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THE ROLE OF 4,5-DIOXOVALERIC ACID IN PORPHYRIN AND VITAMIN B_{12} FORMATION BY CLOSTRIDIA

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SUMMARY: 4,5-Dioxovalerate, which has been proposed as an intermediate in the newly discovered so-called C_5 pathway that leads from L-glutamate to δ-aminolevulinate, strongly inhibits uroporphyrin formation from δ-aminolevulinate in cells of Clostridium tetanomorphum and in cell-free extracts of this organism, in spite of the presence of L-alanine:4,5-dioxovalerate aminotransferase (aminolevulinate aminotransferase, EC 2.6.1.43). The interference by 4,5-dioxovalerate with porphyrin formation is due to strong inhibition of δ-aminolevulinate dehydratase (EC 4.2.1.24). Since 4,5-dioxovalerate hence effectively prevents the operation of the reaction sequence from L-glutamate to porphyrin, it is concluded that 4,5-dioxovalerate does not function as a physiological δ-aminolevulinate precursor.

INTRODUCTION

It was for many years assumed that ALA, the obligatory precursor of all tetrapyrroles in nature (1), is always formed physiologically via the condensation of succinyl-CoA and glycine in the reaction catalyzed by the enzyme ALA synthase (EC 2.3.1.37). The difficulty or impossibility to detect ALA synthase in many tetrapyrrole-producing organisms (2) was adduced to properties of ALA synthase itself or to inhibitors of this enzyme. Recently, however, it was shown that in plants and other photosynthetic organisms the 5-carbon skeleton of ALA can be formed directly from a 5-carbon compound such as L-glutamate or α-ketoglutarate (reviewed in 3-5). Studies with the anaerobic microorganism, Clostridium tetanomorphum, indicated that it does not utilize glycine or succinate for corrinoid formation (6). Another anaerobe, Eubacterium limosum [Butyribacterium rettgeri], incorporates ^{14}C-labelled carbon from glycine into the dimethylbenzimidazole part but not into the tetrapyrrole part of vitamin B_{12} (7). Since the lack of incorporation of glycine was

Abbreviations: ALA, δ-aminolevulinic acid; DOVA, 4,5-dioxovaleric acid

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not due to dilution of administered label by the internal pool of glycine, ALA synthase appears to be absent from this nonphotosynthetic organism. Initial studies with a partially purified enzyme from wheat leaves showed ALA formation from L-glutamate, provided ATP and a reduced pyridine nucleotide were present (8)

The pathway by which ALA is formed from glutamate or α-ketoglutarate is still unknown. Among a number of C_5 intermediates that have been postulated (3), DOVA has been favored (9-12). DOVA could be demonstrated in the green alga Scenedesmus obliquus (13), in Rhodopseudomonas spheroides and maize leaves (14), and in C. tetanomorphum (15) when the conversion of ALA to porphobilinogen, the next compound on the pathway to tetrapyrroles, is inhibited by levulinic acid. In addition, an enzyme was known that converts DOVA to ALA (16-18). This enzyme, L-alanine:DOVA aminotransferase (EC 2.6.1.43) has been purified to homogeneity from extracts of bovine liver mitochondria (19) and of C. tetanomorphum (15). The physiological precursor function of DOVA has not, however, been clearly established. In this paper the possible role of DOVA as a tetrapyrrole precursor in C. tetanomorphum has been investigated.

MATERIALS AND METHODS

ALA, GTP and coenzyme A were obtained from Sigma; 3,5-dibromolevulinic acid from Porphyrin Products; allylacetic acid from Pfalz and Bauer; succinyl-CoA synthetase from Boehringer Mannhein; Whatman LHP-K silica TLC plates from Pierce. ALA dehydratase was purified from C. tetanomorphum as described previously (20).

C. tetanomorphum (ATCC 15920) was grown on medium 163, American Type Culture Collection, as described before (21). After growth for 18 h at 37°C and harvesting, the bacteria were either used directly, or they were immediately frozen and stored at -20°C. Stock cultures were subcultured at 7-day intervals and stored at 4°C.

DOVA was prepared from 3,5-dibromolevulinic acid by the method of Gnuchev *et al.* (22) as described by Varticovski *et al.* (19), and assayed as the quinoxaline derivative using o-diaminobenzene as described by Jerzykowski *et al.* (23). Solutions were stored at pH 4.5 at -20°C.

