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Biosynthesis of Nitro Compounds. II. Studies on Potential Precursors for the Nitro Group of β-Nitropropionic Acid*

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ABSTRACT: The incorporation of ^{15}N- and ^{18}O-labeled substrates into β-nitropropionic acid by growing cultures of *Penicillium atrovenetum* was investigated. Analyses of the β-nitropropionic acid samples by mass spectrometry of their methyl esters indicated that ammonium ion was used for the synthesis of the nitro group in preference to nitrate. The label from [^{18}O]potassium nitrate was not incorporated into the nitro group. The amino group of aspartic acid was utilized in preference (*ca.* 2: 1) to ammonium ion for the synthesis of the nitro group. [3-^{14}C]- and [4-^{14}C]-aspartic acids were incorporated equally well into β-nitropropionic acid. Dilution of the label was small in spite of a low efficiency of incorporation. L-[4-^{14}C]-Aspartic acid, but not the corresponding D isomer, was incorporated into the nitro compound. It was concluded that both the amino group and the carbon skeleton of aspartic acid are on a direct pathway to β-nitropropionic acid.

Label from tartaric acid, which promotes β-nitropropionic acid synthesis, was not incorporated into the nitro compound.

Previous evidence indicated that a reduced nitrogen compound was required by *Penicillium atrovenetum* for the synthesis of β-nitropropionic acid (Raistrick and Stössl, 1958; Hylin and Matsumoto, 1960; Shaw and Wang, 1964). Results reported by these workers suggested that ammonium ion was the obligatory precursor of the nitro group. Birch *et al.* (1960), on the other hand, proposed that aspartic acid was a direct precursor of β-nitropropionic acid and, furthermore, that the aspartic acid amino group was oxidized *in situ* to the nitro group. This view was supported by results given in a brief report by Gatenbeck and Forsgren (1964) who studied the incorporation of [^{15}N,U-^{14}C]-aspartic acid into β-nitropropionic acid.

In an attempt to resolve the question of the source of the nitro group of β-nitropropionic acid, a series of experiments was undertaken on the utilization of various labeled nitrogen compounds by *P. atrovenetum*. The results of these experiments are the substance of this communication.

Materials and Methods

Labeled compounds were obtained from the following sources: [^{15}N]potassium nitrate, Volk Radiochemical Co.; [^{15}N]ammonium chloride, Bio-Rad Laboratories and Nichem, Inc.; L-[^{15}N]aspartic acid, Merck Sharp & Dohme of Canada, Ltd., and Volk Radiochemical Co.; [^{18}O]potassium nitrate, Yeda Research and Development Co., Ltd.; DL-[3-^{14}C]-aspartic acid, Nuclear Research Chemicals, Inc.;

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and DL-[4-^{14}C]aspartic acid and DL-[1,4-^{14}C]tartaric acid from New England Nuclear Corp.

β-Propiolactone was obtained from Eastman Organic Chemicals and *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald) from the Aldrich Chemical Co. D- and L-aspartic acids were obtained from Nutritional Biochemicals Corp. Silicic acid for column chromatography was Mallinckrodt no. 2847 which had been acid washed, dried, and sieved. The 100–200 mesh fraction was used for all the columns. Merck silica gel G for thin layer chromatography was obtained from the Brinkmann Instrument Co. All reagents and the components of the culture media were prepared from reagent grade chemicals.

Mass spectra were determined with an Atlas CH-4 mass spectrometer, equipped with a gas chromatographic inlet system (Leemans and McCloskey, 1967). Temperatures were: flash heater, 100°; column (3 ft, 1% SE-30), 70°; transfer system including He separators, 175°; and ion source, 250°. Ionizing potential was 20 ev, and the ionizing current was 40 μa. Spectra were recorded in 1–3 sec on the apex of the gas chromatographic peak, as indicated by the chromatogram continuously produced by the total ionization monitor. Radioactivity measurements were made on a Packard liquid scintillation spectrometer. All calculations of isotope content in β-nitropropionic acid were made with the consideration that only the L isomer of aspartic acid was utilized by the fungus for synthesis of the nitro compound.

