAc, 40 ml 70% $HClO_4$ are added slowly, and the solution is diluted to 220 ml with HOAc. For detection of ALA after paper electrophoresis, the paper was dried, then sprayed with a satd soln of ethyl acetoacetate in 1 M Na–Pi buffer (pH 6.8). After heating for 30 min at 100°, the pyrrole formed was visualized by spraying with Ehrlich spray reagent: 200 mg *p*-dimethylaminobenzaldehyde dissolved in 8 ml EtOH and 2 ml of 2 N HCl [4]. Alternatively ALA was first condensed

with ethyl acetoacetate in soln and the pyrrole product was extracted into Et_2O at pH 3. PC of the pyrrole derivative was performed on Whatman 3MM paper in *n*-BuOH–*n*-PrOH–5% NH_4OH (2:1:1) [4]. The ALA-pyrrole was visualized after chromatography by the Ehrlich spray reagent.

Conversion of ALA to porphobilinogen and uroporphyrin by barley extracts.. Barley seeds were germinated and grown for 7 days in complete darkness as previously described [5]. After 4 hr of light exposure, 25 g leaves were ground in 25 ml ice-cold 50 mM $NaHCO_3$ buffer (pH 7.5) and sand, with a mortar and pestle. The slurry was filtered through Miracloth, and the filtrate was centrifuged at 10000*g* for 15 min. The supernatant was dialysed overnight against bicarbonate buffer containing 1 mM EDTA. The dialysed preparation was incubated for various times at 37° in bicarbonate buffer containing 1 mM EDTA, 5 mM $MgCl_2$, and DOVA-derived ALA. For this experiment, ALA was synthesized by incubating 0.5 ml of DOVA soln, 0.5 ml of 0.5 M glycine, and 4.0 ml of 50 mM EPPS buffer, pH 8.5, at 100° for 30 min. ALA was used without further purification. Enzyme incubations were stopped by the addition of $HClO_4$ to a final concn of 5%. After centrifugation to remove debris, ALA and porphobilinogen were measured by the methods of ref. [30]. Uroporphyrin was measured by the fluorometric method of ref. [16].

Materials. 5-Bromolevulinic acid was a gift of Dr. S. F. McDonald [32]; the other chemicals were of commercial origin.

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