To obtain 4,5-diaminovaleric acid, allylacetic acid was converted to 4,5-dibromovaleric acid (24). This compound was treated with a 10-fold molar excess of conc. ammonia at 115°C for 17 h in a pressure bomb. After cooling and addition of water the material was brought to dryness *in vacuo*. Absolute ethanol was added to the solid residue, the weakly yellow insoluble material was taken up in a small volume of water and brought to dryness *in vacuo*. Yield of buff-colored powder: 0.48 g from 5 g of allylacetic acid.

ALA dehydratase was measured, using a modification of the method of Shemin (25), by incubation of the enzyme in a solution, total vol 0.5 ml, containing 100 mM Tris-HCl, pH 8.2; 40 mM $MgCl_2$; 0.1 per cent β-mercaptoethanol; ALA and inhibitor as specified. After 20 min at 37°C the reaction was stopped by

addition of 0.5 ml of 0.3 M $HgCl_2$ in 2 N HCl. One ml of the modified Ehrlich reagent of Mauzerall and Granick (26) was added to the mixture. Any precipitate was removed by centrifugation, and the absorbance was read at 555 nm. ALA synthase activity was measured as described by Burnham (27). The reaction mixture, total volume 1.0 ml, contained 0.2 ml of enzyme sample and the following: 200 mM Tris-HCl, pH 7.4; 100 mM $MgCl_2$; 5 mM glycine; 5 mM sodium succinate; 0.5 µM pyridoxal phosphate; 8 µM coenzyme A; 20 µM GTP; 0.25 units of succinyl-CoA synthetase. After 3 h at 37°C the incubation was stopped by addition of 0.5 ml of 25 per cent trichloroacetic acid, followed by 1.5 ml of 2 M acetic acid-sodium acetate buffer, pH 4.6, and 20 µl of acetylacetone. Tubes were covered with glass marbles and placed in a boiling water bath for 20 min. After cooling, an equal volume of modified Ehrlich reagent (26) was added and the absorbance was read at 553 nm against suitable blanks.

Bacterial porphyrin synthesis was measured under various conditions, both *in vivo* and *in vitro*. For both types of experiment, the bacteria (2 or 4 g packed cell weight) were suspended in 10 ml of 100 mM phosphate buffer, pH 7.4, containing 0.1 per cent of β-mercaptoethanol. These suspensions were used directly for the *in vivo* experiments, while for the *in vitro* experiments they were passed twice through a French pressure cell at 19,000 psi, and centrifuged to remove debris. In each case the various substances investigated as precursors were added in the above buffer. The final volumes for both *in vivo* and *in vitro* experiments were 15 ml. Incubations were performed for 3 h in the dark at 37°C.

Porphyrins were determined by extraction of either the bacterial suspension or of the crude extract with an equal volume of ethylacetate-acetic acid (4:1 v/v). In both cases the mixture was agitated by means of a Vortex mixer for 60 sec, and centrifuged to break the emulsion. The upper phase, containing the porphyrins, was retained and exposed to light for 30 min to complete the oxidation of porphyrinogens to porphyrins. The porphyrins were then extracted into 2 ml of 2 N HCl by Vortex agitation of the samples for 30 sec. Uroporphyrin could be quantitated directly at 405 nm [A_{mM} = 541] (28), using an aliquot of the 2 N HCl extract. Alternatively, the porphyrin fraction could be brought to dryness *in vacuo*, esterified overnight with 2 ml of H_2SO_4-methanol (1:9, v/v) and extracted into chloroform by addition of 1 ml each of water and chloroform. After removal of the chloroform *in vacuo*, thin layer chromatography was performed on Whatman LHP-K plates, using the solvent benzene-ethylacetate-methanol (85:13.5:1.5 v/v/v) (29). The bands corresponding to uroporphyrin octamethylester were eluted with chloroform-methanol (8:2 v/v) and concentrations were determined at 406 nm [A_{mM} = 215] (28). The results obtained by the two methods were in good agreement. The first and more rapid method was used routinely.

RESULTS

We have extended the work of Burnham and Plane that suggested the absence of ALA synthase in C. tetanomorphum (6). ALA synthase could not be detected in crude extracts or in extracts fractionated on Sephadex G-150. In addition, no uroporphyrin was formed in a crude extract incubated with glycine, succinate, GTP and coenzyme A, with or without added succinyl-CoA synthetase.

Three different approaches were adopted to investigate the possible role of DOVA as a physiological ALA precursor: (1) comparison of DOVA with ALA as precursor of uroporphyrin; (2) effect of DOVA on formation of uroporphyrin from ALA; (3) effect of DOVA on ALA dehydratase.

