ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 93, 542–545 (1960)

The Biosynthesis of 3-Nitropropanoic Acid by *Penicillium atrovenetum*[1]

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Received February 1, 1961

Maximum production of 3-nitropropanoic acid by *Penicillium atrovenetum* occurs when the culture medium contains an auxiliary carbon source and ammonium ion. The most suitable carbon sources are dicarboxylic acids such as fumaric, succinic, and tartaric. Aspartic acid is presumably deaminated prior to its utilization in the biosynthesis. The addition of an oxidized form of ammonia to fumarate is proposed as a possible first step in the biosynthetic sequence.

INTRODUCTION

The material present in *Indigofera endecaphylla* which is toxic to fowl and mammals has been reported to be 3-nitropropanoic acid (3-NPA) (1). The mechanism of this toxicity has, as yet, not been determined. To facilitate an investigation of this toxicity, 3-NPA labeled with C^{14} was deemed desirable. The mold *Penicillium atrovenetum* liberates 3-NPA into the medium on which it is grown (2), and incorporation of suitably labeled carbon sources into the medium would presumably result in the production of the radioactive acid. Birch *et al.* (3) have suggested that aspartic acid is a direct precursor in the biosynthesis of 3-NPA by *P. atrovenetum*. Our preliminary experiments did not confirm this proposal. Therefore, the effectiveness of various carboxylic acids as precursors of 3-NPA was investigated to determine which was most suitable for the synthesis. The results of these investigations together with some conclusions on the biosynthesis of 3-NPA by *P. atrovenetum* are reported in this paper.

EXPERIMENTAL

MATERIALS

The organic acids used in this investigation were obtained from the following sources and were not further purified. Succinic, fumaric, and DL-aspartic acids from Distillation Products Industries; lactic, tartaric, and acetic acids from Merck; β-alanine and L-aspartic acid from Nutritional Biochemicals; and L-malic and oxalacetic acids from Mann Research. The remaining chemicals used were A.C.S. reagent grade. Cultures of *P. atrovenetum* were kindly provided by Prof. J. H. Birkinshaw, London School of Hygiene and Tropical Medicine.

CULTURAL CONDITIONS

P. atrovenetum, strain S.M.683, was maintained, by monthly transfer, on solidified Raulin-Thom medium (2).

Two basal media were used experimentally. These are:

Raulin-Thom Base: 50 g. anhydrous glucose; 0.4 g. $(NH_4)_2HPO_4$; 0.2 g. $(NH_4)_2SO_4$; 0.4 g. K_2CO_3; 0.2 g. $MgCO_3$; 0.05 g. $FeSO_4.7H_2O$; 0.07 g. $ZnSO_4.7H_2O$; water to 800 ml.

Czapek-Dox Base: 50 g. anhydrous glucose; 2.0 g. $NaNO_3$; 1.0 g. KH_2PO_4; 0.5 g. KCl; 0.5 g. $MgSO_4.7H_2O$; 0.01 g. $FeSO_4.7H_2O$; water to 800 ml. To 80 ml. of basal medium was added the required amount of nitrogen in the form of ammonium hydroxide, the solution was adjusted to pH 4.5, placed in a 500 ml. Erlenmeyer flask and sterilized at 120° for 15 min. When the solution had cooled, a sterile solution of carbon precursor, adjusted to pH 4.5, was added to give a final volume of 100 ml. The media were inoculated with a heavy spore suspension from 30-day-old cultures of *P. atrovenetum*. Inoculated flasks were incubated in the dark at 23–28°C. for 5 days.

[1] Published with the approval of the Director of the Hawaii Agricultural Experiment Station as Technical Paper No. 516.

METHODS

Mycelial Dry Weight

The mycelial mat was collected on a tared disk of Whatman No. 1 filter paper, washed with distilled water, and dried to constant weight at 85°C.

Assay for 3-Nitropropanoic Acid

A 5-ml. aliquot of culture filtrate was shaken with 10 ml. ethyl ether for 1 min. Five milliliters of the ether layer was evaporated to dryness, and the 3-NPA was determined by the method of Matsumoto *et al.* (4).

RESULTS

The initial experiments were designed to determine the most suitable precursor from among a series of mono- and dicarboxylic acids. β-Alanine was included because of its structural analogy to 3-NPA. The results in Table I reveal that the best precursors are four-carbon dicarboxylic acids.

The second series of experiments were performed to test the suggestion (3) that aspartic acid was a direct precursor of 3-NPA. The carbon sources were tested with and without added ammonium ion, and the results are presented in Table II. These data confirm the radiochemical evidence (3) that β-alanine is not an intermediate in the biosynthesis of 3-NPA by this organism.

