Studies in the Biochemistry of Micro-organisms

104. METABOLITES OF *PENICILLIUM ATROVENETUM* G. SMITH: β -NITROPROPIONIC ACID, A MAJOR METABOLITE*

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Gorter (1920) reported the isolation, from the root-bark of a Javanese tree, *Hiptage madablota* Gaertn., of a new glucoside, hiptagin. By treating hiptagin in the cold with hydrochloric acid in acetone he obtained, in addition to glucose, ammonia and carbon dioxide, a colourless crystalline substance, m.p. 68°, which he named hiptagenic acid. He showed that this substance is a strong monobasic acid with the empirical formula $C_3H_5O_4N$ but was unable to identify it with any known substance. Carrie (1934) investigated the known glucoside karakin, from the berries of the New Zealand karaka tree (*Corynocarpus laevigata* Forst.), and by following the same hydrolysis procedure as was used by Gorter isolated, in addition to glucose, ammonia and carbon dioxide, a crystalline acid, m.p. 68°, which was shown by determination of a mixed melting point with hiptagenic acid to be identical with it. Karakin was reinvestigated by Carter (1943), who confirmed the empirical formula for hiptagenic acid and attempted unsuccessfully to determine its structure and to synthesize it. Later Carter & McChesney (1949) identified hiptagenic acid as β -nitropropionic acid, $CH_2(NO_2) \cdot CH_2 \cdot CO_2H$. Some years ago the legume *Indigofera endecaphylla* Jacq. ('trailing indigo') was introduced from the Orient as a forage and cover crop into Hawaii and Latin America, but was later found to produce severe toxic symptoms in dairy cattle. Morris, Pagán & Warmke (1954) identified the toxic principle as β -nitropropionic acid and remarked that since this acid has now been isolated from several different species of plants 'it may play an important role in nitrogen metabolism'.

Meanwhile β -nitropropionic acid became of interest from an entirely different biological angle when it was reported as a metabolite of strains in the *Aspergillus flavus-oryzae* group of microfungi. Bush & Goth (1943) investigated flavicin, a penicillin-like antibiotic which they isolated from cultures of *A. flavus* grown on a modified Czapek-Dox glucose-mineral salts medium containing relatively large amounts (20 ml./l.) of concentrated

corn steep liquor. During the purification of crude flavicin Bush, Goth & Dickson (1945) isolated a toxic fraction in colourless crystals, m.p. 67.5–68.5°, equiv. wt. 124, which had no antibiotic activity, in a yield of 900 mg. from 90 l. of culture fluid. This substance was identified as β -nitropropionic acid by Bush, Touster & Brockman (1951), who state 'whether it [i.e. β -nitropropionic acid] is formed by enzymatic decomposition of a complex substance in the corn steep liquor, or by synthesis from simple precursors, is not known'. More recently Nakamura & Shimoda (1954) reported the detection of β -nitropropionic acid in crude preparations of a new antibiotic, oryzacinin, which was isolated by Shimoda (1951) from laboratory cultures of *Aspergillus oryzae*. This species, which is very closely related morphologically to *A. flavus*, was grown on a sucrose (5%)–peptone (2%)–mineral salts medium in which the nitrogen was present exclusively as peptone and 0.7 g. of the crude sodium salt of oryzacinin was obtained from 12 730 l. of this culture medium. Fractionation of 7.0 g. of the crude sodium salt yielded 310 mg. of β -nitropropionic acid.

We have now found that two out of three available strains of *Penicillium atrovenetum* G. Smith produce remarkably large yields of β -nitropropionic acid when grown on Raulin-Thom solution, which is a chemically defined medium containing only mineral salts in addition to glucose and tartaric acid and in which the sole sources of nitrogen are the ammonium salts of tartaric, phosphoric and sulphuric acids. β -Nitropropionic acid is also produced by *P. atrovenetum* when this mould is grown on Czapek-Dox solution, a chemically defined medium in which sodium nitrate is the sole source of nitrogen. Paradoxically, however, the yield obtained was only about 10% of that isolated from Raulin-Thom solution.