Synthesis of [^{15}N]- *and* [^{18}O]-*β-Nitropropionic Acids.* Potassium nitrate, labeled with either ^{15}N or ^{18}O, was reduced to the corresponding nitrite on about a 10-mmole scale with granular, metallic lead by the method described by King (1950) for the preparation of sodium nitrite (yield, 40–58%). Nitrite was determined by diazotization of sulfanilic acid and coupling to β-naphthylamine as described by Shaw and Wang (1964). β-Nitropropionic acid was synthesized from the labeled potassium nitrite and β-propiolactone by the method of Gresham *et al.* (1952). The crude product was purified by chromatography on about a 40-fold excess of silicic acid using unpurified, reagent grade chloroform as the eluent. Elution of β-nitropropionic acid was followed by removing aliquots of the fractions, evaporating the solvent, dissolving the residues in 0.1 N sodium hydroxide, and measuring the optical density of the resulting solutions at 235 mμ in a Bausch and Lomb Model 505 spectrophotometer. Following chromatography, the material was recrystallized twice from chloroform to give β-nitropropionic acid (over-all yield of 23%) having a melting point of 67–68°.

Preparation and Purification of Methyl β-Nitropropionate. All samples of β-nitropropionic acid were converted to methyl esters for analysis in the mass spectrometer. They were esterified with diazomethane according to the procedure of Schlenk and Gellerman (1960). All of the methyl esters except the sample isolated from the cultures grown on [^{18}O]potassium nitrate were purified by chromatography on about a 40-fold excess of silicic acid. The yield of this ^{18}O-labeled product (about 2 mg of the partially purified acid) was too low for further purification of the methyl ester. The silicic acid was packed in benzene and the methyl esters were applied in benzene. Elution of the esters was followed by determining the absorbance at 235 mμ of an alkaline solution as in the chromatography of the free acid. The columns were eluted first with benzene and then with a mixture of benzene–ethyl acetate–acetic acid (90:10:0.1). Methyl β-nitropropionate was eluted near the front of this second solvent. Fractions containing the ester were pooled and evaporated to dryness under a stream of nitrogen until a constant weight was obtained. The methyl esters obtained by this procedure gave single spots on thin layer chromatograms. The methyl β-nitropropionate samples, pale yellow oils, were stored under nitrogen in sealed, glass ampoules until ready for analysis.

At all stages of preparation and purification, the β-nitropropionic acid samples and their methyl esters were monitored by thin layer chromatography on silica gel plates. The solvent system was benzene–ethyl acetate–acetic acid (120:80:20). Spots were detected either by exposure of the developed plates to iodine vapor or by spraying the plates first with 1 N sodium hydroxide, followed by heating at 120° for 5 min. After the plates had cooled to room temperature, they were sprayed with the diazotization mixture used for the nitrite assay and finally with 6 N hydrochloric acid. β-Nitropropionic acid could be detected at a level of about 20 μg and the methyl ester at 10 μg by this latter method.

The following solvent systems were used for the paper chromatography of tartaric acid: ethyl acetate–acetic acid–water (3:1:1), diethyl ether–acetic acid–water (13:3:1), and Ethyl Cellosolve–ammonia–water (16:1:3).

Culture Conditions. Cultures of *P. atrovenetum* were maintained on agar slants and were grown in liquid media under the conditions described previously (Shaw and Wang, 1964). The basal growth medium contained the following components: glucose (45.5 g), sodium sulfate (0.18 g), dipotassium phosphate (0.36 g), potassium carbonate (0.40 g), magnesium carbonate (0.27 g), ferrous sulfate heptahydrate (0.047 g), and zinc sulfate heptahydrate (0.047 g) in a volume of 1 l. The various additions to this basal medium are given at appropriate points in the Results section.

Isolation and Purification of β-Nitropropionic Acid. β-Nitropropionic acid was isolated from the culture filtrates after 5 days of growth on ^{15}N- or ^{18}O-labeled nitrogen sources by previously described methods (Shaw and Wang, 1964), and the products were purified by chromatography on silicic acid columns. The materials from the columns were resublimed (Raistrick and Stössl, 1958) and converted to methyl esters. β-Nitropropionic acid, isolated from cultures containing ^{14}C-labeled substrates, was further purified after silicic acid chromatography by repeated recrystallization from mixtures of ether and hexane to a constant specific activity.

