Degradation, Structure and some Derivatives of Cephalosporin N

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Abraham, Newton & Hale (1954) suggested that cephalosporin N was a penicillin containing an aminodicarboxylic acid in the side chain. This paper gives further information about the structure of the molecule.

Elementary analysis of a barium salt of cephalosporin N, which, though amorphous, was probably not very far from pure (Abraham *et al.* 1954), gave the following result: C, 38.2; H, 5.9; N, 8.7; S, 6.9%. The C:S ratio was therefore 14 or 15:1 and the N:S ratio 3:1. Assuming that cephalosporin N contained a normal penicillin nucleus ($C_9H_{11}O_4N_2S$), five or six carbon atoms and one nitrogen atom remained for the side chain. Since the substance showed only end absorption in the ultraviolet region, the side chain appeared to contain no conjugated double bonds.

These conclusions were supported by the products obtained on degradation of the molecule. The course of the degradations was followed by chromatography and electrophoresis on paper, while ion-exchange columns and the four-compartment cell for ionophoresis described by Syngé (1951) were used in isolating some of the products. The products were usually isolated from partially purified material, but their formation from cephalosporin N itself was confirmed by work with the purest preparation.

When subjected to ionophoresis on paper in a collidine acetate buffer at pH 7.0, cephalosporin N (detected by antibacterial activity or the ninhydrin reaction) migrated towards the anode at almost the same rate as benzylpenicillin (Fig. 1, spots 1 and 2). On heating at 100° in aqueous solution at pH 2 for 1 hr., carbon dioxide was evolved. This solution contained a penilloic acid, which migrated like the antibiotic on ionophoresis (Fig. 1, spot 3), together with some neutral material. Addition of mercuric chloride to this solution at pH 6 yielded a precipitate of a mercaptide, from which crude penicillamine (I) was obtained by decomposition with hydrogen sulphide. On warming with acetone this gave the crystalline hydrochloride of 2:2:5:5-tetramethylthiazolidine-4-carboxylic acid (II) (m.p. 198°; $[\alpha]_D^{20} + 94^\circ$). Decomposition of (I) by boiling with dilute hydrochloric acid resulted in the formation of D-penicillamine hydrochloride (Abraham, Baker, Chain & Robinson, 1949). Addition of 2:4-dinitro-

phenylhydrazine in hydrochloric acid to the supernatant from the penicillamine mercaptide produced only a very small precipitate, part of which was glyoxal bis-2:4-dinitrophenylhydrazone. In this, the degradation of cephalosporin N differed from that of other penicillins. It appeared probable that an aldehyde derived from the penilloic acid of cephalosporin N formed a water-soluble 2:4-dinitrophenylhydrazone.

On more vigorous hydrolysis (N hydrochloric acid for 6 hr.) the penilloic acid from cephalosporin N was broken down. Ionophoresis on paper and development with ninhydrin then showed the presence of a new acidic substance, which had migrated about 1.6 times as fast as the penilloic acid towards the anode (Fig. 1, spot 4), together with an increased amount of neutral material. The new acidic substance (Fig. 2, spot 1) travelled further than glutamic acid on paper chromatograms run with butanol-acetic acid (Woiwod, 1949). It was accompanied by glutamic and aspartic acids in hydrolysates of crude cephalosporin N, but not in hydrolysates of the purest preparation. This acid was separated from the neutral material formed on hydrolysis by ionophoresis in the four-compartment cell. It was then purified by chromatography on the weakly basic ion-exchange resin Amberlite IR 4B, eluting with ammonium acetate buffer. After

Fig. 1. Positions of derivatives of cephalosporin N after ionophoresis on paper, at pH 7.0 in collidine-acetic acid buffer. Spots developed with ninhydrin. 1, Benzylpenicillin; 2, cephalosporin N; 3, cephalosporin N penilloic acid; 4, α -aminoadipic acid hydrochloride; 5, penicillaminic acid; 6, α -aminoadipylmonoaminoacetaldehyde; 7, α -aminoadipylmonoglycine; 8, cephalosporin N penicilloic acid; 9, cephalosporin N penillic acid; 10, cephalosporin N penillamine.

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crystallization from water it was found to have the composition $C_6H_{11}O_4N$ (m.p. 207–208°; $[\alpha]_D^{20} - 26^\circ$ in 6N-HCl). Electrometric titration showed the presence of two acidic groups of pK 2.2 and 4.4 respectively and a basic group of pK 9.8. The properties of the substance indicated that it was D- α -aminoadipic acid (III). An X-ray powder photograph of the substance was reported by Dr D. Hodgkin to be identical with a photograph of synthetic D- α -aminoadipic acid supplied by Dr J. P. Greenstein. The synthetic compound ($[\alpha]_D^{25} - 25^\circ$ in 5N-HCl) is assumed to have the D configuration because its chloroacetyl derivative was prepared by subjecting chloroacetyl-DL- α -aminoadipic acid to the asymmetric action of acylase I, and because it was not oxidized by the L-amino acid oxidase from *Crotalus adamanteus* (Greenstein, Birnbaum & Otey, 1953). Its rotation in 5N-HCl has the same sign as that of D-glutamic acid and is of similar magnitude.