The mould used is a new species of *Penicillium* which was described recently by Mr George Smith of this Department (Smith, 1956). It has been shown by Neill & Raistrick (1957) to produce a new mycelial colouring matter, atrovenetin, yellow-orange plates, $C_{19}H_{18}O_6$, which, along with the

* Part 103: Rosett, Sankhala, Stickings, Taylor & Thomas (1957).

colouring matters herqueinone, $C_{20}H_{20}O_7$, and norherqueinone, $C_{19}H_{18}O_7$, from the morphologically closely related species, *Penicillium herquei* Bainier & Sartory, is believed to be a derivative of 9-hydroxyperinaphthen-1-one (Barton, de Mayo, Morrison, Schaeppi & Raistrick, 1956).

β -Nitropropionic acid was isolated by exhaustive ether extraction of the acidified culture filtrates of *P. atrovenerum*. The ether extract was evaporated to dryness and the largely crystalline residue was sublimed in high vacuum. The colourless crystalline sublimate, m.p. 68–69°, was identified as β -nitropropionic acid by analysis and by comparison with an authentic synthetic specimen of the acid. A mixture of the two specimens showed no depression in melting point.

A series of metabolism experiments on cultures of the type strain of *P. atrovenerum* grown on Raulin–Thom medium has established the following points of interest; in all these experiments the quoted yields of β -nitropropionic acid refer to substantially pure, colourless, sublimed acid with a melting point close to 68°.

(a) Some of the β -nitropropionic acid is formed in the earliest stages of active growth and metabolism, so that it can scarcely be dismissed as a product of autolysis or as a waste end-product. Thus, although no acid could be detected in sterilized but uninoculated medium, 3 mg. was isolated from a single flask (350 ml. of medium) 28 hr. after inoculation and this amount had increased to 26.5 mg./flask after 48 hr. incubation.

(b) The greater part of the β -nitropropionic acid is formed before the mould is fully grown. Thus, after 5 days' incubation, when the dry weight of the mould mycelium/flask was 2.935 g., the yield of acid was 298 mg. as compared with 304 mg./flask after 8 days' incubation, when the weight of the mould had increased to 4.188 g. (see Table 4).

(c) The yields of β -nitropropionic acid are surprisingly large, the maximum yield obtained on normal Raulin–Thom medium being 370 mg./flask (350 ml.) after 8 days' incubation. In a study of the nitrogen balance during growth of the mould on normal Raulin–Thom medium (see Tables 4 and 5), after incubation periods of 2, 3, 5, 8 and 9 days, the following estimations were made: (1) residual ammonia nitrogen; (2) nitrogen in the mould mycelium; (3) calculated nitrogen in recovered β -nitropropionic acid. From these figures it is calculated that an average of 63% (limits 59–68%) of the ammonia nitrogen which is metabolized by the mould in ways other than in the synthesis of its own mycelium is present in the isolated β -nitropropionic acid, 336 mg./flask of which was obtained after 9 days' incubation.

A similar study (see Tables 6 and 7) was made, in which the Raulin–Thom medium used was modified

by the use of three times the normal amount of ammonium tartrate, thus giving a medium containing 450 mg. of ammonia nitrogen/flask as compared with 180 mg./flask in the normal Raulin–Thom medium. The results obtained showed that, after 3, 5, 7, 12 and 18 days' incubation, an average yield, calculated as above, of 68% (limits 62–82%) was obtained and that 848 mg. of β -nitropropionic acid/flask was isolated after 12 days' incubation.

(d) Analysis of the nitrogen balance on the modified Raulin–Thom medium (Tables 6 and 7) confirms the conclusion previously arrived at that β -nitropropionic acid is essentially a product of the earlier stages of growth and metabolism. Thus, after 3 days' incubation, when only one-quarter of the glucose and one-third of the ammonia nitrogen originally present in the culture medium had been metabolized, 60% (512 mg./flask) of the maximum yield (848 mg. after 12 days) of β -nitropropionic acid had been formed.

β -Nitropropionic acid appears to be the first and only aliphatic nitro compound ever isolated from natural sources, though two naturally occurring aromatic nitro compounds have been described, namely the antibiotic chloramphenicol (chloromycetin) from *Streptomyces venezuelae* (Rebstock, Crooks, Controulis & Bartz, 1949) and an unnamed toxic nitro compound, $C_{21}H_{21}O_6N$, which was isolated from cultures of *Streptomyces thioluteus* by Hirata, Okuhara & Naito (1954). Both these compounds contain a *p*-nitrophenyl residue.

