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Biosynthesis of Nitro Compounds. III. The Enzymatic Reduction of β-Nitroacrylic Acid to β-Nitropropionic Acid*

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ABSTRACT: Crude extracts of *Penicillium atrovenetum* catalyze the reduction of β-nitroacrylic acid to β-nitropropionic acid in the presence of reduced nicotinamide–adenine dinucleotide phosphate (NADPH). The enzyme(s) associated with this reaction has been termed β-nitroacrylic acid reductase. None of several unsaturated compounds, aliphatic and aromatic nitro compounds, and inorganic nitrogen compounds could replace β-nitroacrylic acid as a substrate. Reduced nicotinamide–adenine dinucleotide (NADH) could not substitute for NADPH as the reducing agent. β-Nitroacrylic acid reductase has been partially purified, and some of its properties have been investigated. Determinations of the stoichiometry of the reaction indicated that 1 equiv of β-nitroacrylic acid was reduced and 1 equiv of NADPH oxidized/equiv of β-nitropropionic acid formed. At growth-inhibitory levels of β-nitroacrylic acid, β-nitropropionic acid synthesis was inhibited 50%, and the incorporation of radioactivity from [4-^{14}C]aspartic acid into β-nitropropionic acid was nearly completely inhibited. In the presence of unlabeled aspartic acid, [1-^{14}C]-β-nitroacrylic acid was incorporated into β-nitropropionic acid.

These results suggested that β-nitroacrylic acid is a precursor of β-nitropropionic acid and that β-nitroacrylic acid reductase is involved in the biosynthesis of the saturated nitro compound.

It has been reported that growing cultures of *Penicillium atrovenetum* synthesize β-nitropropionic acid only in the presence of ammonium ion and a four-carbon, dicarboxylic acid (Raistrick and Stössl, 1958; Hylin and Matsumoto, 1960; Shaw and Wang, 1964). The results of Birch *et al.* (1960), Birkinshaw and Dryland (1964), Gatenbeck and Forsgren (1964), and Shaw and McCloskey (1967) show that the carbon skeleton of aspartic acid was incorporated intact into β-nitropropionic acid. The role of the aspartic acid amino group as a precursor to the nitro group is still open to question, although considerable evidence has accumulated which indicates that the amino group is used in preference to ammonium ion for synthesis of the nitro group (Gatenbeck and Forsgren, 1964; Shaw and McCloskey, 1967).

In an attempt to identify intermediates between aspartic acid and β-nitropropionic acid, compounds related to β-nitropropionic acid were synthesized and tested as precursors of the nitro compound. The conversion of one of these compounds, β-nitroacrylic acid, to β-nitropropionic acid was catalyzed by extracts of *P. atrovenetum*. The isolation of the enzyme(s) which carries out this reaction and a description of some of its properties are discussed in this communication. In addition, the possible function of β-nitroacrylic acid as an intermediate between aspartic acid and β-nitropropionic acid has been investigated.

Materials and Methods

Materials were obtained from the following sources: DL-[4-^{14}C]aspartic acid and [^{14}C]potassium cyanide from New England Nuclear Corp., acrylic acid and 2-bromoethanol from Eastman Organic Chemicals,

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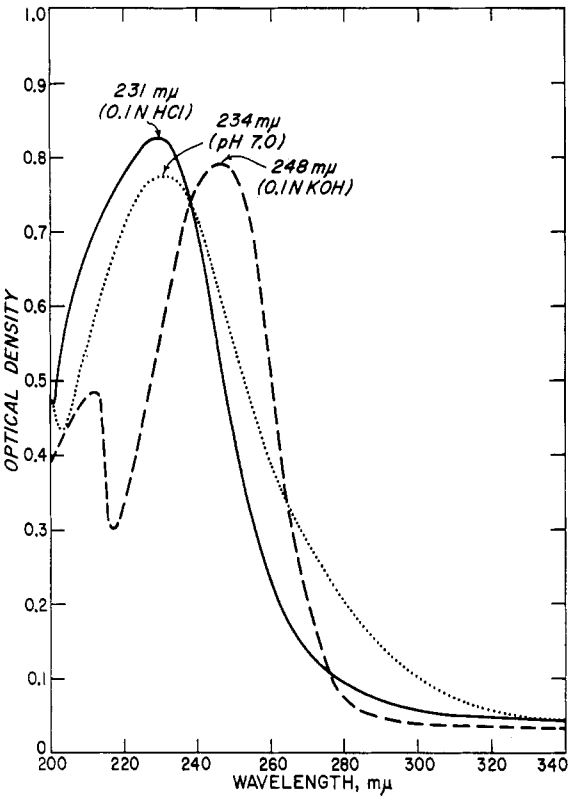