Resolution of DL-[*4-*^{14}*C*]*Aspartic Acid. N*-Acetyl-

TABLE I: Incorporation of ^{14}C-Labeled Substrates into β-Nitropropionic Acid (BNP).[a]

Expt	Substrate	Incorp (%)	Sp Act. (μc/mmole)		BNP:Substrate[b]
			Substrate	BNP	
1	DL-[3-^{14}C]Aspartic acid	0.58	6.47	1.31	0.202
2	DL-[4-^{14}C]Aspartic acid	1.21	5.98	1.38	0.231
3	D-[4-^{14}C]Aspartic acid plus L-aspartic acid	0.02	0.387	0.01	0.027
4	L-[4-^{14}C]Aspartic acid plus D-aspartic acid	3.9	1.24	0.65	0.522
5	DL-[1,4-^{14}C]Tartaric acid	0.022	8.13	0.025	0.0003

[a] For the labeled aspartic acid experiments, the fungus was grown in a modified Raulin–Thom medium (Raistrick and Stössl, 1958) in which the ammonium tartrate concentration was reduced from 8.0 to 2.0 g/l., and disodium tartrate (7.5 g/l.) and aspartic acid (4.12 g/l.) were added. Raistrick's modified Raulin–Thom medium was used in the labeled tartaric acid experiment. In expt 3 and 4, equal quantities of labeled and unlabeled aspartic acid were used. [b] These values represent the ratio of the specific activities of β-nitropropionic acid to the respective substrates.

DL-[4-^{14}C]aspartic acid was prepared in 73% yield by the method of Barker (1953). The racemic mixture was then resolved by the method of Greenstein (1957) with an aspartic acid acylase prepared according to the method described by Birnbaum (1955). The two isomers were diluted with 1.0 g each of the appropriate carrier and purified by two reprecipitations from 90% aqueous ethanol. The yield of L-[4-^{14}C]aspartic acid was 965 mg, $[\alpha]_D^{26}$ +24.6° (*c* 2, 5 N HCl), sp act. 1.24 μc/mmole. The yield of D-[4-^{14}C]aspartic acid was 945 mg, $[\alpha]_D^{26}$ −24.0° (*c* 2, 5 N HCl), sp act. 0.387 μc/mmole.

Results

Incorporation of ^{14}C-Labeled Substrates. The incorporation of DL-[3-^{14}C]aspartic acid and DL-[4-^{14}C]aspartic acid into β-nitropropionic acid by growing cultures of *P. atrovenetum* is shown in Table I (expt 1 and 2). The [4-^{14}C]aspartic acid is incorporated about twice as effectively as the [3-^{14}C]aspartic acid when calculated on a percentage basis; however, this difference represents only a variation in the total amount of β-nitropropionic acid produced. The specific activities of the samples of β-nitropropionic acid are very similar. The product isolated from the medium containing [3-^{14}C]aspartic acid has about 20% of the theoretical specific activity and that from [4-^{14}C]aspartic acid about 23%.

In order to determine whether both or only one of the aspartic acid isomers was utilized for β-nitropropionic acid synthesis, *P. atrovenetum* was grown in media containing equal quantities of D-[^{14}C]aspartic acid plus unlabeled L-aspartic acid and D-aspartic acid plus L-[^{14}C]aspartic acid. The data in Table I (expt 3 and 4) show that the L isomer is incorporated into β-nitropropionic acid with about double the efficiency of the racemic mixture (expt 2). On the other hand, the D isomer is incorporated only 10% as well as the racemic mixture and 5% as well as the L isomer. The traces of radioactivity present in the product from the D-[4-^{14}C]aspartic acid may have been caused by contamination with the L isomer since the observed rotation (−24.0°) was about 4% lower than the value reported (−25.0°) by Birnbaum *et al.* (1952).

