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116. BIOSYNTHESIS OF β -NITROPROPIONIC ACID BY THE MOULD *PENICILLIUM ATROVENETUM* G. SMITH*

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β -Nitropropionic acid has been isolated from four higher-plant and three fungal sources. In higher plants it has been obtained from the bark of the tree *Hiptage madablota* Gaertn. (Gorter, 1920), from the berries of the karaka tree *Corynecarpus laevigata* Forst. (Carrie, 1934), from trailing indigo *Indigofera endecaphylla* Jacq. (Morris, Pagán & Warmke, 1954) and from the roots of the fragrant violet *Viola odorata* (Pailer & Nowotny, 1958). Among fungi it has been found in the culture filtrates of strains of *Aspergillus flavus* (Bush, Goth & Dickson, 1945; Bush, Touster & Brockman, 1951; Marshall & Alexander, 1962), *Aspergillus oryzae* (Nakamura & Shimoda, 1954) and *Penicillium atrovenetum* G. Smith (Raistrick & Stössl, 1958).

Raistrick & Stössl (1958) obtained an exceptionally large yield from *P. atrovenetum*, amounting to 0.85 g./l. of culture filtrate when the yield was nearing maximum after 5 days' incubation. β -Nitropropionic acid accounted for 23 % of the utilized nitrogen, the source being exclusively ammonium present mainly as ammonium tartrate. When the ammonium tartrate concentration was increased threefold, the yield increased to 1.94 g./l. of culture filtrate and accounted for 29 % of the utilized nitrogen. Paradoxically, when a medium containing nitrogen exclusively as nitrate was used, the yield was one-tenth that with ammonium.

The present work was undertaken to test the hypothesis that β -nitropropionic acid is formed from aspartic acid or β -alanine, the nitro group arising by oxidation of the amino group:

While this work was in progress, Birch, McLoughlin, Smith & Winter (1960) in a similar investigation

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showed that ^{14}C from $\text{NaH}^{14}\text{CO}_3$ was incorporated into β -nitropropionic acid by *P. atrovenetum*, 97% of the radioactivity being in C-1 and 1 % in C-2. Similarly, when $[4\text{-}^{14}\text{C}]$ aspartic acid was tracer, 96 % of the radioactivity was in C-1 with none in C-2. Labelled β -alanine was, however, not incorporated. These results indicated that C-1 of β -nitropropionic acid was in equilibrium with aspartic acid, oxaloacetic acid and the C_4 acids of the tricarboxylic acid cycle, but that β -alanine was probably not a precursor.

Hyllin & Matsumoto (1961) confirmed that ammonium was better than nitrate as substrate for β -nitropropionic acid production, but also found that succinic acid, fumaric acid and tartaric acid, when added to a basal medium containing glucose, inorganic salts and ammonium salt, enhanced β -nitropropionic acid production by *P. atrovenetum* more than aspartic acid. Whatever the optical configuration of the aspartic acid, the yield was the same, suggesting deamination before incorporation. This led them to suggest that a possible first step might be reaction of hydroxylamine with fumarate by an aspartase-like reaction. This would not be at variance with the work of Birch *et al.* (1960).

More recently, Marshall & Alexander (1962) have suggested that β -nitropropionic acid may be on the heterotrophic nitrification pathway in *Aspergillus flavus*.

In the present work, $[2\text{-}^{14}\text{C}]$ pyruvate and $[1\text{-}^{14}\text{C}]$ acetate were used as tracers to study β -nitropropionic acid biosynthesis. If it is formed via oxaloacetate or aspartate or both the expected distribution would be as described in Scheme 1. The Δ shows the way in which radioactivity from $[1\text{-}^{14}\text{C}]$ acetate would be incorporated by way of the forward reactions of the tricarboxylic acid cycle. Owing to the asymmetry of the aconitase reaction, the carboxyl carbon of citrate derived from acetate is retained during the course of the cycle and those derived from oxaloacetate are lost, one at the level of oxalosuccinate and the other at the decarboxylation of α -oxoglutarate to succinate. Owing to the symmetry of succinate the radioactivity of the acetate carboxyl group would become equilibrated

Scheme 1. Incorporation of radioactivity from [1-¹⁴C]acetate and [2-¹⁴C]pyruvate into β -nitropropionic acid. Δ , Carbon atoms derived from C-1 of acetate; $\square$, carbon atoms derived from C-2 of pyruvate (other than via acetate); $\blacksquare$, carbon atoms derived from C-2 of pyruvate after recycling of oxaloacetate; $\bullet$, carbon atoms derived from metabolic CO₂.

and would therefore appear in both carboxyl groups of oxaloacetate and so of aspartate (White, Handler, Smith & Stetten, 1959). Recycling would result in the loss of the oxaloacetate carboxyl groups and the pattern would be unaltered. All the radioactivity of β -nitropropionate should be in C-1.

When [2-¹⁴C]pyruvate is the tracer the distribution of radioactivity in β -nitropropionic acid should be the sum of two reactions: (1) decarboxylation of pyruvate, yielding [1-¹⁴C]acetate, leading to incorporation of radioactivity into C-1 as described above; (2) carboxylation of pyruvate by the Wood-Werkman reaction.