FIGURE 1: Ultraviolet spectra of β-nitroacrylic acid. β-Nitroacrylic acid was dissolved in the indicated solvent at a concentration of 8.45×10^{-5} M, and the spectra were run on a Bausch and Lomb Model 505 spectrophotometer. The millimolar extinction coefficients (at λ_{max}) calculated from these spectra are 9.18, 8.50, and 8.94 in 0.1 N hydrochloric acid, 0.1 M potassium phosphate buffer (pH 7.0), and 0.1 N potassium hydroxide, respectively.

NADP[1] and isocitric dehydrogenase from California Biochemical Corp., and isocitric acid trisodium salt from Nutritional Biochemical Corp. Mallinckrodt no. 2847 silicic acid which had been acid washed and sieved was used for column chromatography. The 100–200 mesh fraction was used. Merck silica gel G, obtained from the Brinkmann Instrument Co., was used for thin layer chromatography. All reagents and the components of the culture media were prepared from reagent grade chemicals.

Synthesis of Substrates. 2-Chloro-3-nitropropionic acid was synthesized by the addition of nitryl chloride to acrylic acid by the method of Shechter *et al.* (1952). The product was purified by chromatography on silicic acid columns using chloroform as the eluent followed by recrystallization of this partially purified product from chloroform (mp 78–80°). For large-scale preparations (32 g), the 2-chloro-3-nitropropionic acid from the silicic acid column was dehydrohalogenated without further purification. Schechter's conditions were used; however, in our hands, no reaction occurred unless 2 equiv of glacial acetic acid was added to the reaction mixture. The crude β-nitroacrylic acid was purified by chromatography over silicic acid using chloroform as the eluent and recrystallization from a mixture of ether and hexane (yield 13 g, 54%, mp 134–136°).

β-Nitroacrylic acid is stable when pure and when kept dry and in the dark. It is highly soluble in water and in most organic solvents except chloroform, carbon tetrachloride, and hydrocarbons. It is unstable in solution, particularly in the presence of air and light. Aqeuous solutions turn yellow and then brown in 5–10 min. This apparent decomposition can be minimized by keeping solutions under an atmosphere of nitrogen and in an ice bath. Under these conditions, solutions prepared for use as substrates are stable for 2–3 hr. The ultraviolet spectrum of β-nitroacrylic acid is shown in Figure 1. NADPH was prepared by the reduction of NADP with sodium isocitrate in the presence of isocitric dehydrogenase (Rafter and Colowick, 1957).

[1-^{14}C]Methyl acrylate was synthesized starting from 2-bromoethanol and [^{14}C]potassium cyanide by the method of R. F. Nystrom (personal communication). The ester was saponified by allowing it to stand in 2 N potassium hydroxide for 1 hr at room temperature. The resulting potassium salt (2.05 g, 18.6 mmoles) was diluted with unlabeled acrylic acid (3.00 g, 41.7 mmoles), and the mixture was allowed to equilibrate on a reciprocal shaker for about 18 hr. A portion of the free acid could be removed by vacuum distillation. The residue after distillation was acidified with 1 N sulfuric acid and continuously extracted with ether for 48 hr. After drying and removal of the ether, the residue was combined with the acrylic acid from the distillation (yield 4.3 g, 99% based on the combined acrylic acid and potassium salt). The labeled acrylic acid was converted to β-nitroacrylic acid *via* 2-chloro-3-nitropropionic acid as described previously except that the dehydrohalogenation was accomplished by thermal decomposition *in vacuo* (Shechter *et al.*, 1952). The [1-^{14}C]β-nitroacrylic acid was purified by chromatography and recrystallization as described before; the over-all yield based on the starting [^{14}C]-potassium cyanide was 5%, sp act. 2.4 μc/mmole.