EXPERIMENTAL

Cultures. Three strains of *Penicillium atrovenerum* G. Smith were used: (a) The type strain L.S.H.T.M. (London School of Hygiene and Tropical Medicine), cat. no. S.M. 683; (b) strain S.M. 715; (c) strain M. 467. The history of these strains is given by Neill & Raistrick (1957) and a detailed description of the species is given by Smith (1956). β -Nitropropionic acid was isolated from cultures of strains (a) and (b) but none could be detected from strain (c).

Culture media. (a) *Czapek–Dox solution*: glucose (anhydrous), 50.0 g.; $NaNO_3$, 2.0 g.; KH_2PO_4 , 1.0 g.; KCl , 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $FeSO_4 \cdot 7H_2O$, 0.01 g.; water to 1 l. The only source of nitrogen in this medium is $NaNO_3$ and corresponds to 0.3296 g. of N/l. of medium.

(b) *Raulin–Thom solution*: glucose (anhydrous), 75 g.; tartaric acid, 4.0 g.; ammonium tartrate, 4.0 g.; $(NH_4)_2HPO_4$, 0.6 g.; $(NH_4)_2SO_4$, 0.25 g.; K_2CO_3 , 0.6 g.; $MgCO_3$, 0.4 g.; $FeSO_4 \cdot 7H_2O$, 0.07 g.; $ZnSO_4 \cdot 7H_2O$, 0.07 g.; water to 1.5 l. Nitrogen (0.5258 g. N/l.) is present in this medium solely as ammonium salts.

Production and isolation of β -nitropropionic acid on Raulin–Thom solution

(a) *By strain S.M. 683.* Raulin–Thom solution was distributed in 350 ml. amounts in forty 1-l. conical flasks which were plugged with cotton wool and sterilized at 100° for 30 min. on each of three successive days. The contents of

the flasks were then inoculated with a spore suspension in water, prepared from malt-agar slopes, of the type strain of *Penicillium atrovenetum* S.M. 683, four flasks being sown from each slope. The flasks were incubated in the dark at 24° and were harvested in four batches of ten after 7, 8, 9 and 10 days' incubation respectively. The mycelia in each case covered the surface of the culture medium and had a grey, heavily wrinkled surface with a bright orange-yellow reverse. The clear light-yellow culture fluids were separated by filtration from the mycelia, which were pressed as dry as possible. Each batch of filtrate (pH 3.0, 3.0, 3.2, 3.2) was then extracted without acidification thrice with 0.5 vol. of ether. The ether extracts from each batch were combined, filtered, evaporated at 50°, dried *in vacuo* and weighed (see Table 1, column 2). The residues, consisting of a mass of colourless long needles set in a brown semi-solid oil, were transferred separately to a 250 ml. distillation flask fitted with a 'cold finger condenser' and exhaustively sublimed in high vacuum (about 10^{-4} mm. Hg) with a bath temp. of 60–65°. All the colourless crystalline sublimates melted in the range 65–69° (see Table 1, column 3).

The results summarized in Table 1 indicate that a total of 9.26 g. of sublimed material was isolated from 40 flasks of cultures containing originally a total of 14 l. of Raulin-Thom solution, which is equivalent to 231.5 mg. of sublimates/flask.

The dried crude ether extracts are not readily purified by crystallization from any solvent, though small amounts of the metabolic product may be crystallized from light petroleum (b.p. 60–80°) or cyclohexane, in neither of which is it very soluble. However, the substance sublimes very readily and almost quantitatively in high vacuum at 60–65°, the sublimation being conveniently carried out in a vessel immersed in the vapour of boiling CHCl_3 . By

repeated crystallization or sublimation it was obtained as colourless needles with a constant m.p. of 68–69° (corr.). It contains C, H, O and N, but halogens, sulphur and phosphorus are absent [Found: C, 30.1, 30.2; H, 4.11, 4.26; N, 11.8, 11.9; O (by direct estimation), 54.3, 54.2%; mol.wt. (Rast), 123. Calc. for $\text{C}_7\text{H}_5\text{O}_4\text{N}$: C, 30.3; H, 4.23; N, 11.8; O, 53.75%; mol.wt. 119].