The □ indicates the radioactivity of [2-¹⁴C]-pyruvate and its position in oxaloacetate and derived compounds when formed in this way.

Recycling of this oxaloacetate leads to equilibration of the radioactivity between the methylene carbon atoms of succinate and fumarate, and β -nitropropionic acid should have some labelling in C-2, indicated by ■. Metabolic carbon dioxide

would also have radioactivity (denoted by ●), which would appear in C-4 of oxaloacetate and aspartate, and so in C-1 of β -nitropropionate. The radioactivity in β -nitropropionate when [2- 14 C]-pyruvate is tracer should, therefore, be mainly in C-1 and C-3 with some in C-2.

It was assumed that the glyoxylate cycle would not operate, as the pyruvate and acetate were used in tracer quantities (7 and 0.8 $\mu\text{g./ml.}$ of culture medium respectively) and glucose was the main substrate (Collins & Kornberg, 1960).

MATERIALS AND METHODS

Cultures. *P. atrovenerum* G. Smith, London School of Hygiene and Tropical Medicine catalogue no. S.M. 683 (C.M.I. 61837), was grown in 1 l. flasks containing 350 ml. of the modified Raulin-Thom medium, practically as described by Raistrick & Stössl (1958), of composition: tartaric acid, 2.67 g.; ammonium tartrate, 8.0 g.; MgCO_3 , 0.27 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.047 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.047 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.17 g.; K_2CO_3 , 0.4 g.; $(\text{NH}_4)_2\text{PO}_4$, 0.4 g.;

glucose monohydrate, 54.5 g.; distilled water to 1 l. The steam-sterilized flasks were inoculated with a suspension of spores in distilled water.

Tracers. Tracers were obtained from The Radiochemical Centre, Amersham, Bucks. In the [^{14}C]pyruvate experiment, 100 μC (2.4 mg.) of sodium [^{14}C]pyruvate dissolved in 5 ml. of sterile distilled water was added to each of two flasks at day 0. In [^{14}C]acetate Expt. I, 150 μC (0.3 mg.) of sodium [^{14}C]acetate dissolved in 5 ml. of sterile distilled water was autoclaved and added to a single flask at day 3; in [^{14}C]acetate Expt. II, 100 μC (0.2 mg.) was added to each of two flasks at day 2.

Preparation of β -nitropropionic acid. Flasks were harvested after 8 days for the pyruvate, and after 7 days for the acetate experiments. Crude β -nitropropionic acid was prepared by ether extraction and sublimation as described by Raistrick & Stössl (1958). In the pyruvate experiment the acid was purified by subliming on to a jacketed 'cold finger' nine times; in acetate Expt. I by extracting the crude sublimate in a Soxhlet for 4 hr. with light petroleum (b.p. 40–60°) and subliming the residue repeatedly; and in acetate Expt. II by recrystallizing the crude sublimate from light petroleum (b.p. 60–80°) and subliming five times. Pyruvate and acetate Expt. I samples were diluted with carrier and resublimed before use. The acetate Expt. II sample was used undiluted. All three samples were sublimed to constant radioactivity and melting point. For the pyruvate experiment and acetate Expt. II the m.p. was 68–70° uncorr., and for acetate Expt. I 67.5–70°.

Degradation of β -nitropropionic acid: method 1. (i) Net reaction. 17 N-H₂SO₄ (7 ml.) was placed in a 500 ml. round-bottomed flask, fitted with a two-way adaptor and cooled in an acetone-solid CO₂ bath at -15°. A dropping pipette containing β -nitropropionic acid (421 mg.), dissolved immediately before use in 2 N-NaOH (3.5 ml.), was inserted through a rubber bung in one opening and the other was stoppered to prevent entry of acetone vapour. The H₂SO₄ was stirred vigorously with a magnetic stirrer and the β -nitropropionic solution was added dropwise. The stopper was loosened during the addition and replaced immediately. The temperature was taken down to -20° and 90 ml. of Brady's reagent (0.3% DNP-hydrazine in 2 N-HCl) was added. After standing in the refrigerator for 2–3 hr., the precipitated malonic semialdehyde DNP-hydrazone was filtered off, washed with water and transferred complete with filter paper to a flask fitted with gas inlet, separating funnel and reflux condenser. The outlet from the condenser was connected to a wash bottle and bubbler containing Brady's reagent. Then N₂ scrubbed through 10 N-NaOH, baryta, pyrogallol solution and conc. H₂SO₄ was passed through the system for 15 min., and two empty bubblers after the Brady's bubbler were filled with a saturated solution of baryta. Then 50 ml. of 5 N-H₂SO₄ was added through the separating funnel and the mixture was boiled for 1 hr. while a steady stream of N₂ swept acetaldehyde and CO₂ into the absorption train. The BaCO₃ was filtered rapidly from the solution of excess of baryta and washed thoroughly with boiling water followed by acetone. The cake was dried in the oven for at least 15 min. at 100° before being weighed and counted. This was the general method for all BaCO₃ samples. Acetaldehyde DNP-hydrazone was filtered off and recrystallized once from ethanol.