Culture Conditions. P. atrovenetum cultures were maintained on agar slants as described previously (Shaw and Wang, 1964). The fungus was grown in the dark on liquid Raulin–Thom medium as modified by Raistrick and Stössl (1958) unless otherwise noted. The medium was used either in 100-ml portions in shaken cultures (Shaw and Wang, 1964) or, for large-scale isolation of mycelium, the organism was grown in 10-l. batches in a New Brunswick Model FS-314 fermenter. In this latter case, the temperature was maintained at 26° and the agitation rate at 200 rpm. Various aera-

[1] Abbreviations used: NADPH, reduced nicotinamide–adenine dinucleotide phosphate; NADP, nicotinamide–adenine dinucleotide phosphate; NADH, reduced nicotinamide–adenine dinucleotide; FMN, flavin mononucleotide; FAD, flavin–adenine dinucleotide.

tion rates from 1 to 10 l./min were used. There was little effect on the enzyme level under these various conditions, although the fungus grew much faster at the higher aeration rates.

Mycelium was harvested near the middle of the log phase of growth. For the shake cultures this was about 3 days and for the fermenter grown cultures about 3–4 days depending on the aeration rate. The mycelium was washed with water and then either used immediately for the preparation of enzyme or stored in 10-g batches at −30° for future use.

Preparation and Purification of Cell-Free Extracts. Crude mycelial extracts were prepared by two methods. Portions of mycelium (10 g) were homogenized in 30 ml of 0.05 M potassium phosphate buffer (pH 7.3). This suspension was forced through a French pressure cell (American Instrument Co.) at 15,000–20,000 psi. In the second method, the mycelial homogenate was mixed with 25 g of glass beads (15–20-μ diameter, obtained from the Minnesota Mining and Manufacturing Co.) which had been previously washed with hot 6 N hydrochloric acid, water, and methanol. This mixture was ground in a small colloid mill (Eppenbach Corp.) for 20 min at maximum speed and at a gap of 15–25 μ. The crude extracts prepared by either method were centrifuged at 3000*g* for 15 min to remove glass beads and/or whole cells and cell debris. The supernatant solution from this centrifugation was the crude extract. An extract prepared from 30 g of mycelium was centrifuged at 33,000*g* for 1 hr. The supernatant solution from this centrifugation was then treated with solid ammonium sulfate (24.2 g/100 ml) to 40% saturation. This mixture was centrifuged and the precipitate was discarded. The supernatant fraction was treated with additional solid ammonium sulfate (18.8 g/100 ml) to 65% saturation and again centrifuged. The precipitate from this centrifugation was resuspended in 10 ml of 0.05 M potassium phosphate buffer (pH 7.3) and an aliquot containing 83 mg of protein was chromatographed over a column containing 1.7 g of DEAE-Sephadex A 50 (Pharmacia Fine Chemicals, Inc.) which had been equilibrated and packed in this same buffer. The column was eluted with a solvent gradient made up from 50 ml of 0.05 M potassium phosphate (pH 6.5) in the mixing chamber and 50 ml of 0.4 M potassium phosphate (pH 6.5) in the reservoir. Elution of protein was followed by absorption at 280 mμ and enzyme activity was followed by the assay method described in Table I. Most of the enzyme activity was eluted in three fractions, and these were combined. The results of this purification are shown in Table I. This procedure gave about an 11-fold purification although the enzyme was still quite impure as judged by acrylamide gel electrophoresis. Protein concentrations were determined by the biuret method (Layne, 1957).

Chromatography. Methyl β-nitropropionate and methyl β-nitroacrylate were prepared on a micro scale by the method of Schlenk and Gellerman (1960). Gas chromatography of methyl β-nitropropionate and methyl β-nitroacrylate was carried out on an F & M Model 609 with a hydrogen flame detector. An 8-ft stainless-steel column ($^3/_{16}$-in. i.d.) containing 3% XE-60 on 80–100 mesh Diatoport S (F & M Scientific Corp.) was used. The following gas-flow rates were used (cubic centimeters per minute): helium, 41; hydrogen, 36; and oxygen, 144. The esters were chromatographed at a column temperature of 100°. Under these conditions, methyl β-nitroacrylate had a retention time of 2.8 min and methyl β-nitropropionate, 7.3 min. The methyl esters could be determined quantitatively by measuring the areas of the peaks with a planimiter and comparing the results to standards of known concentrations. β-Nitropropionic acid, β-nitroacrylic acid, and their methyl esters were separated by thin layer chromatography under conditions described by Shaw and McCloskey (1967).

