We are greatly indebted to Dr J. W. Minnis for C, H and N analyses, to Dr P. G. Walker for a gift of D-galactosamine hydrochloride and to Dr P. W. Kent for specimens of *N*-benzoyl- and *N*-toluene-*p*-sulphonyl-glucosaminolactones.

One of us (J.F.) is in receipt of a grant from the Medical Research Council.

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Crystalline *p*-Aminobenzylpenicillin: Preparation and some Properties

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(Received 25 November 1957)

Attempts to prepare *p*-aminobenzylpenicillin were made shortly after the role of precursors in penicillin fermentations became known. These attempts apparently failed (see Behrens, 1949, for review). The formation of *p*-aminobenzylpenicillin was reported by Corse, Jones, Soper, Whitehead & Behrens (1948), but no material was isolated. Brewer & Johnson (1953) described the preparation of a partially purified *p*-aminobenzylpenicillin by using *p*-aminophenylacetic acid as precursor. The levels of penicillin obtained in the fermentation were not given, the units/mg. of the material obtained were not indicated and it was stated that attempts at further purification had failed. It was reported, however, that the rate of decrease in concentration of *p*-aminobenzylpenicillin in the blood of dogs was slower than that of benzylpenicillin (penicillin G), that the rate of renal excretion was only one-third as fast as that for benzylpenicillin and that the material was somewhat more stable to acid than benzylpenicillin.

Owing to the presence of its aromatic amino group, *p*-aminobenzylpenicillin would be amenable

to treatments yielding several types of derivatives. This fact, together with the properties indicated by Brewer & Johnson (1953), initiated, in our Laboratories, a search for a method of preparing pure *p*-aminobenzylpenicillin in sufficient quantities for a systematic study.

The work of Brewer & Johnson (1953), using *p*-aminophenylacetic acid as precursor, was repeated. Penicillin titres of about 1000 units/ml. were obtained, and the active material when isolated was a yellow-to-brown amorphous powder which assayed at about 300 units/mg. Attempts to further purify the material were not successful.

p-Aminobenzylpenicillin is more hydrophilic than *p*-hydroxybenzylpenicillin (penicillin X) and hence cannot be separated from impurities found in the fermentation liquors by the conventional methods of solvent extraction commonly used with benzylpenicillin. Similar difficulties have been encountered in the purification of another hydrophilic penicillin, aminocarboxybutylpenicillin (cephalosporin N, or synnematin B; Newton & Abraham, 1954).

Since *p*-aminobenzylpenicillin is intractable to purification by solvent extraction of fermentation

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liquors, it was thought that it might more readily be obtained in the pure state by chemical conversion from a readily purifiable non-hydrophilic bio-synthetic penicillin. Accordingly, it was found that the non-hydrophilic *p*-nitrobenzylpenicillin was readily produced in the crystalline state and that on hydrogenation it gave a good yield of *p*-aminobenzylpenicillin.

RESULTS

p-Nitrobenzylpenicillin

p-Nitrobenzylpenicillin has been described by Behrens *et al.* (1948), but the amount obtained was not sufficient for recrystallization, and their methods of purification involved tedious chromatographic techniques. Their material assayed about 1700 units/mg.

In our work penicillin titres as high as 3400 units/ml. were obtained and the crystalline *p*-nitrobenzylpenicillin was isolated as the potassium salt by the usual techniques of purification. The material was identified by comparing its ultraviolet-absorption spectrum with that of *p*-nitrophenylacetic acid and by identification of the *p*-aminobenzylpenicillin produced by hydrogenation.

p-Aminobenzylpenicillin

Hydrogenation of *p*-nitrobenzylpenicillin at atmospheric or slightly higher pressures gave *p*-aminobenzylpenicillin. Among the various combinations of catalyst and solvent that were tried, palladium on charcoal as the catalyst and propan-2-ol as the solvent gave the best results. Evaporation *in vacuo* of the solution obtained on hydrogenation yielded crystalline *p*-aminobenzylpenicillin.