(ii) Degradation of acetaldehyde DNP-hydrazone. Kuhn-Roth oxidation was employed as described by

Stickings & Townsend (1961) for 2-methylbutanaldehyde DNP-hydrazone. The resulting sodium acetate was decarboxylated by the Schmidt reaction and the resulting methylamine oxidized as described by Phares (1951).

Degradation of β -nitropropionic acid: method 2. (i) Schmidt decarboxylation. This was based on the method of Phares (1951) for acetate, and was carried out similarly except that NaN₃ was added to the solution of β -nitropropionic acid in 100% H₂SO₄ to start the reaction and the quantity of acid/m-mole of β -nitropropionic acid was 1.07 ml.

(ii) Bromopierin from nitromethylene group. Ba(OH)₂·8H₂O (32 g.) was dissolved in water (60 ml.), and the solution was filtered and treated with bromine (2.5 ml.) at 0°. The ice-cold hypobromite solution was added to a solution of β -nitropropionic acid (119 mg.) in saturated baryta (7 ml.) cooled to 4°. After 30 min. the mixture was heated to 40° and then placed in the refrigerator for 2 hr. Bromopierin was collected by steam-distillation. Excess of water was removed and the oily globule was frozen before being transferred to a flask for wet combustion with KIO₃ (0.1 g.) and Van Slyke-Folch oxidizing mixture (20 ml.) (Van Slyke & Folch, 1940). The CO₂ was swept out of the vessel with a stream of purified N₂ and scrubbed with conc. H₂SO₄, granulated zinc and 1 N-AgNO₃ before precipitation in baryta.

Wet combustion of β -nitropropionic acid, acetate and aspartic acid. This was carried out as described for bromopierin.

Preparation of aspartic acid from mycelium. The two dried ether-extracted mycelia from the [^{14}C]pyruvate experiment were hydrolysed by refluxing gently with 6 N-HCl for 24 hr. The hydrolysate was extracted with ether, evaporated to dryness and, after being redissolved, the humin was filtered off. The filtrate was repeatedly evaporated to dryness to remove HCl and treated with decolorizing charcoal. De-Acidite E, supplied by The Permutit Co. Ltd., was ground and sieved to 100–150 mesh and prepared in the acetate form as described by Partridge & Brimley (1949) for De-Acidite B. The mycelium hydrolysate was adjusted to pH 4.0 with N-NaOH and applied to the column, and eluted with 0.25 N-HCl: 25 ml. fractions were collected and analysed by paper chromatography in propan-1-ol-water (7:3, v/v). Samples were neutralized when necessary with 2 N-NH₃.

The fractions containing crude aspartic acid were evaporated to dryness, redissolved in 10 ml. of water and purified on a column (2 cm. × 150 cm.) of Dowex 50 (pyridine-formate form) as described by Liebster, Kopoldová & Dobiášová (1961) and Liebster, Dobiášová, Kopoldová & Ekl (1961). The column was packed as described by Moore, Spackman & Stein (1958). Elution was carried out with pyridine-formate buffer, pH 3.1, at a flow rate of 20 ml./hr. at 37°: 10 ml. fractions were collected and analysed as above.

Four fractions containing pure aspartic acid were combined, evaporated to dryness several times to remove pyridine and then were treated with decolorizing charcoal and the volume was adjusted to 1 ml. The aspartic acid was crystallized by adding an equal volume of ethanol to the hot solution. The radioactivity of the chromatographically pure material was measured after diluting tenfold with pure carrier, by counting at infinite thickness. The remaining almost pure fractions were treated as above and aspartic acid was crystallized with carrier. Some was also recovered from a few impure fractions by paper chromatography. All

samples were mixed together and recrystallized with more carrier, and used for degradation after determination of purity.

Degradation of aspartic acid. (i) C-1 and C-4 by ninhydrin decarboxylation. Aspartic acid (40.6 mg.) was dissolved in water (2.5 ml.) and chilled, and 300 mg. of ninhydrin was added. After sweeping out with CO_2 -free N_2 , the solution was boiled for 30 min. The liberated CO_2 was collected in baryta.

(ii) C-4 by microbiological decarboxylation. *Clostridium welchii*, strain S.R. 12 obtained from the National Type Culture Collection, was used according to the method of Meister, Sober & Tice (1951). The bacteria were grown on the artificial medium of Marshall & Kelsey (1960). The washed cells and incubation mixture were equilibrated separately at 37° in connected flasks while the system was swept with CO_2 -free N_2 . The cells were tipped in and the CO_2 liberated was collected in baryta.

(iii) C-2 and C-3. Aspartic acid was treated with hypochlorite as described by Cutinelli, Ehrensvar, Reio, Saluste & Stjernholm (1951). Acetaldehyde was collected after steam-distillation as the DNP-hydrazone, which was further degraded as described above under method 1 for β -nitropropionic acid.

Measurement of radioactivity. Samples were mounted on planchets 1.5 cm. in diameter by pressing material in with a plunger tooled to fit them. Count rates were measured at infinite thickness with a Panax end-window Geiger-Müller counter and compared with a polymethyl methacrylate standard, 2.5 cm. in diameter, of nominal radioactivity $1 \mu\text{C/g.}$ (count rate 1130/min.). Correction was made for background radiation (about 15 counts/min.).