Tartaric acid, which promotes β-nitropropionic acid synthesis, was very poorly incorporated into the nitro compound (expt 5). The radioactivity present in the culture filtrates, after the β-nitropropionic acid had been removed, accounted for about 31% of the radioactivity which had been added to the cultures. Paper chromatography of these filtrates in three solvent systems gave single radioactive spots corresponding to tartaric acid. Thus, nearly 70% of the labeled tartaric acid was taken into the fungal mycelium, but only 0.022% was utilized for β-nitropropionic acid synthesis.

Mass Spectra of Synthetic Methyl β-Nitropropionate. Mass spectrometry is uniquely suited for studies involving the biological incorporation of stable isotopes, since mass spectra yield not only the mole percentage of each labeled species but may also frequently be used to confirm or determine the exact location of the label within the molecule. Interpretation of the mass spectrum of methyl β-nitropropionate thus allows the unambiguous, rapid, and accurate determination of ^{15}N and ^{18}O in the nitro group and requires only a few micrograms of sample. The structural identities of the major peaks discussed below are consistent with the mass spectra of synthetic ^{15}N- and ^{18}O-labeled compounds and the analogous ethyl ester.

The spectrum of methyl β-nitropropionate (Figure 1A) consists of essentially four significant peaks: *m/e* 55, 59, 87, and 102. The molecular ion (mass 133) is not sufficiently stable to be observed; this is characteristic of aliphatic nitro compounds (Aplin *et al.*, 1965). Instead, facile loss of a nitro radical yields *m/e* 87 (see Figure 1A), a poorly stabilized primary carbonium

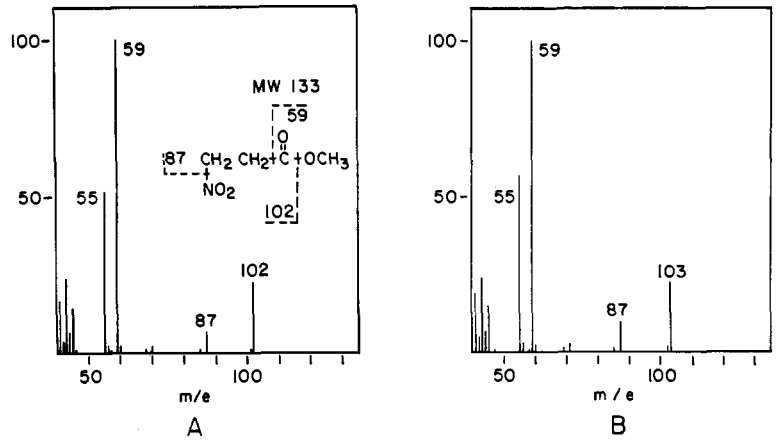
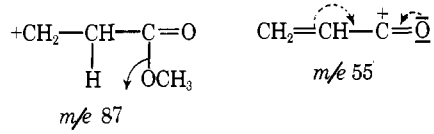


FIGURE 1: Mass spectra of unlabeled methyl β-nitropropionate (A) and [^{15}N]methyl β-nitropropionate (B).

ion which is present therefore in rather low abundance. Further elimination of methanol resulting in *m/e* 55 is indicated by a metastable transition at *m/e* 35.1 (34.8 calcd). Although the mechanism of this process cannot be rigorously established without deuterium labeling on either the α- or β-carbon atoms, a 1,2 elimination appears most likely, providing the driving force for formation of an ion which would be highly resonance stabilized (dotted arrows). Simple cleavage of the C-1,C-2 bond, with charge retention and stabili-



zation by the carbonyl group, yields *m/e* 59. Of greatest interest, however, is *m/e* 102, which is due to loss of the methoxy group and which is the only major ion still retaining the nitro group. Therefore, in the spectrum of synthetic [^{15}N]methyl β-nitropropionate (Figure 1B), *m/e* 102 shifts to *m/e* 103, leaving the other major peaks unchanged. Likewise, in the spectrum (not shown) of methyl β-nitropropionate labeled with ^{18}O in the nitro group, *m/e* 102 shifts 2 mass units to *m/e* 104. Measurements of the intensities of *m/e* 103 and 104 relative to *m/e* 102 are therefore used for calculation of ^{15}N or ^{18}O content.[1]