The neutral fraction from the four-compartment cell yielded an acidic amino acid on oxidation with bromine water (Fig. 1, spot 5). This was separated from residual neutral material by further iono-

phoresis in the four-compartment cell and on crystallization from aqueous acetone yielded prisms of penicillaminic acid (β -sulphovaline) (IV).

The penilloic acid formed by hydrolysis of cephalosporin N in hot aqueous solution at pH 2 had an isoelectric point in the neighbourhood of pH 3.5. When kept at this pH it remained in the specimen compartment of the four-compartment cell, while impurities of stronger acidity migrated into the acetic acid compartment. Oxidation of the penilloic acid with bromine water resulted in the formation of penicillaminic acid and a neutral aldehyde (Fig. 1, spot 6) which were separated by ionophoresis. Oxidation of the aldehyde with freshly precipitated silver oxide yielded an acid (Fig. 1, spot 7) which migrated into the acetic acid compartment of the four-compartment cell. This compound crystallized from aqueous ethanol in small white prisms, m.p. 213–214°. Its composition and equivalent weight were consistent with the formula $C_9H_{14}O_5N_2$. It contained a basic group with pK 9.9 and two acidic groups with pK values of 3.8 and < 3.0 respectively. On paper chromatograms run with butanol-acetic acid and developed with

ninhydrin (Fig. 2, spot 3) it appeared in a position close to that of glycine. On hydrolysis with 6*N* hydrochloric acid at 105° it yielded about equimolecular amounts of a neutral substance and an acidic substance which behaved like glycine and α -aminoadipic acid respectively on paper chromatograms. After treatment with 1:2:4-fluorodinitrobenzene (FDNB) (Sanger & Thompson, 1953), hydrolysis resulted in the formation of glycine and of a substance which behaved like dinitrophenyl- α -aminoadipic acid (DNP- α -aminoadipic acid) when chromatographed on paper in the system of Blackburn & Lowther (1951). The original acid was therefore an α -aminoadipylmonoglycine, and the aldehyde from which it was derived was an α -aminoadipylmonoaminoacetaldehyde.

The dissociation constants of the α -aminoadipylmonoglycine, compared with those of α -aminoadipic acid and other model compounds, indicated that the δ -carboxyl group of α -aminoadipic acid was involved in a peptide bond. The pK of the basic group of an α -amino acid is reduced by about 1.5 units when the α -carboxyl group is involved in a peptide linkage, because the effect of the electronegative CO group is no longer opposed by the charge on the carboxylate ion (Neuberger, 1937). The influence of the CO group, however, diminishes rapidly when it is removed from the α -position. Thus the pK values of the amino groups of glutamic acid, γ -L-glutamyl-L-alanine, and α -L-glutamyl-L-valine were found to be

Fig. 2. Single dimension paper chromatogram of derivatives of cephalosporin N and reference amino acids in butanol-acetic acid. Spots developed with ninhydrin. 1, α -Aminoadipic acid hydrochloride; 2, penicillaminic acid; 3, α -aminoadipylmonoglycine; 4, cephalosporin N penillamine; 5, cephalosporin N *S*-benzylpenillamine; 6, cephalosporin N *S*-benzylpenillamine sulphone; 7, *S*-benzylpenicillamine sulphone; 8, glycine; 9, glutamic acid; 10, valine; 11, leucine.

9.7, 9.6, and 8.1 respectively. Since the pK of the amino group of the α -aminoadipylglycine was very close to that of α -aminoadipic acid, the former appeared to be D- δ -amino- δ -carboxyvaleryl-glycine (V) and the aldehyde from which it was derived to be D- δ -amino- δ -carboxyvaleryl-aminoacetaldehyde (VI).

These results indicate that the penilloic acid has the structure (VII) and that cephalosporin N is therefore (D-4-amino-4-carboxy-*n*-butyl)penicillin (VIII). This structure is in accordance with the fact that the purest preparations of cephalosporin N react with FDNB to form a DNP-derivative which yields DNP- α -aminoadipic acid on hydrolysis, and that the pK of the basic group of cephalosporin N (Fig. 3) is very close to that of α -aminoadipic acid itself.

In alkaline solution above pH 11, cephalosporin N was rapidly inactivated, and electrometric titration revealed that a new ionizable group had appeared with pK 4.7. Ionophoresis on paper at pH 7.0 showed that the product migrated faster towards the anode than α -aminoadipic acid (Fig. 1, spot 8). The new compound was presumably the penicilloic acid (IX), containing three acidic groups, one strong basic group and one weak basic group that was un-ionized at pH 7.0.

When an aqueous solution of cephalosporin N is kept at pH 2.7 and at 37° for 2 hr. there is a loss of antibacterial activity, an increase in dextrorotation, and a change in the ultraviolet absorption spectrum which results in the appearance of strong absorption at 240 m μ . (Fig. 4). Electrometric titration shows that a new basic group has been formed with a pK of about 7.6. These changes are typical of those occurring in a penicillin $\rightarrow$ penicilloic acid transformation.