The BaCO_3 samples, prepared as described under the Nef reaction, ideally weighed 100 ± 2 mg. but never less than 70 mg. β -Nitropropionic acid samples weighed 120 mg., aspartic acid samples 100 mg. and acetaldehyde DNP-hydrazone samples 90–130 mg.

In β -nitropropionic acid degradations samples were counted to at least 20 000–30 000 counts, and standard proportional error in count rate minus background was not more than $\pm 1\%$. For the aspartic acid degradation, owing to the rather low specific radioactivity of the starting material samples were counted repeatedly alternately with the background and $1 \mu\text{C/g.}$ standard over 10 min. periods. The results were analysed statistically as recommended by Francis, Mulligan & Wormall (1957), and the standard proportional errors, always less than $\pm 2\%$, are shown in Table 3. Samples were also counted nearer to the window of the Geiger-Müller tube, giving the $1 \mu\text{C/g.}$ standard a count rate of 2730/min.

RESULTS

Method 1 of degrading β -nitropropionic acid was devised so that C-1, C-2 and C-3 could be obtained separately. This is shown in Scheme 2. The conditions described by Mahler (Noland, 1955) were used for the Nef reaction. The unstable malonic semialdehyde was trapped as the DNP-hydrazone, which could be filtered off and dried *in vacuo* without decomposition. On boiling with 5N-sulphuric acid for 1 hr. acetaldehyde (collected as the DNP-hydrazone) and carbon dioxide (collected as barium

Scheme 2. Method 1 for degrading β -nitropropionic acid.

carbonate) were liberated in a 1:1.08 molar ratio. Chromatography in 95 % (v/v) methanol showed it to have R_f 0.48 [Den, Robinson & Coon (1959) reported R_f 0.46] and that it was pure except for a trace of an unknown compound giving the characteristic blue colour with alkali of a bis-DNP-hydrazone. Carter (1943) obtained small quan-

ties of Nastvogel's osazone (I) when β -nitropropionic acid was allowed to stand in contact with phenylhydrazine, and the slight contamination here may be due to such a reaction. Further purification was not possible owing to the instability of malonic semialdehyde DNP-hydrazone, and the material was hydrolysed with boiling acid, yielding C-1 as carbon dioxide and C-2 and C-3 as acetaldehyde. The DNP-hydrazone prepared from the latter was shown to be free from bis-DNP-hydrazone by chromatography, and when recrystallized from ethanol had m.p. 151–152° (uncorr.) (an authentic sample had m.p. 150–151°; mixed m.p. 151–152°). Owing to the solubility of malonic semialdehyde DNP-hydrazone, additional acetaldehyde could be steam-distilled from the Nef reaction liquors. The DNP-hydrazone formed from this was shown to be pure, as above, and the recrystallized material was added to the rest for further degradation as shown in Scheme 2. Carbon dioxide liberated simultaneously could not be used for counting owing to the action of hot mineral acid on unchanged β -nitropropionic acid yielding malonic acid, which would decarboxylate randomly (Lippincott & Hass, 1939):

To confirm that the slight contamination of malonic semialdehyde DNP-hydrazone did not affect the result, C-3 and C-1 of [2- ^{14}C]pyruvate-derived β -nitropropionic acid were obtained as outlined in Scheme 3 (method 2). It might be argued that the conditions for Schmidt decarboxylation of β -nitropropionic acid would also lead to the formation of malonic acid. However, in blank experiments where sodium azide was omitted, carbon

dioxide was not evolved. Lippincott & Hass (1939) also report that in the presence of 100 % sulphuric acid the intermediate hydroxamic acid is formed and is only hydrolysed to the free carboxylic acid in the presence of water.

Radiochemical incorporation of radioactivity from [2- ^{14}C]pyruvate into β -nitropropionic acid was 6.6 %. The results of degrading it are shown in Table 1. Degradations I and II were carried out on a sample of β -nitropropionic acid from a single experiment, but for degradation II it was diluted with further carrier. There was good agreement between the radioactivities obtained for C-3 by the two methods (41.3 and 40.2 % of the total radioactivity by method 1 and 40.0 % by method 2). Percentages are calculated by taking the total radioactivity of oxidized β -nitropropionic acid counted as barium carbonate as 100 %. The distribution of radioactivity among C-1, C-2 and C-3 agrees with that expected if aspartate or oxaloacetate or both are precursors, C-1 and C-3 being approximately equally radioactive and C-2 about half this.

Radiochemical incorporation of radioactivity from [1- ^{14}C]acetate was 1.4 % in Expt. II. Degradations I and II of β -nitropropionic acid were carried out on material from two separate Expts. I and II. The results are summarized in Table 2. Method 1 only was used, and radioactivities for C-1 were 93.2 and 93.4 % of the total and for C-2 + C-3 2.3 and 3.2 %. These results are also in agreement with the suggested biosynthetic pathway.

To confirm that the distribution of radioactivity from tracer [2- ^{14}C]pyruvate was the same in aspartic acid as in the proposed corresponding β -nitropropionic acid, the mycelia that had produced the sample used for degradation were hydrolysed and aspartic acid was isolated. The aspartic acid used for degradation was chromatographically pure, and had a nitrogen content determined by Kjeldahl digestion of 10.50 % (authentic sample 10.49 %; theoretical 10.52 %).