Incorporation of Stable Isotopes. The incorporation of nitrogen into the nitro group of β-nitropropionic acid from media containing ammonium and nitrate ions is shown in Table II. In expt 1, in which [^{15}N]-ammonium chloride was the only nitrogen source, β-nitropropionic acid which had about 94% of the theoretical ^{15}N content was obtained. This 6% dilution is probably a result of the addition of unlabeled nitrogen compounds in the culture inoculum. When unlabeled potassium nitrate is substituted for one-half of the [^{15}N]ammonium chloride (expt 2), the incorporation of the label is still 80%. This is 85% of the control value. When this same experiment was run with unlabeled ammonium chloride and [^{15}N]potassium nitrate (expt 3) the β-nitropropionic acid contained only 16% of the ^{15}N. This is 17% of the control value. The addition of hydroxylamine hydrochloride to a medium which contained [^{15}N]ammonium chloride as the nitrogen source had no significant effect on the incorporation of the label (expt 4). When *P. atrovenetum* was grown on a medium containing [^{18}O]potassium nitrate as a

TABLE II: Incorporation of ^{15}N-Labeled Substrates into β-Nitropropionic Acid.[a]

Expt	N Source	Atom % of Isotope	Isotope Content in BNP	Fraction of BNP from Labeled N Source
1	$^{15}NH_4Cl$	49	46	0.94
2	$^{15}NH_4Cl + KNO_3$	98	78	0.80
3	$NH_4Cl + K^{15}NO_3$	96	15	0.16
4	$^{15}NH_4Cl$ + $H_2NOH \cdot HCl$	49	47	0.96
5	$KN^{18}O_3$	10	0.5	0.05

[a] The fungus was grown in the basal medium with the addition of 2.2 g of disodium tartrate dihydrate and 3.17 g of tartaric acid/l. and the indicated nitrogen sources. The final concentration of nitrogen was 0.097 M in all experiments except expt 4. In this case an additional 2 μmoles/ml of nitrogen was present as hydroxylamine. In expt 2 and 3, the two nitrogen sources were present in equimolar amounts.

[1] In the case of the ^{15}N determinations, the contribution at *m/e* 103 (approximately 0.9%, not shown in Figure 1A) owing to the presence of naturally occurring heavy isotopes must be subtracted. For a detailed discussion of isotopic distribution calculations, see Biemann (1962).

nitrogen source, essentially none of the label was incorporated into the nitro group (expt 5).

In order to determine if either ammonium ion or the amino group of aspartic acid were preferentially utilized for the formation of the nitro group, a series of growth studies were run in media which contained varying ratios of ammonium ion to aspartic acid. In two preliminary experiments with unlabeled substrates, and in the experiment with ^{15}N-labeled substrates, the production of β-nitropropionic acid increased linearly with increasing ammonium ion concentrations. The magnitude of this increase, however, varied in the three experiments. The β-nitropropionic acid was isolated from the media containing ^{15}N-labeled substrates, and purified and the samples were converted to methyl esters. The ^{15}N content of the esters was determined by mass spectrometry, and the results of these determinations are shown in Figure 2. In these experiments ammonium chloride was the labeled nitrogen source. If the nitrogen from the ammonium ion and the amino group of aspartic acid were utilized equally well, the ^{15}N content of the β-nitropropionic acid would be proportional to the concentration of the labeled precursor. A theoretical line based on that assumption is given by the dashed line in Figure 2. The solid line shows the actual ^{15}N content of the β-nitropropionic acid plotted against the mole fraction of the total nitrogen of the medium present as ammonium chloride. The control flask contained only [^{15}N]ammonium chloride (43 atom % ^{15}N) as a nitrogen source. The actual values for the ^{15}N content of the β-nitropropionic acid were about 35% lower than the theoretical curve until the ammonium ion concentration reached a value which amounted to 75% of the total nitrogen. At higher ammonium ion concentrations, the ^{15}N content of the β-nitropropionic acid approached the theoretical values. The growth medium which contained only ammonium ion gave β-nitropropionic acid which had 42 atom % ^{15}N (97 % of theoretical).

