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On the Biogenesis of β-Nitropropionic Acid

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The incorporation of aspartate-^{15}N-^{14}C(U) into β-nitropropionic acid has been studied and evidence obtained for an *in situ* oxidation of the amino group of aspartate to the level of a nitro group in *Penicillium atrovenetum*. From the culture medium of the same organism hydroxylamine has been isolated and its presence is discussed in relation to the biosynthesis of β-nitropropionic acid.

In studies of the biosynthesis of β-nitropropionic acid produced by *Penicillium atrovenetum*, Birch [1] observed that β-alanine-1-^{14}C was not incorporated into the carbon skeleton of nitropropionic acid whereas aspartate-4-^{14}C labelled the carboxyl group. This group was also found to be labelled from sodium bicarbonate-^{14}C. These results have been confirmed in our laboratory and further extended by including uniformly labelled aspartate-^{14}C as tracer substrate. The demonstration of a uniform labelling in the three carbon atoms of the β-nitropropionic acid formed (C-1, 4.6×10^3; C-2, 4.2×10^3; C-3, 5.1×10^3 cpm/mmole) from the uniformly labelled aspartate emphasizes the origin of the carbon chain from a dicarboxylic acid of the citric acid cycle. These experiments, however, cannot contribute to the elucidation of the possible direct relationship between the nitrogen of aspartate and that of nitropropionic acid. Hylin and Matsumoto [2] have, however, studied the substrate dependence of nitropropionic acid formation; they suggested that the amino group of aspartate is not oxidized *in situ* to form the nitro group, and that aspartate is only a good source for furnishing the dicarboxylic acid, presumably fumaric acid.

To obtain further information on this point we have undertaken some experiments with aspartate labelled with ^{15}N as well as with ^{14}C. Even though the initial ratio of isotope content (^{14}C/^{15}N) of the added aspartate was not retained in the isolated nitropropionic acid in these experiments (Table 1), the level of ^{15}N content in the nitro group still exceeded the average ^{15}N excess of the culture medium. Apparently the amino group of aspartate had been utilized for the formation of the nitro group in preference to the ammonium ions which were the major competing nitrogen source of the medium. Because of the rapid exchange of the amino group of aspartate in different transamina-

Table 1. Incorporation of aspartic acid-^{15}N-^{14}C into β-nitropropionic acid.

Sample	Radioactivity cpm/mg C	^{15}N content, atom %	^{14}C/^{15}N
Standard (ammonium tartrate)	—	0.022	—
Added aspartic acid	23 800	11.540	1995
Isolated β-nitropropionic acid	1 335	2.493	536
N from the culture medium	—	1.360	—

tion reactions, the ^{14}C/^{15}N ratio even for aspartate itself, cannot be expected to last for more than a short period of time. It is, however, possible that the amino group of aspartate is only a more accessible form of the nitrogen than the ammonium ions for the formation of the nitro group so that the results do not definitely prove that the amino group is oxidized while still being attached to the α-carbon of aspartate.

In experiments where the ammonium ions have been substituted with glutamate a much higher dilution effect of ^{15}N in the nitro group was observed after a period of 2 h (Table 2). On the other hand aspartate labelled with ^{14}C was found to be taken up from the medium and metabolized to 99 % in less than 5 min, indicating that nitropropionic acid could very well be formed directly from aspartate despite the rapid exchange of ^{15}N in the presence of glutamate.

Another source of nitrogen tested as a precursor for the nitro group in a similar experiment has been hydroxylamine which could possibly add to fumarate, or form an oxime with oxaloacetate which could be subsequently oxidized to the level of a nitro group. It was found that hydroxylamine had a slight diluting effect on the nitrogen of nitropropionic acid but the effect was to low to consider seriously hydroxylamine as a precursor of the nitro group. However, in the culture medium of the organism growing on substrate containing ammonium ions as the nitrogen source a production of free hydroxylamine was observed by using colorimetric methods and paper chromatography. The hydroxylamine formation ran parallel to the nitropropionic acid production

Table 2. Dilution effects on ^{15}N content of β-nitropropionic acid.

Substrate	Atom % ^{15}N in β-nitropropionic acid
0.25 mM aspartic acid-^{15}N + 1.00 mM hydroxylamine HCl	3.9
0.25 mM » + 1.00 mM glutamic acid	1.9
0.25 mM » + endogenous N (Added aspartic acid containing 11.5 atom % ^{15}N).	4.7

Table 3. Production of β-nitropropionic acid and hydroxylamine.

Days	pH	β-nitroprop. acid μg/ml	NH_2OH μg/ml	β-nitroprop. acid/NH_2OH
0	4.0	—	—	—
2	2.6	13	0.06	217
3	2.1	450	1.1	409
4	1.8	670	1.6	418

(Table 3) and it was found to be dependent of the concentration of ammonium ions in the medium so that an increased amount of ammonium ions gave rise to a higher level of hydroxylamine.