The method of degradation is summarized in Scheme 4 and was based on those of Ehrensvar, Reio, Saluste & Stjernholm (1951) and Moses (1957). The results are shown in Table 3. Owing to the rather low specific radioactivity of the starting material (66.36 $\mu\text{C}/\text{mole}$) samples were counted repeatedly alternately with the background and the results were treated statistically. The standard pro-

Scheme 3. Method 2 for degrading β -nitropropionic acid.

Table 1. *Degradation of [2- 14 C]pyruvate-derived β -nitropropionic acid*

Experimental details are given in the text.

Carbon of β -nitropropionic acid	Product counted and origin	Degradation I			Degradation II		
		Radio- activity (μ C/mole)	Radioactivity/ mole of β -nitro- propionic acid (μ C/mole)	Percentage of total radioactivity of oxidized β -nitro- propionic acid	Radio- activity (μ C/mole)	Radio- activity/ mole of β -nitro- propionic acid (μ C/mole)	Percentage of total radioactivity of oxidized β -nitro- propionic acid
C-3 $\text{CH}_2\cdot\text{NO}_2$	BaCO_3 (C-1 of acetate)	154.6	154.6	41.3	72.2	72.2	40.2
C-2 CH_2	BaCO_3 (bromopierin)	149.7	149.7	40.0	—	—	—
	BaCO_3 (C-2 of acetate)	75.5	75.5	20.1	34.7	34.7	19.3
C-1 CO_2H	BaCO_3 (Schmidt)	144.4	144.4	38.6	—	—	—
	BaCO_3 (malonic semialdehyde DNP- hydrazone)	—	—	—	70.2	70.2	39.2
Radioactivity accounted for (C-1 + C-2 + C-3)	—	—	—	99.3	—	—	98.7
Oxidized β -nitro- propionic acid	BaCO_3	124.7	374.1	100	59.8	179.4	100
β -Nitropropionic acid	β -Nitropropionic acid	350	350	93.5	160.4	160.4	91.5
Acetaldehyde (C-2 + C-3)	Acetaldehyde DNP-hydrazone	211.1	211.1	56.4	102.4	102.4	57.1
C-2 + C-3	BaCO_3	—	230	60.7	—	106.9	59.6

portional error in counts is given in column 3 and does not exceed the minimum overall standard error of $\pm 3\%$ inherent in experiments of this kind. Insufficient acetaldehyde DNP-hydrazone was obtained from the hypochlorite treatment for further degradation, so it was diluted with pure carrier and the values obtained for C-2 and C-3 as barium carbonate shown in square brackets (column 4) were corrected by the dilution factor.

Table 4 compares the radioactivity in C-2, C-3 and C-4 of aspartic acid expressed as percentage of their sum with the distribution in β -nitropropionic acid. The specific radioactivity of undiluted aspartic acid from mycelium was $1312.5\mu\text{C}/\text{mole}$. Subtracting 28% of the radioactivity present in C-1, the remainder of the molecule has a specific radioactivity of $938.4\mu\text{C}/\text{mole}$. The specific radioactivity of undiluted β -nitropropionic acid was $903.5\mu\text{C}/\text{mole}$. Although specific radioactivity determined at infinite thickness for a given compound is theoretically the same whether it is counted as the compound or after wet combustion as barium carbonate, this has not been found to be exactly so.

When the specific radioactivity/mole of compound determined after wet combustion by counting barium carbonate was taken as 100%, the specific radioactivities determined on [2- 14 C]-pyruvate-derived aspartic acid and β -nitropropionic acid were found to be 97.3 and 91.5% respectively. More accurate comparison would be obtain-

ed by correcting these radioactivities to 100%. Thus that of aspartic acid minus C-1 becomes $964.4\mu\text{C}/\text{mole}$ and that of β -nitropropionic acid $976.7\mu\text{C}/\text{mole}$.

DISCUSSION

In several micro-organisms, the distribution of radioactivity in aspartate both from [1- 14 C]acetate (Wang, Thomas, Cheldelin & Christensen, 1952; Ehrensvar *et al.* 1951; Moses, 1957) and [2- 14 C]-pyruvate (Wang *et al.* 1952) has been shown to be clearly in agreement with its direct derivation from oxalacetate formed via the tricarboxylic acid cycle. The results of the present work show that the pattern in aspartate is repeated, with the loss of C-1, in β -nitropropionate. The relationship between percentage distribution of radioactivity in β -nitropropionic acid and C-2, C-3 and C-4 of aspartic acid is close enough within the limits of accuracy of experiments of this kind. Table 4 shows that the fit would be even closer if C-2 and C-4 of aspartate were reversed and C-4 corresponded to C-3 of β -nitropropionate. However, this would not be possible in view of the work of Birch *et al.* (1960) showing that radioactivity from $\text{NaH}^{14}\text{CO}_3$ and [4- 14 C]-aspartate was incorporated almost exclusively into C-1 of β -nitropropionate, and our own results showing the same for radioactivity from [1- 14 C]acetate. These three carbon atoms of aspartic acid also have almost the same specific radioactivity as the

β -nitropropionic acid molecule. Raistrick & Stössl (1958) have shown that incorporation of nitrogen into β -nitropropionic acid and mycelium is rapid at first and reaches a stationary maximum in both at approximately the same time. Tracer was added at day 0 and flasks were harvested during this stationary period, so that, whether β -nitropropionic acid is formed directly from oxaloacetic acid, or, after amination, from aspartic acid, this close relationship would be expected.