In other experiments, L-[^{15}N]aspartic acid (95.2 atom % ^{15}N) was diluted with carrier DL-aspartic acid to a final ^{15}N content of 55.3 atom %. This was added along with unlabeled ammonium ions to cultures of *P. atrovenetum* growing in the media described in Figure 2. When the labeled aspartic acid represented 16.2% of the total nitrogen, the isolated β-nitropropionic acid contained 19 atom % ^{15}N. When the aspartic acid represented 32.2% of the nitrogen, the nitro compound contained 27 atom % ^{15}N. These data show that, at the lower aspartic acid concentration, 35% of the β-nitropropionic acid was derived from the amino acid amino group and, at the higher concentration, 49%.

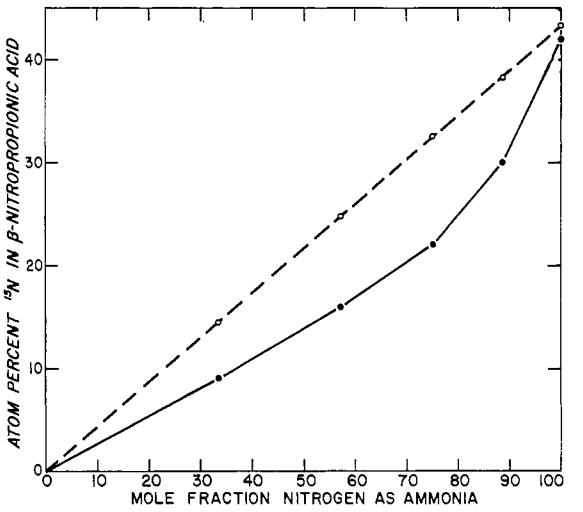


FIGURE 2: Synthesis of β-nitropropionic acid in media containing ammonium chloride and aspartic acid as nitrogen sources. The fungus was grown on the basal medium with the addition of 14.2 g of disodium tartrate dihydrate/l. plus ammonium chloride and/or DL-aspartic acid to give a final nitrogen concentration of 0.0615 M. The solid line represents the actual ^{15}N content of the β-nitropropionic acid formed from the given mole fractions of [^{15}N]ammonium chloride (43 atom % ^{15}N). The dashed line represents the theoretical ^{15}N content of β-nitropropionic acid assuming complete exchange between the ammonium ions and the aspartic acid amino group.

Discussion

The importance of a reduced nitrogen source for the synthesis of β-nitropropionic acid has been amply demonstrated. It has been assumed that because ammonium ion promoted synthesis and nitrate did not, ammonium ion, or possibly other reduced nitrogen compounds, was the obligatory precursor to the nitro group. This assumption has been verified by our present studies. In competition experiments with labeled substrates, ammonium ion was utilized about five times more readily for β-nitropropionic acid synthesis than was nitrate. The β-nitropropionic acid synthesized in the presence of equimolar quantities of these two nitrogen sources was derived about 84–85% from ammonium ion and 15–16% from nitrate. These results indicate that either nitrate is unable to furnish sufficient quantities of the proper nitrogenous precursor to the nitro group or that the over-all metabolism of the fungus is so altered in the nitrate medium that a precursor of the β-nitropropionic acid carbon skeleton is unavailable. The addition of hydroxylamine (2 μmoles/ml) to cultures growing in [^{15}N]ammonium chloride did not decrease the incorporation of label into β-nitropropionic acid. At this level, hydroxylamine had been shown to stimulate β-nitropropionic acid synthesis (Shaw and Wang, 1964); however, the stimulation is apparently not due to the incorporation of hydroxylamine into the nitro group but must be the result of some indirect action.