Some properties of p-aminobenzylpenicillin

Chemical and physical properties. The potassium salt of *p*-aminobenzylpenicillin, with an activity of 1100 units/mg., was a white crystalline powder, readily soluble in water but sparingly soluble in organic solvents. The product withstood a temperature of 110° for several hours without loss of potency. On chromatographic analysis 100% of the activity was found in the zone where penicillin X normally appears. The crystalline potassium salt was readily converted into the crystalline sodium salt by 'salting out' from a 20% (w/v) aqueous solution with sodium sulphate. It was not precipitated from concentrated aqueous solution by procaine, which forms a sparingly soluble salt with benzylpenicillin. However, like benzylpenicillin (Szabo, Edwards & Bruce, 1951), it was precipitated from aqueous solution by *NN*-dibenzylethylenediamine to give a crystalline product which was very sparingly soluble in water (about 1 mg./ml.).

It was more stable in aqueous acid solution (pH 2.3, 24°) than benzylpenicillin. Thus 29% of its activity remained after 167 min., whereas only 4% of the activity of benzylpenicillin was left after this time. *p*-Aminobenzylpenicillin is destroyed by preparations of penicillinase at a slightly lower rate than is benzylpenicillin (M. Goldner, personal communication, 1957).

Elementary analysis of the potassium salt gave the following result: C, 49.6; H, 4.5; N, 10.1; S, 8.2. $C_{16}H_{18}O_4N_2SK$ requires C, 49.6; H, 4.7; N, 10.8; S, 8.3%; $[\alpha]_D^{25} = +267^\circ$ in water (7 mg./ml.). This material showed ultraviolet-absorption maxima at 238 m μ ($\log \epsilon = 4.02$) and 285 m μ ($\log \epsilon = 3.16$). The maxima for *p*-aminophenylacetic acid are 236 and 285 m μ . We are indebted to Eli Lilly and Co., Indianapolis, U.S.A., for data on one of our early preparations of *p*-aminobenzylpenicillin. They reported that the infrared spectrum of the material demonstrated that the lactam structure, amide grouping and other characteristic penicillin functions were present. Their ultraviolet-absorption spectrum analysis was in substantial agreement with that obtained in our Laboratories. They also showed that a basic group (pK_a 4.6) and an acid group (pK_a 2.6) were present.

Antibacterial properties. The antibacterial properties of *p*-aminobenzylpenicillin were compared with those of benzylpenicillin against several micro-organisms. The results are given in Table 1.

The two penicillins seem to be about equally effective against staphylococci and streptococci, but it would seem that *p*-aminobenzylpenicillin is somewhat more effective (one to four times) against *Salmonella* organisms. This is of some interest in view of the activity of cephalosporin N against these organisms (Abraham, Newton & Hale, 1954; Heatley & Florey, 1953). According to the results of Heatley & Florey, and after correcting for impurities, cephalosporin N varies in its relative effectiveness as compared with benzylpenicillin from about 0.2 to 0.5 times for *Salmonella paratyphi* C to six times for *Salmonella enteritidis*. It appears, then, that *p*-aminobenzylpenicillin is about as effective as cephalosporin N against *Salmonella* when tested *in vitro*.

The amino group of *p*-aminobenzylpenicillin (pK_a 4.6) is not as basic as that of cephalosporin N (pK 9.8), but this lower basicity does not seem to alter appreciably its effectiveness against *Salmonella*. But, whereas cephalosporin N shows low activity against staphylococci and streptococci, *p*-aminobenzylpenicillin is about as active as benzylpenicillin against these organisms.

A clinical trial with synnematin B (cephalosporin N) showed that this drug was efficient for the treatment and control of typhoid fever and that it was more effective than chloramphenicol in

clearing *Salmonella typhi* from faeces (Benavides, Olson, Varela & Holt, 1955). However, the economic difficulties associated with its commercial production at present prevent its free use in medicine. It is possible that the more easily obtained *p*-aminobenzylpenicillin might be useful in the treatment of typhoid fever.

Derivatives of *p*-aminobenzylpenicillin. *p*-Aminobenzylpenicillin reacted with certain acid halides to form active derivatives which were less hydrophilic than was the parent compound and showed different solubility properties. It also reacted with *p*-nitrobenzenediazonium fluoborate to give a red, active reaction product. In each case reaction gave rise to an increase in the number of units of penicillin and also to a displacement of the position in which the activity appeared on chromatographic analysis.