The purified product is readily soluble in water, ether, ethanol and benzene. Its aqueous solution is acid to Congo red, gives no precipitate with Brady's reagent (0.32% 2:4-dinitrophenylhydrazine in aq. 2N-HCl), does not absorb Br_2 from bromine water or liberate I_2 from aqueous KI. Its aqueous solution, after neutralization with NaOH to phenolphthalein, gives a buff-coloured precipitate with aqueous FeCl_3 .

The product gave a typical red colour reaction for a primary nitro group in a Victor Meyer nitrosation test with conc. NaNO_2 in 20% aqueous NaOH (quoted in Sidgwick, 1942). It was identified as β -nitropropionic acid, $\text{C}_3\text{H}_5\text{O}_4\text{N}$, $\text{CH}_2(\text{NO}_2)\cdot\text{CH}_2\cdot\text{CO}_2\text{H}$, by failing to depress the m.p., 67–68.5°, of an authentic synthetic specimen, on admixture with it. β -Nitropropionic acid was synthesized, substantially as described by Gresham *et al.* (1952), and purified by sublimation. β -Propionolactone was slowly treated with aqueous NaNO_2 solution at room temp. After standing for 2.5 hr. at 2° the mixture was acidified with conc. HCl and extracted with ether. The crude product obtained on removal of the solvent was purified by repeated sublimation in high vacuum as previously described, giving synthetic β -nitropropionic acid as long colourless needles, m.p. 67–68.5°.

In a duplicate experiment designed to determine the maximum recoverable yield of β -nitropropionic acid and the optimum time of harvesting, 40 flasks of Raulin-Thom medium (350 ml. in each) were inoculated with *P. atrovenetum* type strain S.M. 683 grown on malt-agar, and batches of five flasks each were harvested after 5, 7, 8, 9, 11, 22 and 28 days' incubation at 24°. The culture fluids were collected as in the previous experiment but in this case were acidified by the addition of conc. HCl (5 ml.) to each batch (pH about 1.6). They were then extracted with ether in a highly efficient continuous extraction apparatus for a total period of about 48 hr., the ether extracts being collected from time to time. The crude extracts from each batch were combined, evaporated, dried *in vacuo* and weighed (Table 2, column 3). Each extract was then re-extracted with sodium-dried ether and separated by filtration from undissolved material consisting of tartaric acid in the early stages and semi-solid resins in the later stages of

Table 1. Isolation of β -nitropropionic acid from the type strain S.M. 683 of *P. atrovenetum* grown on normal Raulin-Thom solution

Experimental details are given in the text.

Incubation period (days)	Wt. of dried ether extract (mg./flask)	Wt. of sublimed β -nitropropionic acid (mg./flask)
7	351	242
8	350	231
9	344	236
10	343	217

Table 2. Production of β -nitropropionic acid by strain S.M. 683 grown on normal Raulin-Thom solution

Experimental details are given in the text.

Incubation period (days)	pH	Crude ether extract (mg./flask)	Material not extracted by dry ether (mg./flask)	Material extracted by dry ether (mg./flask)	Wt. of sublimed β -nitropropionic acid (mg./flask)
0	3.9	—	—	—	—
5	3.2	618	210	408	280
7	3.0	681	118	563	349
8	3.0	756	139	617	367
9	3.2	617	95	522	309
11	3.2	665	85	580	284
22	3.9	549	75	474	266
28	4.2	525	22	503	259

incubation (wts. in Table 2, column 4). The ether extracts were evaporated to dryness in the sublimation flask (Table 2, column 5; wt. = column 3 - column 4) and sublimed in high vacuum giving colourless crystalline sublimates (Table 2, column 6) similar to those described in the previous experiment. The results given in Table 2 indicate that the optimum time of harvesting is about 8 days and that, after this incubation period, 0.367 g./flask of pure sublimed β -nitropropionic acid can be obtained.