The lack of incorporation of ^{18}O from [^{18}O]potassium nitrate offers preliminary evidence suggesting that nitrate must be completely reduced to ammonia before being converted to the nitro group. However, these

results are not conclusive because, under the experimental conditions, it was not completely possible to rule out exchange reactions with the water of the medium. It has been demonstrated that under these conditions neither the oxygen of the nitrate ion (Klein and Friedel, 1950) nor that of the β-nitropropionic acid exchanges with oxygen from water; however, the possibility of an oxygen exchange between intermediates from nitrate to the nitro group and water cannot be eliminated because the structures of these intermediates are unknown.

Results with [^{14}C]aspartic acids (Table I) indicate that the carbon skeleton of this amino acid can furnish the carbons for synthesis of β-nitropropionic acid. Furthermore, in spite of the differences in over-all incorporation, the similarity of the ratios of the specific activities of β-nitropropionic acid isolated from growth media containing [3-^{14}C]aspartic acid and [4-^{14}C]-aspartic acid indicates that carbons 3 and 4 are incorporated as a unit. This conclusion is in agreement with those of Birch *et al.* (1960) and Gatenbeck and Forsgren (1964). In addition, Birkinshaw and Dryland (1964) have reported that [2-^{14}C]pyruvate gave rise to aspartic acid and β-nitropropionic acid in which the labeling pattern of carbons 1, 2, and 3 of the β-nitropropionic acid corresponded very closely to carbons 4, 3, and 2, respectively, of the aspartic acid. Thus there seems to be no doubt that carbons 4, 3, and 2 of aspartic acid are incorporated intact into β-nitropropionic acid. The organism is apparently unable to utilize D-aspartic acid for β-nitropropionic acid synthesis. When L-[4-^{14}C]aspartic acid was substituted for the racemic mixture, the ratio of the specific activities of the β-nitropropionic acid to that of the aspartic acid was approximately doubled. This result would be predicted if only the L isomer was utilized. The D isomer gave only a trace of incorporation, and this could be accounted for by small amounts of contamination by the L isomer.

The amino group of aspartic acid would appear to be utilized in preference to ammonium ions for the formation of the nitro group of β-nitropropionic acid. The data in Figure 2 show that in media in which ammonium ions account for 75% or less of the total nitrogen, the incorporation of ^{15}N from the labeled ammonium ions is about 35% less than would be expected if the two nitrogen sources were in equilibrium. A comparison of the data from the ^{15}N and ^{14}C incorporation studies indicates that at equimolar concentrations of aspartic acid and ammonium ions, 66% of the β-nitropropionic acid nitro group and 52% of the carbon skeleton is derived from aspartic acid. These results are generally in agreement with those of Gatenbeck and Forsgren (1964); however, the lack of certain experimental details in that paper makes a direct comparison difficult.

The preferential utilization of the aspartic acid amino group as a source for the nitro group of β-nitropropionic acid would appear to be at variance with the results reported previously by Hylin and Matsumoto (1960) and Shaw and Wang (1964). These workers showed that aspartic acid, even when present in sufficient amounts for normal growth of the organism, did not allow synthesis of β-nitropropionic acid unless the medium was supplemented with either ammonium ions or a four-carbon dicarboxylic acid. These apparently conflicting results suggest that aspartic acid may be metabolized by several pathways and that the composition of the growth medium has an influence on the availability of that amino acid for β-nitropropionic acid synthesis. It is possible that there is a critical intracellular level of aspartic acid at which synthesis of β-nitropropionic acid occurs and above or below which synthesis is inhibited. One enzyme which may be involved in controlling the level of aspartic acid is oxalacetate–glutamate transaminase. This enyzme has been found in *P. atrovenetum* extracts (P. D. Shaw, unpublished data).

The role of tartaric acid in β-nitropropionic acid synthesis is difficult to interpret. It can serve the same function as other four-carbon dicarboxylic acids in promoting synthesis of β-nitropropionic acid, it is extensively metabolized by the organism, but it is not incorporated into the nitro compound. It is probably not dehydrated to oxalacetate as it is in the bacterial system described by Rosenberger and Shilo (1961). Bentley and Keil (1962) have reported that tartaric acid was also required for the synthesis of penicillic acid by *Penicillium cyclopium*, but labeled tartaric acid was not incorporated into the product.

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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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