EXPERIMENTAL

Microbiological assay. The penicillins were assayed by the Oxford cup-plate method with *Micrococcus pyogenes* var. *aureus*, strain 209 P, as the assay agent. The response curve obtained with benzylpenicillin was employed to assay *p*-nitrobenzylpenicillin, *p*-aminobenzylpenicillin and derivatives of *p*-aminobenzylpenicillin. Response curves for these penicillins differed slightly from each other as well as from that for benzylpenicillin.

Antibacterial assay. Solutions were assayed in Medium no. 3 (Grove & Randall, 1955), twofold dilutions being used.

Each tube, containing 0.5 ml. of the penicillin dilution, was seeded with 0.5 ml. of a 10^{-4} dilution of a 24 hr. culture of the organism. Readings were made after incubation for 24 hr.

Chromatographic analysis. The method was that reported by Macmorine (1957) and found to give a good resolution of the polar penicillins.

Ultraviolet-absorption spectra. These were determined on a Beckman spectrophotometer model DK-2.

Hydrogenation. Stainless-steel cylindrical containers were used. These were equipped with a lid which could be bolted on and which was supplied with inlet and outlet openings and a pressure gauge. A Neoprene gasket was inserted between the container and the lid. Magnetic stirring bars covered with polytetrafluoroethylene (Teflon) were employed. Mild-steel containers are not satisfactory because, under the conditions which obtain, the β -lactam ring is opened by alcoholysis. The catalyst was 5% palladium on carbon (activity 980, Baker & Co. Inc., 113 Aston St., Newark 5, N.J., U.S.A.).

Fermentation. Vegetative inocula of *Penicillium chrysogenum* UV15-4 for seeding medium in 20 l. fermenters were obtained according to Farrell (1953). The *p*-nitrophenylacetic acid was prepared according to Robertson (1932). The laboratory fermenter consisted of a standard 20 l. Pyrex bottle equipped for aeration, agitation, sampling, addition of anti-foam and temperature control. The equipment was described by Lane & Collingsworth (1948) and modified by Goldsmith (1951).

The components (% w/v) of the medium used were: corn-steep liquor (50% of solids), 6.8; lactose 3.2; Cerelease (refined dextrose; Canada Starch Co., Cardinal, Ont.,

Table 1. *Comparative antibacterial titres of p-aminobenzylpenicillin and benzylpenicillin*

Organism	Lowest concn. (μ g./ml.) preventing visible growth for 24 hr.		Activity of <i>p</i> -aminobenzylpenicillin
	<i>p</i> -Aminobenzylpenicillin	Benzylpenicillin	Activity of benzylpenicillin
<i>Micrococcus pyogenes</i> var. <i>aureus</i> (A.T.C.C. 653 P)	0.06	0.06	1
<i>M. pyogenes</i> var. <i>aureus</i> (resistant)	7.8	7.8	1
<i>Streptococcus pyogenes</i> (Dochez, N.Y. 5)	0.2	0.1	0.5
<i>Salmonella typhi</i>	7.8	15.6	2
	7.8	7.8	1
	3.9	15.6	4
	1.95	7.8	4
<i>S. enteritidis</i>	3.9	7.8	2
	3.9	7.8	2
	3.9	7.8	2
<i>S. paratyphi</i> A	3.9	15.6	4
	3.9	15.6	4
	3.9	7.8	2
<i>S. paratyphi</i> B	3.9	3.9	1
	1.95	3.9	2
	3.9	3.9	1
<i>S. paratyphi</i> C	0.97	1.95	2
	0.97	3.9	4
	0.97	1.95	2
<i>S. typhimurium</i>	7.8	15.6	2
	7.8	15.6	2
	7.8	15.6	2
<i>S. suispestifer</i>	15.6	31.2	2
	7.8	31.2	4
	7.8	31.2	4

Canada), 0.45; CaCO₃, 0.9; KNO₃, 0.136; KH₂PO₄, 0.23; *p*-nitrophenylacetic acid, 0.182; lard oil, 0.25.

Production of *p*-nitrobenzylpenicillin. Fermentation medium (12 l.) was prepared in the fermenter and the pH was adjusted to 5.65 with 50% (w/v) NaOH. The medium was autoclaved (0.5 hr. at 121°), cooled and the bottle transferred to the fermentation bath. Seeding was carried out with 500–700 ml. of vegetative inoculum, and the temperature of the bath maintained at 26.5°, with an agitator speed of 400–500 rev./min. Samples were withdrawn at intervals for assay and pH determination. Antifoam (6% Alkaterge C; Commercial Solvents Corp., 260 Madison Avenue, New York 16, N.Y., U.S.A.) in lard oil was added when needed. An average of 26 ml. was necessary.