The penillic acid from cephalosporin N (Fig. 1, spot 9) was purified by chromatography on a buffered column of Amberlite IR 4B. It was obtained as an amorphous white powder ($[\alpha]_D^{20} + 354^\circ$

Fig. 3. Electrometric titration curves at 20°. ●—●, Cephalosporin N barium salt (58 units/mg.); x—x, cephalosporin N penillic acid.

in water; $\lambda_{\max}$ 240 $m\mu$, $E_{1\text{cm}}^{1\%}$ 140). It contained three acidic groups, the weakest having a pK of 3.1, and two basic groups with pK values of 7.6 and 10.3 respectively (Fig. 3). The results of elementary analysis were in approximate agreement with those expected of a hydrate of $C_{14}H_{21}O_6N_3S$ (X).

The penillic acid yielded the same products as cephalosporin N on hydrolysis with N hydrochloric acid at 100° . A paper chromatogram of a hydrolysate which had been treated with bromine water showed the presence of penicillaminic acid and α -aminoadipic acid in approximately equimolecular proportion, together with a trace of glycine (Fig. 5). A hydrolysate of sodium benzylpenicillin to which one molecular proportion of α -aminoadipic acid had been added behaved in a very similar manner (Fig. 5).

Fig. 4. Ultraviolet absorption spectra in water. $\odot-\odot$, Cephalosporin N barium salt (58 units/mg.); $\times-\times$, cephalosporin N penillic acid.

Addition of mercuric chloride to a solution of the penillic acid resulted in the immediate precipitation of a white mercaptide. Decomposition of the latter with hydrogen sulphide yielded an amorphous neutral substance (Fig. 1, spot 10), which was laevorotatory, gave a strong positive test for a free thiol group, and showed a spot on paper chromatograms run with butanol-acetic acid which had an R_f value slightly less than that of α -aminoadipic acid (Fig. 2, spot 4). This compound had the properties of a penillamine (XI). It could be precipitated from aqueous solution as a picrate, which facilitated its preparation from crude penillic acid.

Benylation of the penillamine yielded an S -benzylpenillamine which crystallized from warm

Fig. 5. Chromatogram of products formed from cephalosporin N penillic acid and benzylpenicillin on hydrolysis with N -HCl and oxidation with bromine water. 1, Cephalosporin N penillic acid; 2, benzylpenicillin plus α -aminoadipic acid; 3, benzylpenicillin. The top spot is penicillaminic acid and the bottom spot α -aminoadipic acid.

water in fine needles, m.p. 196–198°. The equivalent weight and elementary composition of this substance agreed with the formula $C_{20}H_{27}O_4N_3S, \frac{1}{2}H_2O$. Its ultraviolet absorption spectrum showed only end absorption and a plateau in the region 250–265 m μ . ($E_{1\%}^{1\text{cm}}$ 9.0) due to the presence of a benzyl group. Titration showed the presence of two acidic groups with pK values of 2.5 and 2.2, and two basic groups with pK values of 7.0 and 10.0 respectively (Fig. 6). On paper chromatograms run with butanol–acetic acid its R_f value was slightly less than that of valine (Fig. 2, spot 5). In the ninhydrin–carbon dioxide method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) for the determination of amino acids, it yielded one molecular proportion of carbon dioxide.

The *S*-benzylpenillamine remained largely unchanged on treatment with hot dilute hydrochloric acid for several hours. On oxidation with hydrogen peroxide in formic acid (Sanger & Thompson, 1953) it formed a compound (presumably a sulphone) with an R_f value slightly greater than that of glutamic acid (Fig. 2, spot 6). On treatment with bromine water, both the *S*-benzylpenillamine and its sulphone were broken down. Paper chromatograms of the products showed the presence of one spot in

Fig. 6. Electrometric titration curve of cephalosporin N *S*-benzylpenillamine at 20°.

the position of benzylpenicillamine sulphone (Fig. 2, spot 7) and one spot in the position of α -aminoadipic acid.

Crude preparations of cephalosporin N yielded aspartic and glutamic acids as well as α -aminoadipic acid on hydrolysis. It therefore seemed possible that aspartyl and glutamyl derivatives of the penicillin nucleus were formed by the *Cephalosporium* (Newton & Abraham, 1953). Further work, however, has produced no evidence of the existence of more than one active substance in crude material, and it is perhaps significant that the glutamic acid from this material, unlike the aminoadipic acid, has the L configuration. D- α -Aminoadipic acid does not appear to have been isolated previously from a natural source although it has been prepared synthetically (Greenstein *et al.* 1953), but L- α -aminoadipic acid is involved in the metabolism of lysine in the guinea pig (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1948) and in *Neurospora* (Mitchell & Houlahan, 1948). In crude cephalosporin N, α -aminoadipic acid is present in compounds other than the antibiotic itself. These compounds are being investigated further.

Cephalosporin N reacts readily with certain acid halides, acetic anhydride, and phenyl isocyanate, in sodium bicarbonate solution, to form derivatives which do not contain a free amino group. These derivatives still contain the penicillin nucleus because they are rapidly inactivated by penicillinase, but they differ greatly from the parent compound in solubility and antibacterial activity. They can be extracted from an aqueous solution at pH 2.5 into ethyl acetate, or butanol, and re-extracted into water at pH 6.0. They have a higher activity than cephalosporin N against *Staphylococcus aureus* and a lower activity against *Salmonella typhi*. None of the derivatives has yet been purified, but Table 1 shows the change in antibacterial activity of an aqueous solution of crude cephalosporin N (28 units/mg.) when they are formed, and the relative activities of the products after passage through an organic solvent.