TABLE I: Purification of β-Nitroacrylic Acid Reductase.[a]

Fraction	Total Units	Total Protein (mg)	Sp Act.	Act. Recovd (%)
Crude extract	57	740	0.077	100
33,000*g* supernatant	60	496	0.121	103
40–65% $(NH_4)_2SO_4$	52	89	0.575	86
DEAE-Sephadex	39	45	0.866	69

[a] The assay mixture contained potassium phosphate (pH 5.0) (100 μmoles), NADPH (0.3 μmole), β-nitroacrylic acid (3.0 μmoles), enzyme, and water to a final volume of 1.0 ml. One unit of enzyme activity is defined as that amount which catalyzes the oxidation of 1 μmole of NADPH/min. Specific activity is defined as units per milligram of protein.

Results

Properties of β-Nitroacrylic Acid Reductase. When β-nitroacrylic acid is incubated with crude extracts of *P. atrovenetum* and NADPH, the reduced pyridine nucleotide is rapidly oxidized. The substrate requirements for this reaction are shown in Table II. In the absence of β-nitroacrylic acid or when a boiled enzyme preparation is used, there is little oxidation of NADPH. NADH cannot replace NADPH. A typical assay is shown in Figure 2. The change in optical density is linear for the first 3 min and then falls off to zero as the NADPH is depleted. An enzyme preparation, partially purified as described in the Experimental Section, was used in this and all subsequent experiments. At substrate saturation levels, the rate of NADPH oxidation is proportional to enzyme concentration (Figure 3). The oxidation of NADPH is most rapid at about pH 5 (Figure 4). Figure 5 shows the effect of NADPH and β-nitroacrylic acid concentrations on the rate of NADPH oxidation. The reaction rate is maximum at about 2.5×10^{-4} M NADPH and 1.9×10^{-3} M β-nitroacrylic acid. The K_m values calculated

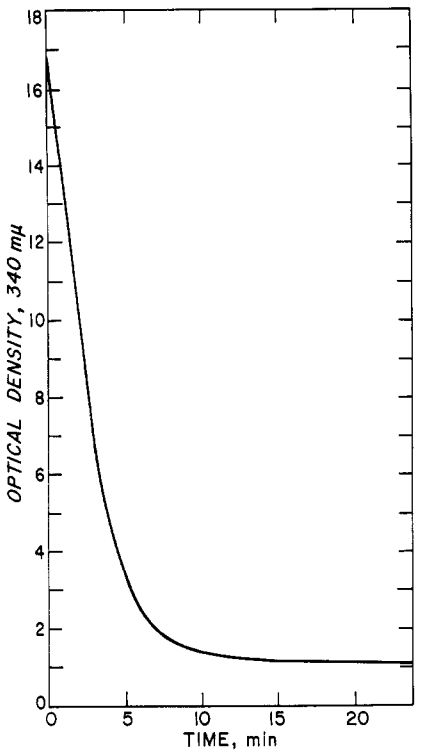

FIGURE 2: NADPH oxidation in the presence of β-nitroacrylic acid. Assay conditions are given in Table I.

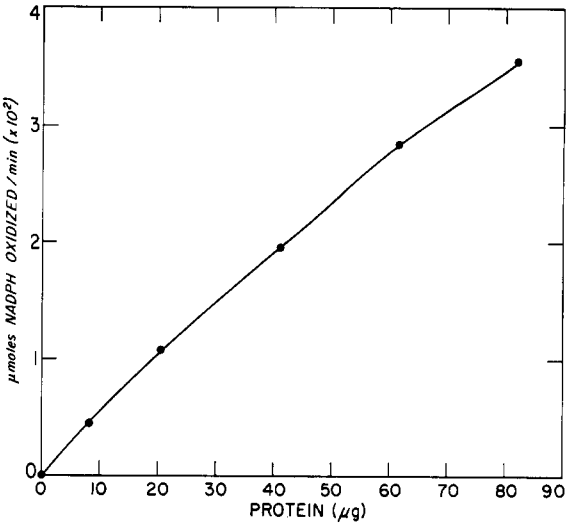

FIGURE 3: Effect of enzyme concentration on NADPH oxidation. Assay conditions are those given in Table I except for the variations in protein concentration.

from these data are 3.7×10^{-5} for NADPH and 1.6×10^{-4} for β-nitroacrylic acid.