(b) *By strain S.M. 715.* Twenty-five flasks of Raulin-Thom solution were inoculated with *P. atrovenetum* strain S.M. 715 and incubated at 24°. Batches of five flasks were harvested after 5, 7, 9, 14 and 21 days. The mycelia, which were grey and heavily wrinkled after 5 days, quickly developed a blue-green colour with a dirty-yellow reverse. The culture filtrates, which were bright yellow to clear orange-yellow in colour up to 14 days, changing to light brown after 21 days, were collected as with strain S.M. 683. Portions (500 ml.) from each batch were extracted a number of times with 250 ml. of ether each time. None of the batches was acidified before extraction except the batch which was harvested after 21 days, which was made acid to Congo red with HCl. The results obtained are summarized in Table 3 and indicate that the yields of β -nitropropionic acid, m.p. 67–69°, obtained from *P. atrovenetum* strain S.M. 715 are of the same order as those obtained from strain S.M. 683.

(c) *By strain M. 467.* Ten flasks of *P. atrovenetum* strain M. 467, grown on Raulin-Thom solution for 7 days, were harvested in the usual manner. The culture filtrate, pH 2.9, was extracted 5 times with 0.5 vol. of ether, giving 0.571 g. of light-brown oil from which no crystals, but only a trace of colourless oil, were obtained on attempted sublimation at 62° in high vacuum.

*Production of β -nitropropionic acid on
Czapek-Dox solution by strain S.M. 683*

Twenty flasks of Czapek-Dox solution were inoculated with the type strain S.M. 683 of *P. atrovenetum* and incubated at 24°. Two batches of 10 flasks each were harvested after 7 and 10 days' incubation respectively. The mycelia were different in appearance from those on Raulin-Thom solution, having a blue-green upper surface and an orange reverse. The culture filtrates, light green-brown in colour and somewhat opalescent, were acidified with conc. HCl (5 ml.) and extracted five times with 0.5 vol. of ether. The experimental results follow. Incubation period, 7 and 10 days; pH 4.5 and 4.3; glucose (%), 3.04 and 2.46; total wt. of dried ether extract from 10 flasks, 0.934 and 0.999 g.;

total wt. of sublimate from 10 flasks, 0.309 and 0.271 g. The sublimate was not nearly as pure as those obtained from Raulin-Thom solution, but, after resublimation, 527 mg. of the combined sublimate gave 513 mg. of off-white needles, m.p. 60–67°, mixed m.p. 64–69° with crystals obtained from Raulin-Thom solution. The yield of β -nitropropionic acid obtained on Czapek-Dox solution was thus only about one-tenth of that from Raulin-Thom solution.

In an experiment designed to investigate the water-soluble, ether-extractable metabolites of *P. atrovenetum* other than β -nitropropionic acid, 100 flasks of Czapek-Dox solution were inoculated with strain S.M. 683 and incubated at 24° for 21 days. The mycelium (dry wt. 328 g.), which was then deep blue-green in colour with an orange reverse, was separated by filtration from the greenish brown, somewhat opalescent culture fluid of pH 4.1 and containing 0.79% of residual glucose (by polarimeter). The filtrate was acidified with conc. HCl (200 ml.) to pH about 1.6, kept at 0° for 12 days and refiltered. This filtrate was adjusted to pH 6.6–7.0 with aqueous NaOH, evaporated *in vacuo* to 1.5 l., reacidified with conc. HCl (200 ml.) and again filtered. The chocolate-brown precipitates (6 g.), probably containing atrovenetin, which were separated by filtration, were discarded.