These derivatives of cephalosporin N resemble benzylpenicillin more closely than cephalosporin N itself in some of their physical and antibacterial

Table 1. Changes of antibacterial activity in the formation of derivatives of cephalosporin N

Derivative formed from	Product	Final/initial activity of solution against		(Activity against <i>Staph. aureus</i> /Activity against <i>Salm. typhi</i>) of extracted product
		<i>Staph. aureus</i>	<i>Salmonella typhi</i>	
—	Benzylpenicillin	—	—	140
—	Cephalosporin N	—	—	1
FDNB	DNP-cephalosporin N	5	—	1000
Benzoyl chloride	Benzoylcephalosporin N	2.9	0.10	31
Benzoyloxycarbonyl chloride	Benzoyloxycarbonylcephalosporin N	2.1	0.35	7
Phenyl isocyanate	Phenylcarbamylcephalosporin N	2.2	0.23	11
Acetic anhydride	Acetylcephalosporin N	1	0.14	7

properties. Their absolute activity against either *Staph. aureus* or *Salm. typhi*, however, appears to be less than that of benzylpenicillin. This fact may be associated with the presence of a carboxyl group in the side chain. Coghill, Stodola & Wachtel (1949) found that azo derivatives of *p*-hydroxy-benzylpenicillin in which the substituent carried an acidic group were less active against *Staph. aureus* than derivatives in which the substituent was non-polar.

EXPERIMENTAL

Ionophoresis on paper. This was carried out in an apparatus similar to that described by Flynn & Mayo (1951) using sheets of Whatman no. 1 paper 36 cm. long. The buffer (pH 7.0) was prepared by adding 36.3 ml. of commercial collidine (distilled under N_2 ; b.p. $54^\circ/12$ mm.) and 185 ml. of *N* acetic acid to 3.86 l. of water. The apparatus was run at 500 v for 3 hr. Substances with no net charge at pH 7.0 were carried about 1 cm. towards the cathode by end-osmosis.

Four-compartment cell. This was similar to that described by Synge (1951) and was fitted with a cooling coil carrying cold tap water. In most cases the potential was raised to 500 v and the current fell to about 35 ma towards the end of the experiment.

Electrometric titrations. These were carried out in the manner described by Newton & Abraham (1952).

Antibacterial activity. This was measured by a diffusion method on plates seeded with the test organism (Abraham *et al.* 1954).

Isolation of penicillamine. Cephalosporin N (500 mg.; 42 units/mg.) was dissolved in water (8 ml.) and 2 *N*-HCl (0.63 ml.) was added to bring the pH of the solution to 2.0. The solution was refluxed in a stream of N_2 for one hr. Passage of the N_2 through 0.3 *N*-Ba(OH) $_2$ resulted in the precipitation of BaCO $_3$. Titration of the remaining Ba(OH) $_2$, using phenolphthalein as an indicator, showed that CO $_2$ corresponding to 8.8 ml. of 0.1 *N*-HCl had been evolved. The pH of the hydrolysate was adjusted to 6.0 and a saturated aqueous solution of HgCl $_2$ (25 ml.) was added. Within a few minutes a flocculent precipitate formed which was separated by centrifuging. After washing with 5 ml. of a solution of HgCl $_2$ (70% saturated) the precipitate was suspended in water (5 ml.) and decomposed with H $_2$ S. The HgS was removed at the centrifuge and the supernatant solution evaporated *in vacuo*. The residue (68 mg.) gave a strong positive reaction for a free thiol group with sodium nitroprusside. It was heated under reflux with acetone (10 ml.) for 30 min., and the hot solution filtered and evaporated to dryness. A crystalline residue remained which consisted of 2:2:5:5-tetramethylthiazolidine-4-carboxylic acid (33 mg.). This was redissolved in hot acetone, the solution concentrated, and 0.05 ml. of 11 *N*-HCl added. The hydrochloride of 2:2:5:5-tetramethylthiazolidine-4-carboxylic acid (20 mg.) separated from the solution in prisms, m.p. 198° ; $[\alpha]_D^{20} + 94^\circ$ in water (*c*, 1.0). (Found: C, 40.6; H, 7.7; N, 6.0; S, 12.4; Cl, 14.8. $C_8H_{15}O_2NS \cdot HCl$, $\frac{1}{2}H_2O$ requires C, 40.9; H, 7.3; N, 6.0; S, 13.6; Cl, 15.1%.) An X-ray powder photograph was identical with a photograph of an authentic sample of the thiazolidine.

The thiazolidine (5 mg.) was dissolved in *N*-HCl (1 ml.) and the solution boiled for 2 min. Evaporation *in vacuo* yielded a residue which crystallized in long needles and gave

a strong positive reaction for a free thiol group with sodium nitroprusside. An X-ray powder photograph of the compound was identical with a photograph of authentic penicillamine.