Compounds such as acrylic acid, crotonic acid, fumaric acid, and cinnamic acid in which the nitro group of β-nitroacrylic acid is replaced by hydrogen, methyl, carboxyl, and phenyl groups, respectively, do not serve as substrates for the enzyme, nor are these compounds inhibitors of the reaction. The following compounds were also tested as possible substrates and found to be inactive: 2-hydroxy-3-nitropropionic acid, shikimic acid, aconitic acid, itaconic acid, pyruvic acid, phosphoenolpyruvate, cytochrome *c*, several aromatic and saturated aliphatic nitro compounds, and a variety of inorganic nitrogen compounds. The addition of FMN or FAD had no effect on the enzymatic activity and neither azide (1×10^{-3} M), fluoride (5×10^{-3} M), atebrine (1×10^{-4} M), nor anaerobiasis was inhibitory. The enzyme was inhibited by cysteine and other sulfhydryl compounds, however, when they were present in concentrations equivalent to β-

TABLE II: Substrate Requirements for β-Nitroacrylic Acid Reductase.[a]

Incubn Condn	μmoles of NADPH Oxidized/min (× 10^3)
Complete	42.3
Complete minus β-nitroacrylic acid	1.9
Boiled extract	6.0
NADH in place of NADPH	4.8

[a] The complete incubation mixture contained potassium phosphate (pH 5.0) (100 μmoles), β-nitroacrylic acid (10 μmoles), NADPH (0.3 μmole), and protein (40 μg) in a final volume of 1.0 ml.

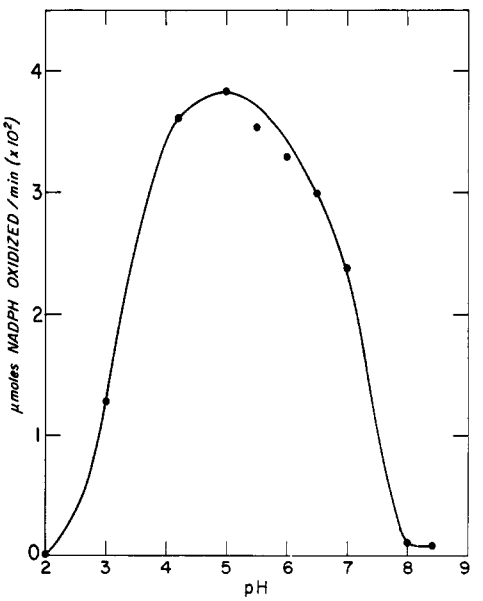

FIGURE 4: Effect of pH on β-nitroacrylic acid reductase. Assay conditions are those given in Table I except for the pH of the potassium phosphate buffer.

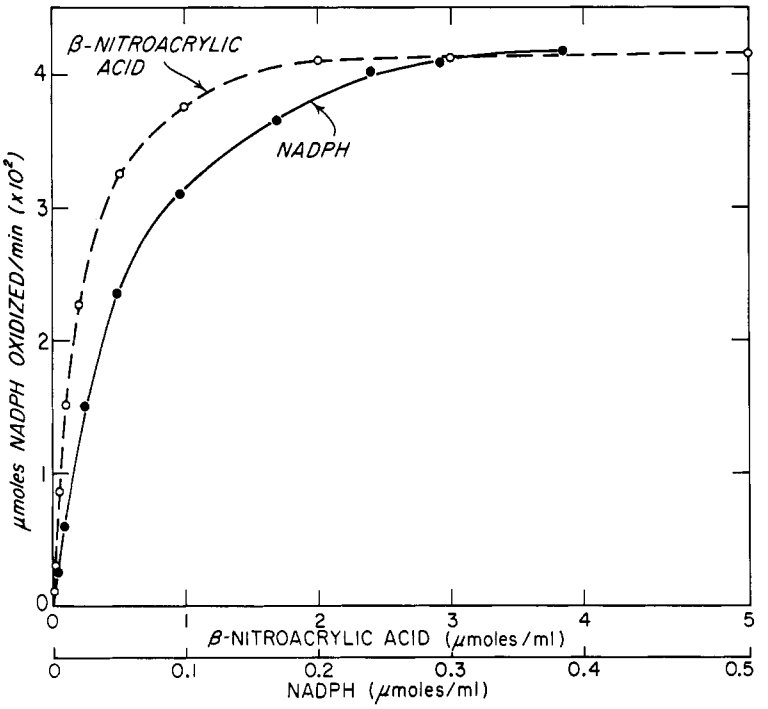

FIGURE 5: Effect of substrate concentrations on β-nitroacrylic acid reductase. Assay conditions are those given in Table I except for the variation in substrate concentrations.