The reddish coloured filtrate was extracted twice with ether (200 ml. + 500 ml.). The extract, on removal of the solvent, gave 6.1 g. of dark-brown gum which did not crystallize. The aqueous phase was then further extracted with ether in two highly efficient continuous extractors and gave the following fractions: (a) after 14 hr., 14.0 g. of partially crystalline gum; (b) after a further 70 hr., 32.7 g., mostly crystalline; (c) after concentration of the aqueous phase to 900 ml., filtration and extraction for 84 hr., 31.1 g. of good crystals, m.p. 130–150°. Fractions (a) and (b) were combined and were shown to consist essentially of somewhat impure citric acid, as follows. The mixture was washed with cold ethyl acetate to remove gum, crystallized from boiling ethyl acetate (Soxhlet) + charcoal, giving 28.6 g. of off-white crystals, m.p. 148–152°, and then from dry ether (Soxhlet), giving colourless crystals, m.p. 152–153°, mixed m.p. 151.5–153° with anhydrous citric acid, m.p. 151.5–153°; titration equivalent, 64.28, calc. for anhydrous citric acid $C_6H_8O_7$, titrating as a tribasic acid, 64.04; trimethyl ester, via methanol + dry HCl, colourless crystals, m.p. 76–78° (literature, 78.5–79°); citramide, from the above trimethyl ester + NH_3 , colourless crystals, m.p. 207° (literature, 207–208°). Fraction (c) consisted of only slightly impure citric acid. The total yield of citric acid obtained was thus about 60 g.

Table 3. *Production of β -nitropropionic acid by P. atrovenetum, strain S.M. 715, grown on normal Raulin-Thom solution*

Experimental details are given in the text.				
Incubation period (days)	pH	No. of extractions	Crude ether extract (mg./flask)	Wt. of sublimed β -nitropropionic acid (mg./flask)
5	3.3	5	368	276
7	3.3	7	355	255
9	3.4	13	389	273
14	3.7	6	281	194
21	4.1	11	390	273

*Nitrogen balance sheets for cultures of
P. atrovenetum strain S.M. 683*

A. *Grown on normal Raulin-Thom medium.* A batch of fifty 1 l. flasks, each containing 350 ml. (accurately measured) of Raulin-Thom solution [see culture medium (b)], were sterilized at 100° for 30 min. on each of three successive days. Twenty-four flasks were inoculated with an aqueous suspension of spores of *P. atrovenetum* strain S.M. 683 prepared from 45-day-old cultures on tubes of malt-agar, three flasks being sown from each tube. A further 24 flasks were similarly inoculated with 13-day-old cultures of the same strain of *P. atrovenetum*. Two flasks were reserved for analysis.

The inoculated flasks were incubated at 24° in the dark and batches of four flasks each were harvested after 2, 3, 5, 8 and 9 days' incubation. Each batch was filtered on a Büchner funnel, the combined mycelia were drained at the pump and flasks and mycelia were washed with three successive portions of water. The combined filtrates and washings were adjusted to 1.5 l. and analysed as described below. The combined mycelia were dried to constant weight (Table 4, column 3) *in vacuo* at room temp. over conc. H_2SO_4 and then over silica gel.

The mould, which grew well on this medium, was white after 2 and 3 days' incubation, grey with an olive-yellow reverse after 5 days and blue-green with an olive reverse after 8 and 9 days. The culture solution became increasingly

yellow with age and was finally bright yellow and slightly opalescent after 9 days.

The following estimations, the results of which are assembled in Tables 4 and 5, were carried out on the culture filtrate and mycelium. (i) pH by comparator (Table 4, column 2). (ii) Residual ammonia N (Table 5, column 2). A rapid stream of NH_3 -free air was aspirated at room temp. through 10 ml. of the filtrate mixed with 2N-NaOH (5 ml.) and capryl alcohol (octan-2-ol) (10 drops) and the liberated NH_3 was absorbed and titrated in 0.02N- H_2SO_4 containing methyl red. The medium theoretically contained 184.1 mg. of NH_3 -N per flask; found, on medium after sterilization but not inoculated, 179.6 mg. of NH_3 -N per flask. (iii) N as β -nitropropionic acid (Table 5, column 4). The filtrate (1.4 l.) was acidified to pH 1.5 with conc. HCl (5–8 ml.) and was exhaustively extracted with ether in a continuous extractor for 12–18 hr. The ether extract was filtered, evaporated, dried *in vacuo* over silica gel and weighed (A). The dried extract was thoroughly re-extracted with dry ether, and the undissolved portion, consisting essentially of tartaric acid, was separated by filtration, dried and weighed (B). The difference between weights (A) and (B) gives the weight of the ether-soluble extract (Table 4, column 6) which was evaporated, allowed to crystallize and sublimed in high vacuum at 60–65° as described above. The colourless crystalline sublimate of almost pure β -nitropropionic acid was collected and weighed (Table 4, column 5), and from this weight its theoretical

Table 4. *General cultural details of P. atrovenetum, strain S.M. 683, grown on normal Raulin-Thom medium*

Experimental details are given in the text.