In 120 hr. titres of 2000–3400 units/ml. were reached. There was considerable evaporation of water during the course of the fermentation. The volume of filtered broth was 5–7 l./bottle.

To 13.82 l. of filtered broth (pH 7.5) was added 8 l. of commercial amyl acetate (mixed acetates; McArthur Chemical Co., 140 Liberty St., Toronto, Canada). The mixture was stirred vigorously and a solution of 20% (w/v) H₃PO₄ was added until the pH of the aqueous phase fell to 2.3. The 'amyl acetate' phase was separated in a Sharples centrifuge (laboratory model) and the aqueous phase washed with a small amount of 'amyl acetate'. The combined 'amyl acetate' phase (7.59 l.) was extracted with 0.4N-KHCO₃ soln. until the pH of the aqueous phase reached 6.8. The aqueous phase (281 ml.) assayed at 89 600 units/ml. It was treated with 1.5 g. of Darco KB charcoal (Atlas Powder Co. Can. Ltd., Brantford, Ont., Canada) and the carbon removed by filtration to give a concentrate of *p*-nitrobenzylpenicillin.

Ammonium sulphate was added (30 g./100 ml. of concentrate); the mixture was stirred for 15 min. and the precipitated ammonium salt of *p*-nitrophenylacetic acid was removed by filtration.

Crystalline potassium *p*-nitrobenzylpenicillin. The aqueous concentrate, after removal of precursor, was extracted at pH 2.2 with 0.5 vol. of 'amyl acetate' and the 'amyl acetate' phase was then extracted with 1.2N-KHCO₃ soln. until the pH rose to 7.1. The concentrated solution was again adjusted to pH 2 and extracted into an equal volume of 'amyl acetate'. The 'amyl acetate' phase was separated and dried with anhydrous Na₂SO₄. To the 'amyl acetate' concentrate was added, with vigorous stirring, a solution of KOH in butan-1-ol (40 g./l.) until the pH of the reaction mixture as measured with a Beckman meter (model G) reached 8.35. The mixture was put in the refrigerator (5°) overnight and the crystals were then removed by filtration and washed twice with butan-1-ol and three times with ether.

The yield from 13.82 l. of filtered broth was 5.45 g. of slightly yellow material assaying 1965 units/mg. Chromatographic analysis showed 96.7% of the activity in the G zone with 3.3% in the dihydro F zone and traces in the F and K zones. The product had an absorption maximum at 278 mμ in water (log ε = 4.01). $[\alpha]_D^{25} = +290$ in water (3.5 mg./ml.).

Crystalline potassium *p*-aminobenzylpenicillin. Potassium *p*-nitrobenzylpenicillin (10 g.) was added to a 1.6 l. stainless-steel reactor and to this was added a slurry of 5 g. of 5% palladium-charcoal catalyst in 36 ml. of water.

Propan-2-ol (256 ml.) was added with stirring under N₂. The reactor was sealed, flushed with N₂ and filled with H₂ to a pressure of 28 lb./in.².

Stirring was started. The pressure dropped rapidly at first, reaching 15 lb./in.² in about 20 min. and slowly thereafter until by 7 hr. very little H₂ was being taken up. The temperature of the reaction was kept at about 24° by means of a water-cooled jacket. After 7 hr. the reactor was flushed with N₂ and propan-2-ol (325 ml.) was added. The spent catalyst was removed by filtration and the filtrate was put in the refrigerator (5°) overnight. The next day there was a slight turbidity in the liquid. This was removed by filtration and the filtrate was distilled at about 20 mm. Hg and with a bath temperature of 40°.

The volume of liquid was reduced to about 60 ml., by which time well-formed needle-shaped crystals appeared. The crystals were removed by filtration, washed with propan-2-ol and dried at 100° *in vacuo*. The yield was 5.5 g. of slightly yellow-coloured material which assayed at 1000 units/mg. and gave 90% of the activity in the X zone on chromatographic analysis. When heated for 17 hr. at 100–110°, the product showed no loss of potency and no significant change in the zone at which the penicillin activity appeared on chromatographic analysis.