TABLE I

Formation of uroporphyrin from DOVA and L-alanine

Suspensions of fresh cells of Clostridium tetanomorphum (4 g) or extracts from 4 g of frozen bacteria were incubated with additions indicated and worked up as described in Materials and Methods.

Additions	Uroporphyrin Formed (nmoles)
Intact Cells	
None	0.31
DOVA (5.3 mM) + L-Alanine (6.7 mM)	0.37
ALA (6.7 mM)	89
Crude Extract	
None	2.4
DOVA (5.3 mM) + L-Alanine (6.7 mM)	2.3
ALA (6.7 mM)	48

Both intact resting C. tetanomorphum cells and cell-free extracts of C. tetanomorphum did not convert DOVA in the presence of L-alanine to uroporphyrin (Table I). It will be noted that under identical conditions added ALA did lead to uroporphyrin (Table I). Moreover, DOVA actually inhibited the formation of uroporphyrin from ALA by resting cells of C. tetanomorphum and by cell-free extracts of this organism (Table II), even in the presence of 40-fold excess of ALA (Table III).

In order to determine the basis for the inability of DOVA itself to be converted to uroporphyrin and for the interference of DOVA with porphyrin formation from ALA in spite of the presence in this organism of a DOVA aminotransferase, the effect of DOVA on ALA dehydratase was investigated. DOVA was found to be a strong inhibitor of ALA dehydratase (Fig. 1). The inhibition was of the mixed type, similar to inhibition by levulinic acid, the first widely used inhibitor of the enzyme (Fig. 2). DOVA (K_I =0.4 mM) is in fact a much stronger inhibitor of ALA dehydratase than levulinic acid (K_I = 5 mM). It inhibits as strongly as succinylacetone, a powerful competitive inhibitor (K_I = 0.4 mM) of ALA dehydratase (21).

TABLE II

Formation of uroporphyrin from ALA in the presence of DOVA

Suspensions of cells, or extracts from 2 g of frozen Clostridium tetanomorphum were incubated with additions indicated and worked up as described in Materials and Methods.

Additions	Uroporphyrin Formed (nmoles)
Intact Cells	
None	0.20
ALA (6.7 mM)	37
DOVA (5.3 mM) + L-Alanine (6.7 mM) + ALA (6.7 mM)	2.4
Crude Extract	
None	0.45
DOVA (5.3 mM) + L-Alanine (6.7 mM)	0.35
ALA (6.7 mM)	8.2
DOVA (5.3 mM) + L-Alanine (6.7 mM) + ALA (6.7 mM)	0.70

A compound suggested by Beale (3), 4,5-diaminovaleric acid, was also inactive, and in fact inhibited porphyrin formation from ALA (Table IV).

DISCUSSION

The candidacy of DOVA as a porphyrin precursor in C. tetanomorphum is considerably weakened by the marked inhibitory effect of DOVA on uroporphyrin formation from ALA and by the inability of DOVA to serve as a porphyrin precursor with intact cells or with cell-free extracts. The inhibitory effect of DOVA on tetrapyrrole formation is almost certainly entirely due to its inhibition of ALA dehydratase. DOVA could function as a porphyrin precursor only if its K_M for the transaminase were much lower than its K_I for the dehydratase. In C. tetanomorphum, however, these two values for DOVA are very similar [0.26 mM vs. 0.4 mM] (15, 20). A further argument against a precursor role of DOVA is given by the fact that for the ALA dehydratase the K_I of DOVA [0.4 mM] is actually less than the K_M of ALA [0.8 mM] (20).

Various other observations support the conclusion that DOVA is not a porphyrin precursor. Thus it has been shown very recently that L-alanine:DOVA

TABLE III

Effect of different DOVA concentrations on uroporphyrin formation from excess ALA

Suspensions of 2.4 g aliquots of Clostridium tetanomorphum from the freezer were incubated for 4 h at 37°C in 15 ml of 100 mM phosphate buffer, pH 7.4, containing 0.1 per cent of β-mercaptoethanol, 12 mM ALA, 11 mM L-alanine and varying amounts of DOVA. Uroporphyrin was extracted and read directly as given in Materials and Methods.

DOVA (mM)	Absorbance (402 nm)	Uroporphyrin (nmoles)
0	0.462	95.5
0.33	0.442	91.2
0.67	0.435	89.8
1.33	0.364	75.2
2.67	0.240	49.5
4.00	0.126	25.9
5.33	0.066	13.5

aminotransferase is identical to L-alanine:glyoxylate aminotransferase (EC 2.6.1.44) from bovine liver mitochondria (30). In fact the rate of transamination by this enzyme is 60-fold greater with glyoxylate than with DOVA.