Incubation period (days)	pH	Wt. of mycelium (g./flask)	N content of mycelium (%)	Wt. of sublimed β -nitropropionic acid (mg./flask)	Total wt. of ether-soluble extract (mg./flask)
0	3.9	—	—	—	—
2	3.6	0.627	6.07	72.1	95.9
3	3.3	1.491	4.52	168.0	244.0
5	3.2	2.935	3.36	297.6	417.1
8	2.9	4.188	2.78	303.8	487.2
9	3.2	4.115	2.68	336.3	535.6

Table 5. *Nitrogen balance sheet for P. atrovenetum, strain S.M. 683, grown on normal Raulin-Thom medium (see Table 4)*

Incubation period (days)	N as residual NH_3 (mg./flask)	Mycelial N (mg./flask)	β -Nitropropionic acid N (mg./flask)*	Residual N (mg./flask)	N accounted for (mg./flask)
0	179.6	—	—	3.2	182.8
2	127.6	38.1	8.5 (61%)	3.8	178.0
3	82.0	67.3	19.8 (65%)	8.3	177.4
5	29.9	98.5	35.0 (68%)	10.6	174.0
8	4.9	116.2	35.7 (61%)	12.6	169.4
9	2.1	110.3	39.6 (59%)	16.3	168.3

* Values in parentheses are explained in the text.

content of N was calculated (Table 5, column 4). The figures given in parentheses in this column are calculated as follows. The sum of N in residual NH_3 (column 2) + N in the mycelium (column 3) is deducted from the N present as NH_3 in the original medium (179.6 mg.). The N present in the isolated β -nitropropionic acid is then expressed as a percentage of this figure, which may be described as the percentage of the yield of β -nitropropionic acid theoretically obtainable from the NH_3 -N that is metabolized by the mould in ways other than in the synthesis of its own mycelium.

(iv) Residual nitrogen in filtrate other than ammonia nitrogen (Table 5, column 5). The ether-extracted filtrate was freed from ether by bubbling air through it, and its volume was readjusted to its initial value of 1.4 l. by the addition of water. Its total N content was then determined on a portion by the Kjeldahl method. Deduction of the NH_3 -N (Table 5, column 2) from this Kjeldahl figure then gives 'Residual N in filtrate other than NH_3 -N'. (v) Mycelial N (Table 5, column 3). This was determined by the Kjeldahl method on weighed portions of the dried mycelium. (vi) N accounted for (Table 5, column 6). This figure is the sum of the items given in Table 5, columns 2-5.

The results obtained with the second batch of 24 flasks, which were inoculated with cultures of *P. atrovenetum* strain S.M. 683 aged 13 days, were in general very similar to those just described when cultures aged 45 days were used for inoculation. The main difference noted was that, whereas the older cultures gave a maximum yield of

336.3 mg. of β -nitropropionic acid/flask after 9 days, the younger cultures gave a maximum yield of 369.9 mg./flask after 8 days.

B. *Grown on modified Raulin-Thom medium.* The medium used in this experiment differed from normal Raulin-Thom medium in two respects: it contained (a) 5% of glucose monohydrate, (b) three times the amount of ammonium tartrate, i.e. 12.0 g./1.5 l. The medium thus theoretically contains 467.9 mg. of NH_3 -N/flask; found, on medium after sterilization but not inoculated, 450.4 mg. of NH_3 -N. The discrepancy between the NH_3 -N calculated and found occurs in both media A and B and is probably caused, in part, by the fact that solid diammonium tartrate 'slowly loses some NH_3 on exposure to air and then has an acid reaction' (Merck Index, 1952).

Twenty flasks, each containing 350 ml. of this medium, were sterilized and inoculated with a spore suspension of *P. atrovenetum* strain S.M. 683 in distilled water, prepared from five test-tube cultures on malt-agar and aged 32 days. The inoculated flasks were incubated at 24° in the dark and batches of four flasks each were harvested after 3, 5, 7, 12 and 18 days respectively.