This product was recrystallized as follows: 1 g. was dissolved in water (1 ml.) and butan-1-ol (24 ml.) was added. The mixture was left in the refrigerator (5°) overnight. Next day the crystalline material was removed by filtration, washed with butan-1-ol, then with ether and dried. The yield was 0.62 g., which assayed at 1055 units/mg.

This product (0.6 g.) was dissolved in water (0.6 ml.) and propan-2-ol (7.5 ml.) was added. The crystalline precipitate (white needles) was removed by filtration, washed with propan-2-ol, then with ether and dried at 65° *in vacuo*. The yield was 0.38 g., which assayed at 1100 units/mg. and gave 100% of the activity in the X zone.

Reaction with benzoyl chloride. Potassium *p*-aminobenzylpenicillin (520 mg., 955 units/mg.) and KHCO₃ (200 mg.) were dissolved in water (2 ml.). Benzoyl chloride (0.15 ml.) was added and the mixture was shaken at room temperature until the odour of benzoyl chloride was no longer present. Water was added to a volume of 25 ml. This solution assayed at 28 600 units/ml. (91%, G zone). Thus from 496 600 units of *p*-aminobenzylpenicillin (almost completely in X zone) there was obtained 715 000 units (91%, G zone).

Reaction with 3:5-dinitrobenzoyl chloride. Potassium *p*-aminobenzylpenicillin (520 mg.) and KHCO₃ (250 mg.) were dissolved in water (2 ml.). To the solution was added, in small portions, with shaking and cooling, 3:5-dinitrobenzoyl chloride (300 mg.). Shaking was continued 0.5 hr. after evolution of gas had ceased, by which time a crystalline precipitate had formed. The mixture was placed in the refrigerator (5°) for 3.5 hr. and was then so thick it would not flow. Addition of water (18 ml.) gave a clear solution. Ethyl acetate (50 ml.) was added. The mixture was stirred vigorously and H₃PO₄ (10%, w/v) was added with stirring until the aqueous phase was pH 2.57. The ethyl acetate extract was shaken with 0.2N-KHCO₃ soln. (10 ml.) to yield an aqueous solution (10.5 ml., pH 7.8) which assayed at 67 550 units/ml. (100%, G zone). To this concentrate (4 ml.) water (4 ml.) was added and saturated aqueous potassium acetate soln. (1.9 ml.) to give an oily precipitate which crystallized on standing. This was separated by

centrifuging, washed with propan-2-ol and with ether and dried. Yield was 160 mg. of white powder, which assayed at 890 units/mg. (100%, G zone) and which was contaminated with potassium acetate.

Reaction with *p*-nitrobenzenediazonium fluoborate. Potassium *p*-aminobenzylpenicillin (2 g.) and potassium acetate (3 g.) were dissolved in water (20 ml.). The solution was cooled to 0° and to it was added a cold solution of *p*-nitrobenzenediazonium fluoborate (1.22 g.) in water (100 ml.). The resulting viscous, red liquid was stirred for 2 hr. and then acetic acid (10 ml.) was added. In about 5 min. the viscous liquid changed to a jelly-like mass. This was left in the refrigerator (5°) overnight, was filtered, washed several times with water and pressed to a dark-red cake with a rubber dam. After drying there was obtained a brick-red powder (2.5 g.), which assayed at about 1400 units/mg. The activity of this material was found in an elongated zone between the F and K zones.

SUMMARY

1. With *p*-nitrophenylacetic acid as a precursor, *p*-nitrobenzylpenicillin was produced in fermentation media in titres as high as 3400 units/ml.

2. Crystalline potassium *p*-nitrobenzylpenicillin was isolated by the usual techniques employed in the purification of benzylpenicillin.

3. Hydrogenation of *p*-nitrobenzylpenicillin yielded *p*-aminobenzylpenicillin, which was isolated as the crystalline potassium salt. This is about as effective as cephalosporin N against *Salmonella* organisms *in vitro*.

4. *p*-Aminobenzylpenicillin reacts with certain acid chlorides and with *p*-nitrobenzenediazonium fluoborate to form active derivatives. These derivatives have greater activity than *p*-aminobenzylpenicillin when tested against *Micrococcus*

pyogenes by the cup-plate method, and have altered solubility properties.

Our thanks are due to Mr G. D. Graham and Mr A. Luik, who were responsible for the microbiological and antibacterial assays.

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