Isolation of D- α -aminoadipic acid. Cephalosporin N (500 mg.; 28 units/mg.) was hydrolysed in *N*-HCl for 7 hr. at 110° under N_2 . The hydrolysate was evaporated to dryness *in vacuo* and the residue separated into acidic and neutral fractions in the four-compartment cell. The acidic fraction (210 mg.) was found by paper chromatography to be a mixture of α -aminoadipic, glutamic and aspartic acids. This material, together with about 50 mg. of similar material from another experiment, was chromatographed on a column (4 $\times$ 27 cm.) of Amberlite IR4B (200–320 mesh). The amino acids were eluted with 0.2 *M* ammonium acetate buffer, pH 5.0 (Hirs, Moore & Stein, 1952), the eluate being collected in 20 ml. fractions. The rate of flow was adjusted to 20 ml./25 min. The fractions containing the amino acids were located roughly by spotting samples of each fraction (3 $\times$ 5.3 μ l.) on a sheet of filter paper, heating to 110° for 10 min. to remove ammonium acetate, spraying with ninhydrin solution, and then heating at 105° for 5 min. Blue spots developed from fractions 50–59 and 61–70. Much weaker spots were obtained from fractions 82–86.

Samples (0.05 ml.) from every other fraction over the region where the amino acids had been located, were placed into test tubes (3 $\times$ 0.5 in.) and evaporated to dryness *in vacuo*. The ammonium acetate was sublimed by placing the tubes in holes drilled into a blackened aluminium block, which was placed in a desiccator containing dishes of P $_2$ O $_5$ and KOH. The desiccator was continuously evacuated with a high-vacuum pump for 8 hr. while the block was heated to 40 – 50° by means of an external infrared lamp (we are indebted to Dr D. Kay for this suggestion). Quantitative ninhydrin determinations on the resulting samples (Moore & Stein, 1948), showed that the mixture had been resolved into three components which accounted for 50% (fractions 50–59), 37.5% (fractions 60–74) and 12.5% (fractions 76–92) respectively of the total. The fractions of the major band, which contained α -aminoadipic acid, were pooled and freed from ammonium acetate buffer (Hirs *et al.* 1952). The residue was dissolved in 4 ml. of hot water and glacial acetic acid was added until the pH of the solution was 4.0. It was then left to crystallize overnight. The crystals were filtered and washed with cold water. The first crop yielded 35 mg. A further 25 mg. were obtained by concentrating the mother liquor and acidifying to pH 3.2. The substance had m.p. 207 – 208° ; $[\alpha]_D^{20} - 26^\circ$ in 6 *N*-HCl (*c*, 1.0). (Found: C, 44.2; H, 7.0; N, 9.0. $C_8H_{11}O_4N$ requires C, 44.8; H, 6.8; N, 8.7%.) The mixed m.p. with an authentic sample of DL- α -aminoadipic acid (m.p. 198°), kindly provided by Dr E. Work, was 188 – 190° . The mixed m.p. with an authentic sample of D- α -aminoadipic acid, kindly provided by Dr J. P. Greenstein, was 207 – 208° .

On descending chromatograms run with butanol–acetic acid, free α -aminoadipic acid was liable to give two separate spots close together. Only the lower spot, however, was obtained when the amino acid was applied to the paper as the hydrochloride, and only the higher spot when it was applied as the ammonium salt.

Isolation of L-glutamic acid. The material recovered from fractions 60–74 of the preceding experiment was dissolved in 2 *N* acetic acid (1.5 ml.) and ethanol (3 ml.) was added to the solution. The L-glutamic acid which separated (33 mg.) had $[\alpha]_D^{20} + 27.9^\circ$ in 6 *N*-HCl (*c*, 0.8). Crystallization from 11 *N*-

HCl yielded L-glutamic acid hydrochloride. (Found: C, 33.2; H, 5.2; N, 8.0; Cl, 19.0. $C_6H_{10}O_4NCl$ requires C, 32.7; H, 5.4; N, 7.7; Cl, 19.3%.)

Oxidation of D- α -aminoadipic acid with chloramine-T. The procedure was similar to that used by Dakin (1917) for the oxidation of glutamic acid. D- α -Aminoadipic acid (16 mg.) from cephalosporin N was dissolved in N-NaOH (1 ml.) and chloramine-T (29 mg.) was added. The mixture was kept for 5 min. at 20° and then for 15 min. at 50°. Addition of 2:4-dinitrophenylhydrazine in 2N-HCl precipitated an acidic 2:4-dinitrophenylhydrazone (10 mg.) which was crystallized twice from aqueous ethanol. This substance had m.p. 130–131°. The mixed m.p. with a substance obtained in a similar manner from authentic DL- α -aminoadipic acid was 130–131°. The substance appeared to be the 2:4-dinitrophenylhydrazone of glutaric acid semialdehyde. (Found: C, 44.8; H, 4.2; N, 18.4. $C_{11}H_{12}O_6N_4$ requires C, 44.5; H, 4.1; N, 18.9%.)