It seems feasible that β -nitropropionate could be formed directly from aspartate by oxidation of the amino group and loss of C-1. If either of the symmetrical C_4 tricarboxylic acid-cycle acids fumarate or succinate were a direct precursor, β -nitropropionate derived from $[2-^{14}C]$ pyruvate would have to have equal labelling in C-2 and C-3. As this has been shown to be otherwise, the aspartase-like reaction between fumarate and hydroxylamine

suggested by Hylin & Matsumoto (1961) seems unlikely. However, there is no evidence about the transformations of the amino group, and, since aspartate is in equilibrium with oxaloacetate, incorporation into oxaloacetate of a form of nitrogen, e.g. hydroxylamine, more oxidized than the amino level, would also be in agreement with the tracer work with ^{14}C . The observations that succinic acid, fumaric acid and tartaric acid increase β -nitropropionic acid production more than aspartic acid, and that stimulation by the latter is the same whatever the optical configuration (Hylin & Matsumoto, 1961), suggest the amino group may not be directly oxidized. Tartrate can be dehydrated in bacteria to oxaloacetic acid (Krampitz & Lynen, 1956; Shilo, 1957).

Recently Marshall & Alexander (1962) have implicated β -nitropropionic acid in heterotrophic nitrification in *Aspergillus flavus*. A nitrifying

Table 2. Degradation of $[1-^{14}C]$ acetate-derived β -nitropropionic acid

Experimental details are given in the text.

Carbon of β -nitropropionic acid	Product counted and origin	Degradation I			Degradation II		
		Radio-activity (μC /mole)	Radioactivity/mole of β -nitropropionic acid (μC /mole)	Percentage of total radioactivity of oxidized β -nitropropionic acid	Radio-activity (μC /mole)	Radio-activity/mole of β -nitropropionic acid (μC /mole)	Percentage of total radioactivity of oxidized β -nitropropionic acid
C-3 $CH_2 \cdot NO_2$	$BaCO_3$ (C-1 of acetate)	—	—	—	5.74	5.74	2.3
C-2 CH_2	$BaCO_3$ (C-2 of acetate)	—	—	—	2.20	2.20	0.9
C-1 CO_2H	$BaCO_3$ (malonic semialdehyde DNP-hydrazone)	164.9	164.0	93.2	230.3	230.3	93.4
Acetate (C-2 + C-3)	$BaCO_3$ (combustion of acetate)	2.04	4.08	2.3	4.00	8.00	3.2
Radioactivity accounted for (C-1 + C-2 + C-3)	—	—	—	95.5	—	—	96.6
Oxidized β -nitropropionic acid	$BaCO_3$	58.94	176.8	100	82.2	246.6	100
β -Nitropropionic acid	β -Nitropropionic acid	164.6	164.6	93.2	220.3	220.3	89.3
Acetaldehyde (C-2 + C-3)	Acetaldehyde DNP-hydrazone	—	—	—	8.00	8.00	3.2
C-2 + C-3	$BaCO_3$	—	—	—	—	7.94	3.2

Scheme 4. Degradation of aspartic acid.

strain formed organically bound hydroxylamine, nitrite and nitrate simultaneously after growth on peptone, amino acid or ammonium medium at neutral pH. β -Nitropropionic acid production on a neutral ammonium medium was also demonstrated and a rather specific enzyme forming nitrite from it could be prepared from the mycelium. This was the only authenticated step although the oxidation states of the detected nitrogen compounds suggested a sequence:

The *A. flavus* strain of Bush *et al.* (1945, 1951) formed no nitrite or nitrate and *P. atrovenerum* only very little on neutral ammonium or peptone medium (Marshall & Alexander, 1961). The nature

of the bound hydroxylamine was not determined and it was uncertain whether it arose by oxidation of an amino group or organic binding of free hydroxylamine. There have been other reports of bound hydroxylamine. Virtanen & Laine (1939) found the oxime of oxaloacetic acid excreted by pea-plant nodules actively fixing nitrogen. Virtanen & Saris (1955) identified the oximes of pyruvic acid, 2-oxoglutaric acid, oxaloacetic acid and glyoxylic acid when *Torulopsis utilis* was grown on nitrite, and the conclusion was that the oximes were formed non-enzymically from free hydroxylamine and the oxo acids. Saris & Virtanen (1957*a, b*) also obtained some complex-bound hydroxylamine from *Azotobacter* with nitrogen, nitrite or ammonium as nitrogen source. It was not formed if the pH was allowed to fall.

Table 3. Degradation of [2- ^{14}C]pyruvate-derived aspartic acid

Experimental details are given in the text.