One criticism of the above experiments is the use of racemic aspartate. It is conceivable that if one isomer were used in the synthesis, the other might behave as an inhibitor and such a situation could explain the results obtained. To overcome this objection an experiment was designed to detect the inhibition (if any) by (*a*) altering the concentration of isomers, and (*b*) using only the L isomer. If the latter were the intermediate and the D isomer an inhibitor, then the use of only the L form should result in an increased yield of 3-NPA. If the converse were true, decreased amounts of 3-NPA would be expected. Similarly, addition of the L isomer to a racemic mixture should cause detectable changes in the amount of 3-NPA synthesized if the stereoisomerism were a significant factor. The data pre-

TABLE I

THE EFFECTS OF VARIOUS CARBON COMPOUNDS ON THE PRODUCTION OF 3-NITROPROPANOIC ACID BY *Penicillium atrovenetum*

Acid added[a]	Mycelial dry wt.	3-NPA/100 ml.	μg. 3-NPA / mg. dry wt.
	mg.	*mg.*	
None	400	0.4	1.0
	681	0.6	0.9
β-Alanine	585	4.7	8.0
L-Lactic	1165	13.3	11.4
Acetic	380	6.8	18.0
Succinic	1145	49.2	43.0
	1061	42.6	40.0
Fumaric	1290	60.1	46.6
	813	37.6	46.3
L-Malic	1078	28.0	26.0
Oxalacetic	875	26.8	30.7
DL-Tartaric	1085	48.2	44.4

[a] Two millimoles/100 ml. Raulin-Thom basal medium containing an extra 2 mmoles NH_4^+.

TABLE II

THE EFFECTS OF AMMONIUM ION AND VARIOUS CARBON COMPOUNDS ON THE PRODUCTION OF 3-NITROPROPANOIC ACID BY *Penicillium atrovenetum*

Acid added[a]	NH_4^+	Raulin-Thom medium		Czapek-Dox medium	
		Mycelial dry wt.	3-NPA/100 ml.	Mycelial dry wt.	3-NPA/100 ml.
	mmoles	*mg.*			
None	0	—	—	590	0.5
	2	449	0.6	772	0.0
		650	0.5	580	0.5
Succinic	0	—	—	608	0.5
	2	632	37.6	—	—
		1061	20.0	762	41.6
Fumaric	0	870	0.0	682	0.6
	2	652	30.4	896	48.2
		813	37.6	784	42.0
DL-Aspartic	0	980	0.7	1259	3.6
		1114	0.8	999	0.5
	2	1056	37.2	1532	37.2
		892	34.0	1380	27.6

[a] Sterilized separately and added aseptically to the basal media at the rate of 2 mmoles/100 ml. final volume.

TABLE III

THE EFFECTS OF ISOMERS OF ASPARTIC ACID ON THE PRODUCTION OF 3-NITROPROPANOIC ACID BY *Penicillium atrovenetum*

Acid added[a]	Amount	NH_4^+	Raulin-Thom medium		Czapek-Dox medium	
			Mycelial dry wt.	3-NPA	Mycelial dry wt.	3-NPA
	mmoles		*mg.*			
DL-Aspartic	2	0	1114	0.8	999	0.5
	2	2	892	34.0	750	37.6
L-Aspartic	2	0	758	0.6	864	0.6
	2	2	824	33.6	934	33.6
DL-Aspartic + L-Aspartic	1 + 1	2	1027	34.8	905	36.3

[a] Separately sterilized and added to basal media to give a final volume of 100 ml.

sented in Table III demonstrate that the configuration of aspartate does not detectably influence the synthesis of 3-NPA.

DISCUSSION

Maximum production of 3-nitropropanoic acid by *P. atrovenetum* requires the presence of ammonium ion and an auxiliary carbon source in the medium. The most suitable compounds are the four-carbon dicarboxylic acids, succinic, fumaric, and tartaric acids. These and other related acids listed in Table I are presumably interconvertible by accepted biochemical reactions, with the exception of tartrate. The metabolism of tartrate by molds is poorly understood, and its participation as an intermediate is based on the experimental evidence presented. Tartrate could be converted to oxalacetate by dehydration, but no evidence is available to support this transformation.

The data presented tend to eliminate aspartate as a significant intermediate in the biosynthesis of 3-nitropropanoate. If it were indeed a direct precursor then presumably the alpha amino group would be oxidized *in situ*. Added ammonium ion should have no effect on the production of 3-NPA, and the amount formed should be similar to that produced in media containing succinate or fumarate plus ammonium ion. The results show that in the absence of ammonium ion almost no 3-NPA is produced regardless of the carbon source. In the presence of ammonium ion fumarate and succinate are as good precursors as aspartate. This must mean that aspartate is deaminated and the carbon chain enters the four-carbon dicarboxylic acid pool, presumably as oxalacetate or fumarate.

It is not known whether the nitrogen of 3-NPA is attached to the four-carbon precursor or to some three-carbon intermediate, and the oxidation state of the nitrogen at the time of its incorporation into the molecule has yet to be determined. Amination of the carbon chain seems to be eliminated by the results obtained with aspartic acid, even though ammonium ion is required for maximum synthesis. Nitrite ion is present in nitrate-containing media, such as Czapek-Dox, but the low yields of 3-NPA obtained with this medium when ammonium ions are absent seems to eliminate direct participation of nitrite ion.

An attractive hypothesis is the addition of hydroxylamine to fumarate analogous to the addition of ammonia catalyzed by aspartase (5). However, when hydroxylamine was incorporated into inoculated media or replacement media no 3-NPA was synthesized. This is not too surprising in view of the toxic properties of the compound. Nevertheless, biosynthesis of 3-NPA by the addition of some nitrogen compound to fumarate is a definite possibility. Of the carbon compounds tested, fumarate is the most acceptable precursor. Not only are the yields of 3-NPA superior when fumarate is the auxiliary carbon source but aspartate can be directly converted to fumarate in a reaction catalyzed by the enzyme aspartase (5). This enzyme has been demonstrated in *Penicillium sp.* (6) and is being sought in the organism used in the present investigation.

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