In later stages of growth, nitrite also appears in the culture medium as well as hydroxylamine (Table 4). The nitrite may be formed either by a further oxidation of hydroxylamine, or by decomposition of the formed nitropropionic acid. The latter reaction has been demonstrated by Little [3] to occur with an enzyme extract from *Neurospora crassa*. In control experiments it was found that both these possibilities occurred, particularly at neutral pH. If the amino group in aspartate is oxidized *in situ* to a nitro group, possible intermediates would be β-oximinopropionic acid or nitrososuccinic acid (the oxime of oxaloacetate). The former compound was prepared labelled with ^{14}C in α- and β-positions and added to the media of growing cultures of *P. atrovenetum* as well as to cultures with the original medium replaced with an aqueous solution of the radioactive oximinopropionic acid. No radioactive nitropropionic acid could be isolated in these experiments and no stimulation of the production of nitropropionic acid was observed by using nonlabelled nitrososuccinic acid in similar experiments.

Table 4. Production of hydroxylamine and nitrite.

Days	Standard medium pH	NH_2OH μg/ml	NO_2^- μg/ml	NP*	Buffered medium pH	NH_2OH μg/ml	NO_2^- μg/ml	NP*
0	3.9	—	—	—	6.4	—	—	—
2	3.6	—	—	—	5.8	—	—	—
3	3.2	0.05	—	—	5.5	0.06	—	—
5	3.1	0.18	0.08	+	6.2	0.07	0.11	—
6	4.0	0.32	0.07	+	6.0	0.09	0.27	—
7	7.2	0.17	0.74	+	6.2	0.06	0.37	—

* NP = β-nitropropionic acid,
+ = NP present, — = NP absent

Negative results from this kind of experiments should be interpreted with utmost caution as so many unknown factors are involved before the substance reaches the intracellular region for further transformations.

Of the results reported here, it seems probable that the isotope experiments are the most likely to provide significant information about the biosynthesis of β-nitropropionic acid. We therefore believe that the results indicate an *in situ* oxidation of the amino group of aspartate to higher levels of oxidation, and we consider that hydroxylamine is a decomposition product of some intermediate in the oxidation.

EXPERIMENTAL

Culture conditions. Penicillium atrovenetum G. Smith L. S. H. T. M. SM 683 was grown as surface culture in Fernbach flasks on Raulin-Thom medium containing: anhydrous glucose, 75 g; tartaric acid, 4.0 g; ammonium tartrate, 15 g; $(NH_4)_2HPO_4$, 0.6 g; $(NH_4)_2SO_4$, 0.25 g; K_2CO_3, 0.6 g; $MgCO_3$, 0.4 g; $FeSO_4.7H_2O$, 0.07 g; $ZnSO_4.7H_2O$, 0.07 g and distilled water to 1.5 l.

Material. β-Alanine-1-^{14}C was prepared from ethylene chlorohydrin and $Na^{14}CN$.[4,5]

DL-Aspartic acid-4-^{14}C was synthesized from CH_3 $^{14}COONa$.[4] Maleamic acid-2,3-^{14}C was prepared from maleic anhydride-2,3-^{14}C as described by Anschütz.[6]

β-Oximinopropionic acid-2,3-^{14}C was obtained from maleamic acid by using the method described by Rinkes.[7]

L-Aspartic acid-^{15}N was biologically prepared with *Escherichia coli* from fumaric acid and $^{15}NH_4NO_3$ as described by Wu *et al.*[8] L-Aspartic acid-^{15}N-^{14}C(U) was obtained by precipitation of an aqueous solution of L-aspartic acid-^{15}N and L-aspartic acid-^{14}C(U) with ethanol.

Nitrososuccinic acid diethylester was synthesized as described by Schmidt and Widmann.[9]

Isolation of β-nitropropionic acid. The culture medium was acidified with hydrochloric acid and extracted with ether. After evaporation of the ether phase to dryness the nitropropionic acid was isolated either by paper chromatography (chloroform-methanol-4 % formic acid, 10/1/1, R_F 0.45) or by sublimation at 10 mm Hg at 100°C. Recrystallisations from xylene and a mixture of chloroform-carbon tetrachloride gave pure β-nitropropionic acid.

Degradation of β-nitropropionic acid. The labelled nitropropionic acid dissolved in 50 % ethanol was hydrogenated with Raney-Nickel as catalyst to β-alanine at atmospheric pressure. The β-alanine formed was decomposed to acrylic acid which was then reduced to propionic acid as described by Lagerkvist.[10] A stepwise Schmidt degradation of the propionic acid allowed the radioactivity of the individual carbon atoms to be measured.

The nitrogen of β-alanine was converted to ammonium chloride by the Kjeldahl procedure before mass analysis.

Determinations of hydroxylamine and nitrite. The amount of hydroxylamine and nitrite in the culture medium were determined colorimetrically by using the methods described by Csáky [11] and Grossowic.[12] In order to isolate hydroxylamine the culture medium was made alkaline with sodium hydroxide and then steam distilled into hydrochloric acid. Evaporation of the acid solution gave a residue mainly consisting of ammonium chloride. The hydrochloride of hydroxylamine was extracted from the residue with butanol. To show the identity of hydroxylamine the extract was chromatographed on Whatman 1 paper in three different solvents: (1) butanol-ethanol-water-conc. HCl (10/10/5/2), R_F 0.43, (2) 95 % ethanol-6 N HCl (70/30), R_F 0.36, (3) butanol-2 N HCl (1/1), R_F 0.22. The hydroxylamine spot was developed with picryl chloride and ammonia as described by Bremner.[13]

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