Carbon of aspartic acid	Product counted and origin	Percentage error in counts	Radioactivity (counts/min./100 mg.) (background subtracted)	Radio-activity ($\mu\text{C}/\text{mole}$)	Radio-activity/mole of aspartic acid ($\mu\text{C}/\text{mole}$)	Percentage of total radioactivity of oxidized aspartic acid
C-1 CO_2H	C-1 + C-4 (ninhydrin) minus C-4 (<i>Cl. welchii</i>)		97	19.45	19.45	28.5
C-2 $\text{CH}\cdot\text{NH}_2$	BaCO_3 (Schmidt)	± 1.0	89 ± 0.9 [61 ± 0.6]* (18)	17.84	17.84	26.2
C-3 CH_2	BaCO_3 (methylamine)	± 1.6	47 ± 0.8 [32 ± 0.5]* (22)	9.42	9.42	13.8
C-4 CO_2H	BaCO_3 (<i>Cl. welchii</i>)	± 1.2	97 ± 1.2 (10)	19.45	19.45	28.5
(C-1 + C-4)	BaCO_3 (ninhydrin)	± 1.2	97 ± 1.2 (9)	19.45	38.90	57.0
Acetaldehyde (C-2 + C-3)	Acetaldehyde DNP-hydrazone	± 0.9	129 ± 1.4 (1)	29.40	29.40	43.1
C-2 + C-3	BaCO_3		136	27.26	27.26	40.0
Oxidized aspartic acid	BaCO_3	± 1.0	85 ± 0.86 (11)	17.04	68.16	100
Aspartic acid	Aspartic acid			66.36	66.36	97.3
Radioactivity accounted for:						
C-1 + C-4 (ninhydrin) + C-2 + C-3		—	—	—	—	97.0
C-1 + C-4 (ninhydrin) + C-2 + C-3 (acetaldehyde DNP-hydrazone)		—	—	—	—	100.10

* Values in square brackets are those actually determined on the BaCO_3 before correction for dilution (see the text).

Table 4. Comparison of percentage distributions of radioactivity in aspartic acid and β -nitropropionic acid

Carbon of aspartic acid	Percentage distribution of radioactivity	Percentage distribution of radioactivity in C-2, C-3 and C-4 of aspartic acid as percentage of their sum	Carbon of β -nitropropionic acid	Percentage distribution of radioactivity in β -nitropropionic acid	
				I	II
C-1 CO_2H	28.5				
C-2 $\text{CH}\cdot\text{NH}_2$	26.2	38.2	C-3 $\text{CH}_2\cdot\text{NO}_2$	41.3	40.2
C-3 CH_2	13.8	20.2	C-2 CH_2	40.0	19.3
C-4 CO_2H	28.5	41.6	C-1 CO_2H	20.1	39.2

There are certain paradoxes between the work with *A. flavus* and *P. atrovenetum*. When the *A. flavus* medium was allowed to become acid, small quantities of free hydroxylamine were formed, but no bound hydroxylamine, nitrite or nitrate. If β -nitropropionic acid were on the nitrifying pathway its production might also be expected to stop, although no test for it under these conditions was reported by Marshall & Alexander (1962). *P. atrovenetum* was grown by Birch *et al.* (1960) and by ourselves on Raulin-Thom medium with a starting pH of about 4.0 falling as low as 2.8, but the yields were excellent.

Also, in the *A. flavus* work, β -alanine was a markedly better nitrogen source for nitrite and nitrate production than peptone, DL- α -alanine or DL-aspartic acid. With *P. atrovenetum* Birch *et al.* (1960) showed that radioactivity from β -alanine was not incorporated into β -nitropropionic acid, although β -alanine is taken up by the mould (Dryland, 1962), and Hylin & Matsumoto (1961) found that β -nitropropionic acid production was enhanced much less by β -alanine added to the medium than by aspartic acid.

It seems clear that the carbon skeleton of β -nitropropionic acid is formed from C-2-C-4 of oxaloacetic acid, C-4 becoming the C-1 of β -nitropropionic acid, but whether the nitro group is formed by direct oxidation of an amino group or by incorporation into oxaloacetate of a more oxidized form of nitrogen such as hydroxylamine remains uncertain.

SUMMARY

1. [$1\text{-}^{14}\text{C}$]Acetate and [$2\text{-}^{14}\text{C}$]pyruvate were used as tracers for studying the production of β -nitropropionic acid by *Penicillium atrovenetum*, and a method of degrading the nitro acid so that each carbon could be obtained separately was developed.

2. The radioactivity incorporated into β -nitropropionic acid from each tracer was shown to be in agreement with the theoretical if these compounds were incorporated by way of the tricarboxylic acid cycle into oxaloacetate, and the β -nitropropionic acid carbon skeleton was derived from this by loss of C-4.

3. Aspartic acid was isolated from the mycelium used for β -nitropropionic acid production with [$2\text{-}^{14}\text{C}$]pyruvate as tracer. The portion of the molecule remaining after subtraction of C-4 was shown to have a specific radioactivity almost identical with that of β -nitropropionic acid and to contain the same distribution of radioactivity.