Isolation of penicillaminic acid. The neutral fraction obtained from the four-compartment cell in the isolation of α -aminoadipic acid was oxidized by passing Br_2 vapour through the solution until Br_2 was present in slight excess. The solution was freeze-dried and the residue dissolved in water (20 ml.). The resulting solution, after adjusting its pH to 3.5 with 2N- NH_3 , was placed in the specimen compartment of the four-compartment cell. Current was then passed through the cell for 3 hr., the potential being gradually raised to 500 v, and the pH of the specimen compartment being maintained at 3.5. The contents of the acetic acid compartment were evaporated to dryness and yielded a crystalline residue of penicillaminic acid (65 mg.). This was dissolved in water, decolorized by treatment with charcoal, and recrystallized by the addition of acetone to the solution. Penicillaminic acid separated in the form of white prisms. (Found: C, 30.6; H, 6.0; N, 6.9. $C_6H_{11}O_6NS$ requires C, 30.7; H, 5.6; N, 7.1%.) An X-ray powder photograph was identical with a photograph of authentic penicillaminic acid.

Isolation of δ -amino- δ -carboxyvalerylglycine. Crude penillic acid was prepared from cephalosporin N (42 units/mg.) in the manner described in the following experiment. It was separated from cations by ionophoresis in the four-compartment cell. The penillic acid (470 mg.) was dissolved in water (25 ml.) and the solution heated at 100° in a stream of N_2 for 2 hr. CO_2 was evolved and penilloic acid formed. The pH of the solution was adjusted to 3.0 with 2N- NH_3 and the solution was placed in the specimen compartment of the four-compartment cell. The current was passed until it fell to 50 ma at 250 v. At pH 3.5 most of the penilloic acid remained in the specimen compartment, while impurities of stronger acidity migrated into the acetic acid compartment.

The contents of the specimen compartment (150 mg.) were oxidized by passing a slight excess of Br_2 vapour into the solution. The solution was then stirred with freshly precipitated Ag_2CO_3 until the pH rose to 3.9. After removing the $AgBr$ and excess of Ag_2CO_3 at the centrifuge, N-HCl was added until no further precipitate of $AgCl$ occurred. The solution was centrifuged, and the supernatant, after adjusting its pH to 4.5 with 2N- NH_3 , was replaced in the specimen compartment of the cell. Current was passed for 4 hr. until it finally fell to 25 ma at 500 v, the pH being maintained at 4.5. Penicillaminic acid had then migrated into the acetic acid compartment and a neutral aldehyde remained in the specimen compartment.

The solution from the specimen compartment was adjusted to pH 7.0 and stirred at 80° for 2 hr. with Ag_2O

freshly precipitated from 400 mg. of $AgNO_3$. Reduction of Ag_2O began almost immediately. The solution was centrifuged and the supernatant was adjusted to pH 3.0 with N-HCl. After removing $AgCl$ at the centrifuge the supernatant was adjusted to pH 7.0 and again placed in the four-compartment cell. Current was passed for 2.5 hr., the potential being raised gradually to 500 v and the pH of the specimen compartment maintained at 7. The contents of the acetic acid compartment were evaporated *in vacuo*, leaving a crystalline residue (20 mg.). This was dissolved in water containing a few drops of glacial acetic acid, and ethanol added until crystallization began. The product separated in clusters of small prisms, m.p. 213–214°. (Found: C, 44.0; H, 6.4; equiv. 230. $C_8H_{14}O_5N_2$ requires C, 44.0; H, 6.4%; equiv. 218.)

The α -aminoadipylmonoglycine could be chromatographed on Amberlite IR4B (200–320 mesh) at pH 3.5. It was eluted from a column (20 $\times$ 1.2 cm.) with 0.8N acetic acid containing enough NH_3 to bring the pH to 3.5. Fractions of 2 ml. were collected and the substance emerged as a single band in fractions 42–48.

Preparation of penillic acid. Cephalosporin N (1 g., 51 units/mg.) was dissolved in water (80 ml.) and the solution adjusted to pH 3.0 with N-HCl (1.4 ml.). The solution was kept at 37° and the rise in the optical density at 240 m μ . was determined at 30 min. intervals. After 3 hr. the value of $E_{1\text{cm}}^{1\%}$ had increased from 37 to a maximum of 87. The solution was immediately freeze-dried.

A portion of the resulting crude penillic acid (870 mg.) was chromatographed on a column (34 $\times$ 4 cm.) of Amberlite IR4B (200–320 mesh). Elution was carried out with 0.2M ammonium formate (pH 3.2) containing 0.5% (v/v) thiodiglycol (Hirs *et al.* 1952). Fractions of 30 ml. were collected at 30 min. intervals. The optical density at 240 m μ . and the ninhydrin colour density after removing ammonium formate by sublimation, were determined on samples taken from selected fractions. The penillic acid was located in fractions 30–45. Fractions 36–40 were freeze-dried and the residue dissolved in water and placed in the specimen compartment of the four-compartment cell. The penillic acid, which migrated into the acetic acid compartment of the cell, was recovered by freeze-drying. The product was dissolved in water (5 ml.) and precipitated by the addition of dry acetone (95 ml.). The sticky precipitate was separated by centrifuging and dried to a fine white powder (110 mg.) by grinding under dry acetone. It had $[\alpha]_D^{20} + 354^\circ$ in water (c, 0.25). (Found: C, 45.3; H, 6.4; N, 10.2; S, 8.2. $C_{14}H_{20}N_2SO_6$, H_2O requires C, 44.6; H, 5.9; N, 11.2; S, 8.5%.)