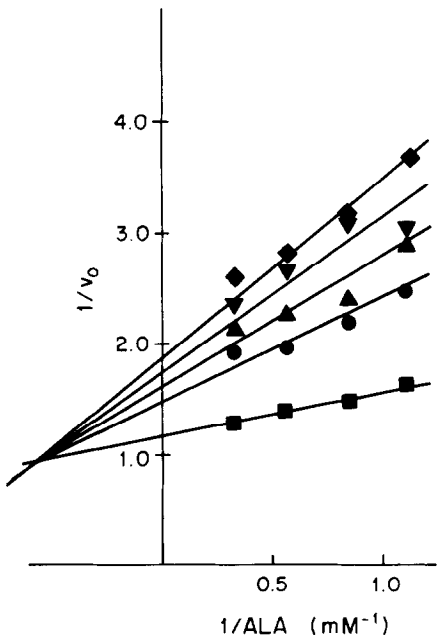

FIG. 1. Inhibition of ALA dehydratase by DOVA. Assays were performed with the purified enzyme from Clostridium tetanomorphum as described in Materials and Methods. Concentrations of DOVA used were: (■) 0, (●) 0.8 mM, (▲) 1.2 mM, (▼) 1.6 mM, (◆) 2.0 mM.

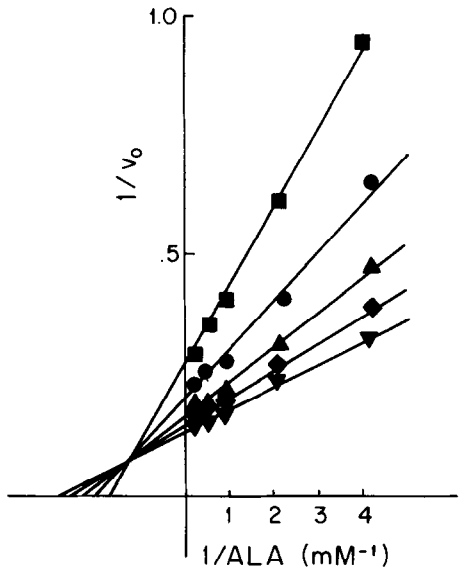

FIG. 2. Inhibition of ALA dehydratase by levulinic acid. Assays were performed with the purified enzyme from Clostridium tetanomorphum as described in Materials and Methods. Concentrations of levulinate used were: (▼) 0, (◆) 0.5 mM, (▲) 1.0 mM, (●) 2.0 mM, (■) 5.0 mM.

The argument that DOVA is a physiological ALA precursor, based on the existence of an enzyme that specifically and irreversibly forms ALA from DOVA, has hence become much less compelling. Furthermore, the observation that DOVA cannot be detected in various organisms in the absence of an added inhibitor of ALA dehydratase (13-15) does not prove that DOVA is a physiological precursor of ALA. Studies with etiolated barley leaves point to a breakdown of ALA to DOVA (31). A variety of rat tissues can also metabolize ALA by transamination (32). An alternative possibility, that DOVA might be formed from ALA by oxi-

TABLE IV

Formation of uroporphyrin from 4,5-diaminovaleric acid

2.0 g aliquots of Clostridium tetanomorphum were suspended in 15 ml of 100 mM phosphate buffer, pH 7.4, containing 0.1 per cent of β-mercaptoethanol and the additions indicated, incubated for 4 h at 37°C and worked up for uroporphyrin as described in Materials and Methods.

Additions	Uroporphyrin Formed (nmoles)
None	0.19
4,5-Diaminovalerate (12 mM)	0.20
ALA (6.7 mM)	40.6
ALA (6.7 mM) + 4,5-Diaminovalerate (12 mM)	9.5

dative deamination, has not been investigated. It is likely, indeed, that DOVA is found not because it is a precursor but because it is a breakdown product of ALA.

DOVA has recently been reported to be a porphyrin precursor in respiring rat hepatocytes, but all activity was lost with hypoxic or with disrupted cells (33). It is unlikely that under these conditions DOVA transaminase would be lost. It is in keeping with the data presented (33) that DOVA is converted to succinyl-CoA in intact respiring mitochondria.

It had previously been suggested (21) that the inhibitory effect of a number of substances such as levulinic acid, succinylacetone and succinylacetone pyrrole on ALA dehydratase is related to the presence of a succinyl group in these compounds, resembling that in ALA. A similar structural correlation for the first two of these inhibitors was advanced independently by Meller and Gassman (34). It is clear that DOVA is yet another substance that must be added to this list.

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