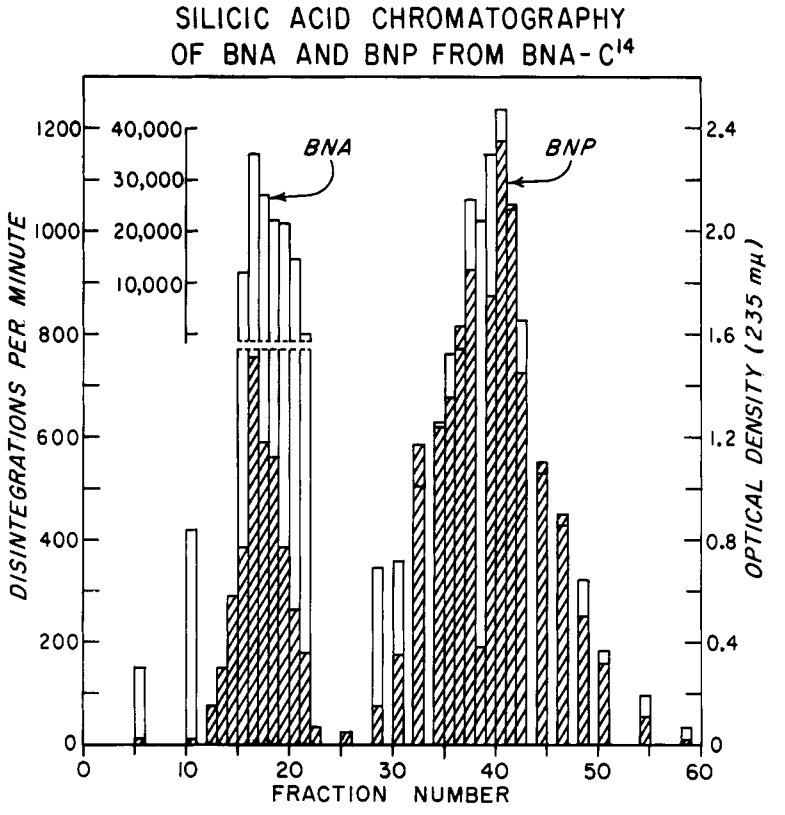

FIGURE 6: Silicic acid chromatography of β-nitropropionic acid from [1-^{14}C]β-nitroacrylic acid. BNA refers to β-nitroacrylic acid and BNP to β-nitropropionic acid. (open area) Disintegrations per minute. (cross-hatched area) Optical density at 235 mμ in 0.1 N KOH. The nitro compounds were obtained from two incubations as described in Table III except only 10.4 mg of carrier β-nitropropionic acid was added.

nitroacrylic acid. This inhibition is probably due to the reaction of the sulfhydryl compounds with the β-nitroacrylic acid rather than an inhibition of the enzyme itself. Evidence for this hypothesis is based on the disappearance of the β-nitroacrylic acid absorption maximum at 230 mμ upon addition of 1 equiv of cysteine and the appearance of a new maximum at 205 mμ having about one-half the extinction of the 230-mμ peak. Thes pectrum of the cysteine-treated β-nitroacrylic acid is very similar to that of β-nitropropionic acid. The nature of the product of this reaction is unknown; however, the nitro group is still present as evidenced by a positive test in the assay system used for the determination of β-nitropropionic acid (Shaw and Wang, 1964). It is possible that the cysteine adds to the very reactive double bond of the β-nitroacrylic acid.

Characterization of Reaction Product. Two incubations were run with [1-^{14}C]β-nitroacrylic acid as substrate. The products of these incubations were dissolved in chloroform and chromatographed over 4.0 g of silicic acid with chloroform as the eluent. The results of this chromatography are shown in Figure 6. The first material eluted from the column was unchanged β-nitroacrylic acid. The second peak represents β-nitropropionic acid, and it can be seen that there is a direct correspondence between the radioactivity and mass as measured by the absorption of the β-nitropropionic acid at 235 mμ in 0.1 N sodium hydroxide. In another experiment, the β-nitropropionic acid was isolated with carrier and recrystallized from chloroform to a constant specific activity. The results of this experiment are shown in Table III; they show that the β-nitroacrylic acid reduction product is β-nitropropionic acid. Vapor phase chromatography of the methyl esters of these nitro compounds and chromatography of the acids on thin layer plates under conditions described by Shaw and McCloskey (1967) confirmed the identification of the reaction product.