In the photometric ninhydrin determination (Moore & Stein, 1948) 100 μ g. of the penillic acid gave the same colour density as 29 μ g. of leucine.

Preparation of an S-benzylpenillamine. Crude penillic acid (400 mg.) from cephalosporin N (40 units/mg.) was dissolved in 20 ml. water and a saturated aqueous solution of $HgCl_2$ (30 ml.) was added. A white precipitate formed and CO_2 was evolved. The precipitate was centrifuged, suspended in water (15 ml.) and decomposed with H_2S . After removing the HgS the supernatant was evaporated *in vacuo*. The amorphous residue (200 mg.) gave a strong positive test for a free thiol group with sodium nitroprusside. The material was dissolved in water (2.5 ml.) and a saturated aqueous solution of picric acid was added until no more precipitate was formed. The oily penillamine picrate was separated from the solution and converted into

the hydrochloride by solution in 0.5 N-HCl (10 ml.) and extraction of the picric acid with ether. Evaporation of the colourless aqueous solution *in vacuo* yielded amorphous penillamine hydrochloride (75 mg.), $[\alpha]_D^{20} - 23^\circ$ in water (c, 1.5).

The penillamine hydrochloride was dissolved in liquid NH_3 (15 ml.) and sodium added in small pieces until the solution developed a permanent blue colour. Benzyl chloride (0.026 ml.) was then added and the NH_3 evaporated. The residue was dissolved in water and the pH of the solution adjusted to 5.5 with 2 N-HCl. The solution was extracted with ether to remove excess of benzyl chloride and placed in the specimen compartment of the four-compartment cell. Current was passed for 2.5 hr., until it fell to 30 ma. at 500 v, and the pH of the solution was maintained at 5 to 6. Evaporation of the contents of the specimen compartment *in vacuo* gave a white crystalline residue (55 mg.). Recrystallized from warm water (1 ml.) it formed fine needles, m.p. 196–198°. (Found: C, 58.1; H, 6.7; N, 9.6; S, 7.9; equiv. 410. $\text{C}_{20}\text{H}_{27}\text{O}_4\text{N}_2\text{S}$, $\frac{1}{2}\text{H}_2\text{O}$ requires C, 58.1; H, 6.8; N, 10.1; S, 7.7%; equiv. 414.)

In the ninhydrin-carbon dioxide reaction of Van Slyke *et al.* (1941), the CO_2 obtained from 10 mg. of the *S*-benzylpenillamine neutralized 0.46 ml. of 0.1 N-Ba(OH) $_2$. The theoretical amount of CO_2 from one α -aminoadipic acid residue would have neutralized 0.49 ml. of 0.1 N-Ba(OH) $_2$. Under the same conditions no CO_2 was obtained from benzylpenillamine, prepared from benzylpenicillin.

Reaction of cephalosporin N with 1:2:4-fluorodinitrobenzene (FDNB). FDNB (1.5 ml.) was added to a mixture of 4% (w/v) NaHCO_3 (24 ml.) and acetone (35 ml.). A sample (2 ml.) of the solution was removed to serve as a blank for determinations of optical density. Cephalosporin N (1 g.; 24 units/mg.) was added to the remainder of the solution at 20°. Samples were removed at intervals and the increase in optical density at 350 $\mu\mu$, relative to the blank solution, was determined. The reaction was completed in about 45 min. The pH of the solution, which was 8.5 (glass electrode) during the reaction, was adjusted to 6.5 with 2 N-HCl and the acetone was then removed *in vacuo*. When assayed against *Staph. aureus* this solution was found to contain 5 times the total activity of the starting material.

The subsequent operations were carried out at 3°. The solution was acidified to pH 2.8 and extracted 3 times with one vol. of ethyl acetate. The extract was shaken twice with one vol. of water, adjusting the pH to 5.5 with N-NaOH. The resulting aqueous solution was brought to pH 7.0 and freeze-dried. The preparation of DNP-cephalosporin N sodium salt (500 mg.) showed an activity of 200 units/mg. when assayed against *Staph. aureus*, and 0.2 units/mg. when assayed against *Salm. typhi*.

Reaction with benzoyl chloride. Crude cephalosporin N (100 mg., 28 units/mg.) was dissolved in 3% (w/v) aqueous NaHCO_3 (6 ml.) and acetone (6 ml.) was added. The solution was cooled to 0°, stirred, and 0.05 ml. benzoyl chloride in acetone (11 ml.) was slowly added. After 20 min. the acetone was removed *in vacuo* and the aqueous solution (adjusted to pH 7.0) extracted with one vol. of ether. The resulting solution (5.5 ml.) contained 1485 units/ml. when assayed against *Staph. aureus*. It was cooled to 0°, its pH adjusted to 2.5 with 2 N-HCl, and extracted twice with one vol. of *n*-butanol. The combined butanol extracts were shaken twice with water (3 ml.), adjusting the pH of the aqueous phase to 6.5. The aqueous solution was extracted with ether and then freeze-dried. The product (65 mg.) showed an activity of

123 units/mg. when assayed against *Staph. aureus* and 4 units/mg. when assayed against *Salm. typhi*.