The mould grew well on this medium and, in the early stages of growth, was very similar in appearance to the flask cultures on normal Raulin-Thom medium described in section A. Sporing commenced on the fifth day and was complete by the seventh day. On the eighteenth day the thick greyish green mycelium was very brittle and showed obvious signs of autolysis. The culture solution, initially

Table 6. *General cultural details of P. atrovenetum, strain S.M. 683, grown on modified Raulin-Thom medium*

Experimental details are given in the text.

Incubation period (days)	pH	Residual glucose (g./flask)	Wt. of mycelium (g./flask)	N content of mycelium (%)	Wt. of sublimed β -nitropropionic acid (mg./flask)	Total wt. of ether-soluble extract (mg./flask)
0	4.5	16.10	—	—	—	—
3	3.8	12.17	1.453	4.85	512	602
5	3.7	7.28	3.078	5.28	679	892
7	3.9	4.79	3.638	5.15	753	965
12	4.8	1.72	3.365	5.14	848	1033
18	7.2	0.67	3.033	5.06	758	1010

Table 7. *Nitrogen balance sheet for P. atrovenetum, strain S.M. 683, grown on modified Raulin-Thom medium (see Table 6)*

Incubation period (days)	N as residual NH_3 (mg./flask)	Mycelial N (mg./flask)	β -Nitropropionic acid N (mg./flask)*	N accounted for (mg./flask)
0	450.4	—	—	450.4
3	306.6	70.5	60.2 (82%)	437.3
5	171.0	162.4	79.8 (68%)	413.2
7	125.0	187.4	88.6 (64%)	401.0
12	117.5	173.1	99.7 (62%)	390.3
18	152.8	153.3	89.2 (62%)	395.3

* Values in parentheses are explained in the text.

clear pale-yellow in colour, gradually darkened to clear orange-yellow after 12 days' incubation.

The quantitative examination of the mould cultures was carried out in a similar way to that described in section A, but with the following variations: (a) The residual N in the culture filtrate other than $\text{NH}_3\text{-N}$ (Table 5, column 5) was not estimated. (b) Residual glucose in solution was estimated polarimetrically after removal of tartaric acid with basic lead acetate solution (Table 6, column 3). The results obtained are given in Tables 6 and 7.

SUMMARY

1. β -Nitropropionic acid, $\text{CH}_2(\text{NO}_2)\cdot\text{CH}_2\cdot\text{CO}_2\text{H}$, has been shown to be a major nitrogenous metabolite of cultures of *Penicillium atrovenetum* G. Smith grown on two chemically defined or 'synthetic' culture media containing, as sole sources of nitrogen, the ammonium salts of tartaric, phosphoric and sulphuric acids in the one medium and sodium nitrate in the other.

2. Over 60% of the ammonia nitrogen that was metabolized by the mould for purposes other than the synthesis of its mycelium was recovered as crystalline β -nitropropionic acid. A maximum yield of 2.4 g. of this acid/l. of culture filtrate was isolated.

3. β -Nitropropionic acid is formed in the earliest stages of growth of the mould, reaches a maximum amount before the mould is fully grown and thereafter decreases.

4. The yield of β -nitropropionic acid obtained from the medium containing nitrate was only about 10% of that isolated from the medium in which ammonium salts constituted the sole source of nitrogen.

5. β -Nitropropionic acid has now been isolated from several different species of plants and micro-organisms, so that it seems probable that the acid plays a significant, but at present undefined, part in their nitrogen metabolism.

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The Separation of Amino Acids and their N-Acetyl Derivatives by Paper Chromatography and Paper Ionophoresis

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The success of any method of analysis by isotopic-dilution techniques in which labelled reagents are employed is dependent upon the complete separation of the derivatives formed both from each other and also from any excess of the reagent used. During the development of such a method for the determination of sub-microgram quantities of

amino acids and peptides, which uses acylating agents labelled with ^3H and ^{14}C and separation of the acetyl derivatives by two-dimensional chromatography, it was found that traces of active acetate ion appeared in a vital area of the chromatogram. A satisfactory separation of the *N*-acetyl amino acids is obtained by chromatography