4. The unequal distribution of radioactivity in C-2 and C-3 of [$2\text{-}^{14}\text{C}$]pyruvate-derived β -nitropropionic acid indicated that fumaric acid, owing to its symmetry, could not be a direct precursor. The level of oxidation of nitrogen introduced into oxal-

acetate, whether amino or more oxidized, e.g. hydroxylamine, and the possible relationship with heterotrophic nitrification, are discussed.

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The Isolation of *S*-Sulphoglutathione from the Small Intestine of the Rat

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Administered inorganic [^{35}S]sulphate is incorporated into sulphated mucopolysaccharides by mammalian intestine *in vivo* (Crevier & Bélanger, 1954; Kent, Whitehouse, Jennings & Florey, 1956). The initial reaction, the activation of sulphate to form adenosine 3'-phosphate 5'-sulphatophosphate (Robbins & Lipmann, 1957), has been shown to occur in mucosal extracts (Kent & Pasternak, 1958; Varandani, Wolf & Johnson, 1960), and adenosine 3'-phosphate 5'-sulphatophosphate itself has been isolated from mouse intestine (Pasternak, 1960). In the course of that isolation it was noted that other ^{35}S compounds of low molecular weight were formed by mammalian intestine and one of these has now been identified as *S* [^{35}S]-sulphoglutathione. Waley (1959) has previously reported the isolation of *S*-sulphoglutathione from mammalian lens.

MATERIALS AND METHODS

S-Sulphocysteine. The sodium salt of *S*-sulphocysteine was prepared by the procedure outlined by Segel & Johnson (1963). The crystalline material gave an infrared-absorption spectrum similar to that published by those authors; $[\alpha]_D^{25} -93 \pm 1^\circ$ (c, 1.0) in water (Found: C, 15.0; H, 3.65; N, 6.5; S, 21.9; Na, 9.4. Calc. for $\text{C}_3\text{H}_6\text{NO}_2\text{S}_2\text{Na} \cdot \text{H}_2\text{O}$: C, 14.9; H, 3.35; N, 5.8; S, 26.6; Na, 9.5%). The material yielded a single orange-red spot with ninhydrin reagent after paper chromatography in solvents *A*, *B* and *C* (see below).

S-Sulphoglutathione. *S*-Sulphoglutathione was prepared by a modification of the method described by Waley (1959). Oxidized glutathione (350 mg.; Sigma Chemical Co., St Louis 18, Mo., U.S.A.) was shaken at 37° with 10 ml. of 1.0*M*-sodium sulphite-0.01*M*-cupric sulphate adjusted to pH 7.0 with nitric acid. After 12 hr. the reaction mixture

had changed from colourless to deep blue and the solution was then applied at 4° to a column (20 cm. $\times$ 1.5 cm. diam.) of Dowex 50 (X_4 ; H^+ form; 200-400 mesh). The column was washed with 20 ml. of water and the total eluate was neutralized with an aqueous suspension of barium hydroxide. The barium sulphate was removed by centrifugation and the barium salt of *S*-sulphoglutathione precipitated with 5 vol. of ethanol. The white precipitate was collected by centrifugation, dissolved in water, filtered through an Oxoid membrane filter (Oxo Ltd., London) and reprecipitated with ethanol five times. The final precipitate (250 mg., 36% yield) was dried under vacuum over anhydrous calcium chloride at room temperature. The barium salt of *S*-sulphoglutathione had $[\alpha]_D^{20} -36 \pm 6^\circ$ (c 0.17) in water (Found: C, 19.8; H, 3.85; N, 7.3; S, 11.95; Ba, 23.4. Calc. for $\text{C}_{10}\text{H}_{15}\text{N}_3\text{O}_6\text{S}_2\text{Ba} \cdot 4\text{H}_2\text{O}$: C, 20.2; H, 3.9; N, 7.1; S, 10.8; Ba, 23.1%). The first sulphur analysis gave a very low value (5.6%). That quoted was obtained by iodine titration of reduced material. It yielded only one ninhydrin-positive spot after paper electrophoresis in 0.1*M*-sodium acetate buffer, pH 5.6, and after paper chromatography in solvents *A*, *B* and *C* (see below). It was hydrolysed by 1.0*N*-hydrochloric acid (at 100° for 30 min.) to yield inorganic sulphate. The pure material gave a positive reaction for free thiol with sodium nitroprusside only after acid hydrolysis.

2,4-Dinitrophenyl-*S*-sulphoglutathione. The barium salt of *S*-sulphoglutathione (6 mg.) in 2.0 ml. of aq. 1% (v/v) triethylamine was mixed with a solution containing 5 ml. of ethanol, 0.05 ml. of 1-fluoro-2,4-dinitrobenzene and 0.05 ml. of triethylamine. The mixture was shaken at 37° for 2 hr. and the reaction terminated by the addition of 4.0 ml. of 2.0*N*-formic acid and 10 ml. of ether. The aqueous phase which separated was extracted with ether and the DNP derivative of *S*-sulphoglutathione so obtained was purified by paper electrophoresis on Whatman 3MM paper in 0.1*M*-sodium acetate buffer, pH 5.6, followed by preparative paper chromatography on Whatman 3MM paper in solvent *B*. The yellow material was eluted from the paper in a few millilitres of water and stored in the dark at -20° .

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