TABLE III: Recrystallization of [^{14}C]β-Nitropropionic Acid.[a]

Recrystallization	Sp Act. (dpm/mg)
Crude	1260
First	357
Second	270
Third	319
Fourth	215
Fifth	227
Sixth	215

[a] Incubation mixture contained potassium phosphate (pH 5.0) (300 μmoles), [1-^{14}C]β-nitroacrylic acid (sp act. 2.4 μc/mmole, 10.3 μmoles), and NADPH (1.63 μmoles) in a final volume of 3.0 ml. After NADPH had been completely oxidized, 0.3 ml of 6 N hydrochloric acid and 50.00 mg of β-nitropropionic acid were added. The mixture was extracted with four equal volumes of ethyl acetate and recrystallized from chloroform.

Stoichiometry of β-Nitroacrylic Acid Reductase. Assays were prepared as described in Table II. The disappearance of NADPH was measured in the usual manner. The disappearance of β-nitroacrylic acid was measured by removing aliquots of the reaction mixtures at 1-min intervals and determining the absorbance at 231 mμ in 0.1 N hydrochloric acid. At this wavelength, the absorbance due to β-nitropropionic acid is negligible in acid solution so it does not interfere with the β-nitroacrylic acid determination. The incubations were allowed to proceed for 10 min at which time the NADPH had been completely oxidized. At this time β-nitropropionic acid was isolated by extraction with ethyl acetate, then converted to a methyl ester, and the methyl β-nitropropionate determined by gas chromatography. The results, representing the average of three determinations, are given in line 1 of Table IV.

TABLE IV: Stoichiometry of β-Nitroacrylic Acid Reductase.[a]

Method	NADPH Oxidized	β-Nitroacrylic Acid Reduced	β-Nitropropionic Acid Formed
1	0.88	0.91	1.2
2	1.63	—	2.0

[a] The values given in method 1 are the average of three incubations run as described in Table II. Other experimental details are given in the text. The values in method 2 were calculated from the results of the final recrystallization reported in Table III.

They suggest a ratio of 1 equiv of oxidized NADPH to 1 equiv of reduced β-nitroacrylic acid to 1 equiv of β-nitropropionic acid formed.

The second line of Table IV gives a comparison of the above results with those obtained using [1-^{14}C]-β-nitroacrylic acid. These data are from the experiment described in Table III. In this experiment the disappearance of β-nitroacrylic acid was not determined, but the values of NADPH and β-nitropropionic acid are in agreement with a 1:1 ratio.

Synthesis of β-Nitropropionic Acid by Whole Mycelium. Cultures of *P. atrovenetum* were allowed to grow in 100-ml portions of a Raulin–Thom medium as described in the Experimental Section. After 36 hr, three flasks were removed and the mycelium was harvested. Aliquots of the brew were removed from each of the remaining flasks and the β-nitropropionic acid concentration was determined. At this time, DL-[4-^{14}C]-aspartic acid (4 μmoles/ml, 34.2 μc/mmole) and varying