Reaction with benzyloxycarbonyl chloride. This reaction was carried out in a similar manner to the previous one. Crude cephalosporin N (100 mg.; 28 units/mg.) yielded a solution (5.5 ml.) which showed an activity of 1060 units/ml. against *Staph. aureus*. The product obtained from the butanol extract (71 mg.) showed an activity of 81 units/mg. against *Staph. aureus* and 11 units/mg. against *Salm. typhi*.

Reaction with phenyl isocyanate. This was carried out in a similar manner to the reaction with benzoyl chloride, using 100 mg. of crude cephalosporin N (28 units/mg.). After the reaction, the solution contained 6160 units when assayed against *Staph. aureus* and 645 units when assayed against *Salm. typhi*. The extract from butanol contained 4060 units against *Staph. aureus* and 370 units against *Salm. typhi*.

Reaction with acetic anhydride. Crude cephalosporin N (35 mg., 28 units/mg.) was allowed to react with acetic anhydride (0.01 ml.) under conditions similar to those used in the reactions with acid chlorides. The resulting solution contained 980 units when assayed against *Staph. aureus* and 135 units when assayed against *Salm. typhi*. The extract from butanol contained 800 units against *Staph. aureus* and 120 units against *Salm. typhi*.

SUMMARY

1. Cephalosporin N is hydrolysed by hot dilute acid to a penilloic acid, from which penicillamine may be obtained by treatment with mercuric chloride. More vigorous hydrolysis results in the liberation of D- α -aminoadipic acid.

2. Oxidation of the penilloic acid with bromine water yields penicillaminic acid and a neutral aldehyde. The latter has been oxidized with silver oxide to an α -aminoadipylmonoglycine. The pK of the amino group of this compound indicates that α -aminoadipic acid is linked to glycine through its δ -carboxyl group.

3. It is concluded from these results that cephalosporin N is probably (D-4-amino-4-carboxy-*n*-butyl)penicillin.

4. Cephalosporin N is transformed to a penillic acid in aqueous solution at pH 2.7. The penillic acid reacts with mercuric chloride to form a penillamine which has been isolated as a crystalline *S*-benzyl derivative.

5. Cephalosporin N reacts with certain acid chlorides, acetic anhydride, and phenyl isocyanate, to form derivatives which contain no free amino group. These derivatives are more active against *Staphylococcus aureus* and less active against *Salmonella typhi* than cephalosporin N itself.

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Site of Inhibitory Action of Cocaine on Cellular Respiration

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It has long been recognized that cocaine depresses cell metabolism. Niwa (1919) demonstrated that cocaine, in concentration sufficient to anaesthetize the sciatic nerve of a frog, reduced the production of carbon dioxide by the isolated nerve; Felloni (1935) observed that cocaine depressed the oxygen uptake of blood, Wortis (1935) that of brain and, more recently, Cook & McDevitt (1945) have shown that cocaine and other local anaesthetics retarded the respiration of yeast. Cocaine inhibits certain esterases (Broekmeyer, 1924), some dehydrogenases (Bach & Lambert, 1938) and the esterification of phenols, including adrenaline (Torda, 1943*a, b*).

Investigations preliminary to a detailed study into the mode of action of cocaine in depressing cellular metabolism have revealed that cocaine inhibits both fermentation and respiration by retarding the metabolism of pyruvate. The results of experiments with *Saccharomyces cerevisiae* showed that depression of alcoholic fermentation could be attributed to inhibition of carboxylase and that growth in the presence of cocaine induced in the yeast a modified response to addition of vitamin B₁ (Ryman & Walsh, 1951, 1952).

Experiments analogous to those with *S. cerevisiae* on fermentation have now been carried out on respiration using *Torulopsis utilis*. Investigations into the effects of cocaine on isolated steps of carbohydrate oxidation have been carried out mainly with enzyme systems prepared from mammalian tissues.

The results here presented show that the toxicity of cocaine towards tissue respiration lies, in part at least, in the ability of the alkaloid to block the entry of two-carbon fragments into the tricarboxylic acid cycle.

MATERIALS AND METHODS

Reagents. Sodium pyruvate was prepared from re-distilled pyruvic acid by the method of Robertson (1942) and recrystallized until pure, as determined iodometrically (Wieland, 1924). Adenosine triphosphate (ATP) (L. Light and Co.) was freshly weighed for each experiment. Diphosphopyridine nucleotide (DPN) was prepared from fresh baker's yeast as described by LePage (1949). Other chemicals were of either B.P. or A.R. quality.

Homogenates. Glass homogenizers of the Potter and Elvehjem type were used, the tubes being cooled by immersion in a freezing mixture.

Yeast. *Torulopsis utilis*, var. *major* (National Collection of Type Cultures, no. 6593).

Spectrophotometric determinations. A Unicam Quartz spectrophotometer was used.

EXPERIMENTAL AND RESULTS

Effects of cocaine on respiration of Torulopsis utilis

Preliminary experiments showed that cocaine (0.004 M) inhibited the respiration of *T. utilis* with glucose as substrate by about 25 %, with pyruvate as substrate by about 80 %; that addition of vitamin B₁ did not modify the effects of cocaine and that, unlike the effects on *Saccharomyces fermenta-*