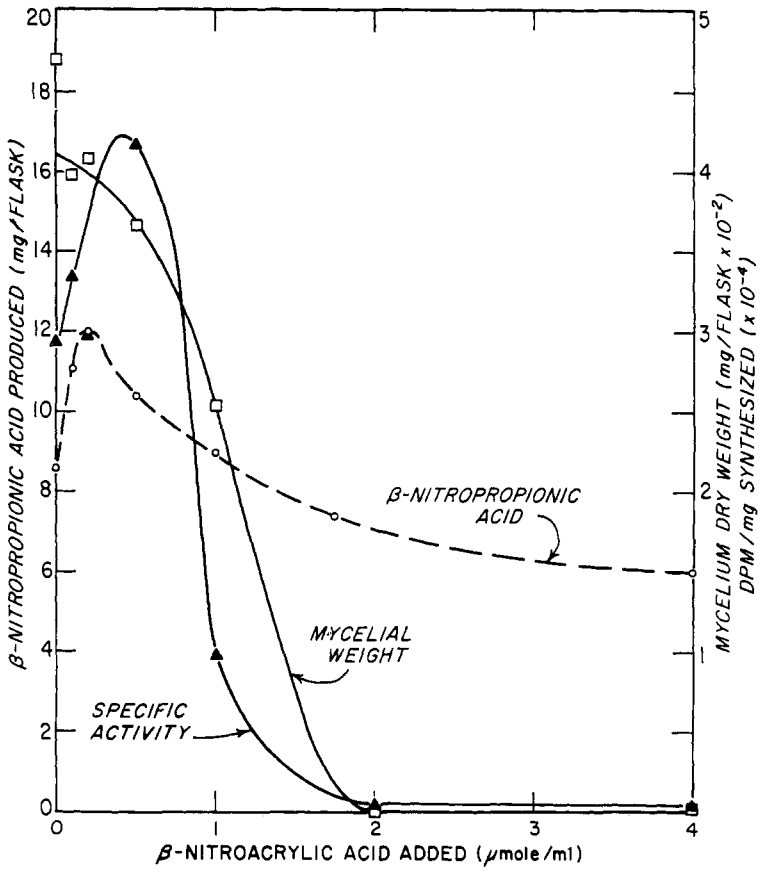

FIGURE 7: Effect of β-nitroacrylic acid on the incorporation of DL-[1-^{14}C]aspartic acid into β-nitropropionic acid. Experimental details are discussed in the text.

amounts of unlabeled β-nitroacrylic acid were added to the cultures. Because of the instability of aqueous solutions of β-nitroacrylic acid, it was added in three equal portions at 4-hr intervals. The total amounts of β-nitroacrylic acid varied from 0 to 4 μmoles/ml. After shaking for an additional 12 hr, the mycelium was removed by filtration, washed with water, and dried to constant weight at 80° in a vacuum oven. β-Nitropropionic acid was isolated from the filtrates, purified, and determined by previously described methods (Shaw and Wang, 1964; Shaw and McCloskey, 1967). The results of this experiment are shown in Figure 7. The values of β-nitropropionic acid and for mycelial dry weight are the results of subtracting the corresponding 36-hr values from the final values for each flask. At values of ≥ 2 μmoles of β-nitroacrylic acid/ml, growth of the organism is completely inhibited. β-Nitropropionic acid synthesis is inhibited 50% and only traces of radioactivity are incorporated into the product.

A similar experiment was run in which unlabeled DL-aspartic acid (4 μmoles/ml) and [1-^{14}C]β-nitroacrylic acid (2.05 μmoles/ml, 0.50 μc/mmole) were added to the 36-hr cultures of *P. atrovenetum*. The labeled β-nitroacrylic acid was again added in three portions at 4-hr intervals. The average specific activity of the β-nitropropionic acid from three experiments was 0.17 ± 0.02 μc/mmole. This is 34% of the theoretical value.

Discussion

An enzyme, which we have termed β-nitroacrylic acid reductase, catalyzed the reduction of β-nitroacrylic acid to β-nitropropionic acid. Although there is no direct evidence for the involvement of a single enzyme, partial purification of the catalytic activity did not indicate a multiple enzyme requirement. The substrate specificity of the enzyme is strict for both the NADPH and β-nitroacrylic acid. The stoichiometry studies indicated that the following equation best describes the reaction catalyzed by β-nitroacrylic acid reductase. Repeated attempts to demonstrate the reversibility of the reaction have been unsuccessful.

$$\begin{array}{l} CH{=}CHCOOH + NADPH + H^+ \longrightarrow \\ | \\ NO_2 \end{array} \qquad \begin{array}{l} CH_2CH_2COOH + NADP^+ \\ | \\ NO_2 \end{array}$$

The reduction of β-nitroacrylic acid is the first demonstration of the synthesis of β-nitropropionic acid in a cell-free system. While it has not been proved that the enzyme is directly involved in the biosynthesis of β-nitropropionic acid by *P. atrovenetum* or that β-nitroacrylic acid is on the pathway of the biosynthesis, it does provide good presumptive evidence for such roles. Furthermore, the high degree of specificity of the enzyme indicates that it is not simply a nonspecific catalyst for the reduction of double bonds.

Further evidence for the role of β-nitroacrylic acid as an intermediate in the biosynthesis of β-nitropropionic acid was provided by experiments in which the incorporation of radioactivity from a known precursor of the β-nitropropionic acid carbon skeleton, aspartic acid, was nearly completely inhibited in the presence of unlabeled β-nitroacrylic acid (Figure 7). This indicated that the β-nitroacrylic acid was used preferentially for the synthesis of β-nitropropionic acid. In a similar experiment with [1-^{14}C]β-nitroacrylic acid and unlabeled aspartic acid, the label from the β-nitroacrylic acid was incorporated into β-nitropropionic acid. The specific activity of the product was, however, only 34% that of the starting material instead of the expected value which should have approached 100%. In spite of this discrepancy, these experiments show that the fungal mycelium, in a nongrowing state, was able to incorporate the unsaturated nitro compounds into β-nitropropionic acid.

Acknowledgments

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