Products of Aminolysis and Enzymic Hydrolysis of the Cephalosporins

By J. M. T. HAMILTON-MILLER, G. G. F. NEWTON* AND E. P. ABRAHAM
Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, U.K.

(Received 5 August 1969)

1. The reaction of cephalosporins with ammonia, amino acids and other simple amino compounds in weakly alkaline aqueous solutions yields labile compounds with $\lambda_{\max}$, 230 nm. The reaction of deacetyl- and deacetoxy-cephalosporins under similar conditions yields compounds with $\lambda_{\max}$, 260 nm. 2. Hydrolysis with a β -lactamase results in the formation of compounds with $\lambda_{\max}$, 230 nm from deacetyl-cephalosporins and cephalosporins, but not from deacetoxycephalosporins. 3. These different compounds decompose to give penaldates and penamaldates derived from the side chain and the carbon atoms of the β -lactam ring. 4. Derivatives similar to those obtained with simple amino compounds appear to be formed when cephalosporins and their analogues react with lysine polymers. 5. Some of the chemical and physical properties of the various derivatives have been studied and tentative structures for them are proposed. 6. Possible implications of the results in relation to the immunological properties of the cephalosporins are discussed.

Despite the resemblance in structure between the penicillins (I) and cephalosporins (II) there are striking differences in the products obtained on cleavage of their β -lactam rings.

Opening of the β -lactam ring of the penicillins by dilute alkali, penicillinase (EC 3.5.2.6) or reaction with amino groups yields mainly D- α -penicilloates. The penicilloate structure is relatively stable, although mutarotation and epimerization may occur to give D- β , D- γ and D- δ diastereoisomers, which differ from the D- α compound in configuration at C-5 and/or C-6 (Mozingo & Folkers, 1949; Batchelor, Dewdney & Gazzard, 1965; Schneider & de Weck, 1965; Sabath, Jago & Abraham, 1965).

The presence of a Δ^3 double bond in the cephalosporin ring system leads to more extensive cleavage on hydrolytic degradation of the molecule. Early experiments with cephalosporin C (II, R = $-\text{O}_2\text{C} \cdot \text{CH}(\text{NH}_3^+) \cdot [\text{CH}_2]_3$, R' = $\text{CH}_3 \cdot \text{CO} \cdot \text{O}$) indicated that this substance yielded no penicillamine (D- β -thiolvaline) on acid hydrolysis and was fragmented when treated with dilute alkali (Newton & Abraham, 1956). Opening of the β -lactam ring of cephalosporin C, cephalothin (II, R = $\text{C}_4\text{H}_9\text{S} \cdot \text{CH}_2$, R' = $\text{CH}_3 \cdot \text{CO} \cdot \text{O}$) or cephaloridine (II, R = $\text{C}_4\text{H}_9\text{S} \cdot \text{CH}_2$, R' = $\text{C}_5\text{H}_9\text{N}$) with a β -lactamase was subsequently found to be accompanied by the expulsion of R' as acetate or pyridine. At the same time the characteristic u.v. absorption of these com-

(I)

(II)

pounds ($\lambda_{\max}$, 260 nm) disappeared. It was replaced by an absorption band with $\lambda_{\max}$, 230 nm that disappeared in turn after several hours (Sabath *et al.* 1965). These results with cephalosporins in which R' was a leaving group, such as acetoxy or pyridinium, contrasted with those obtained with deacetylcephalosporin C lactone (III). On mild hydrolysis the opening of the β -lactam ring of the lactone appeared to occur without other changes and the resulting product (IV), which showed $\lambda_{\max}$, 265 nm, could be regarded as the corresponding 'cephalosporoate' (Abraham & Newton, 1961).

Reaction of cephalosporin C in aqueous solution with ammonia, or with the amino groups of amino acids at pH 11, was briefly reported to yield labile compounds whose u.v.-absorption spectra resembled

* Deceased 1 January 1969.

that of the product resulting from the action of a β -lactamase, whereas no compound absorbing in this region was obtained from deacetoxycephalosporin C (II, R' = H) (Newton, Abraham & Kuwabara, 1968). The compounds with $\lambda_{\max}$ 230 nm were less stable in concentrated than in dilute solution. Their breakdown was followed by the appearance of new chromophores with $\lambda_{\max}$ 270 and 278 nm respectively, which was attributed to a fragmentation of the molecule and the formation of penaldates (V) and penamaldates (VI).

The experiments described in this paper and the following one were designed to extend those of Newton *et al.* (1968) and to throw further light on the changes that occur when different cephalosporins react with amino groups. A more detailed knowledge of these changes was required for a study of the nature of the haptenic groups of antigens obtained by reaction of cephalosporins with proteins and for comparison of these haptens with those obtained from penicillin.

MATERIALS AND METHODS

Penicillins and cephalosporins. Sodium benzylpenicillin was from Glaxo Laboratories Ltd., Greenford, Middx., U.K.; potassium cephalosporin C, potassium deacetoxycephalosporin C, 7-(*n*-valeramido)cephalosporanic acid ('*n*-butylcephalosporin'), deacetoxy-*n*-butylcephalosporin and 7-phenylacetamido- Δ^2 -cephalosporanic acid (' Δ^2 -cephaloram') were kindly given by Glaxo Research Ltd. To convert the latter three compounds into their sodium salts they were dissolved in a solution of NaHCO₃ (1 equiv.). The sodium butylcephalosporins were precipitated by the addition of 30 vol. of acetone and the solution of sodium Δ^2 -cephaloram was freeze-dried. Sodium deacetylcephalosporin C was prepared as described by Jeffrey, Abraham & Newton (1961) and deacetylcephalosporin C lactone (cephalosporin C₆) as described by Abraham & Newton (1961). Sodium deacetyl-*n*-butylcephalosporin was prepared similarly, except that the product was extracted from aqueous solution at pH 2.2 with butyl acetate followed by ethyl acetate, transferred from the extract into water at pH 7 and isolated by freeze-drying.

Cephaloram sodium salt was as described by Loder, Abraham & Newton (1961).

Buffer solutions. Sodium phosphate, McIlvaine's sodium phosphate-citrate and tris buffers were made as described by Dawson & Elliott (1959).

Radioactive compounds. [1-¹⁴C]Glycine (41.4 μ Ci/ μ mol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Lysine polymers. Poly-L-lysine hydrobromide, of molecular-weight range 30 000–50 000, and a 1:1 polymer of L-lysine and L-serine, of molecular weight 35 000, were obtained from Miles-Yeda Laboratories, Rehovoth, Israel.

β -Lactamase preparation. A culture supernatant fraction containing the β -lactamase from *Pseudomonas pyocyanea* N.C.T.C. 8203 was prepared as described by Sabath *et al.* (1965). Samples (5 ml) were freeze-dried and reconstituted for use in 0.1 vol. of 25 mM-sodium phosphate buffer, pH 7.0.

Enzymic hydrolysis of cephalosporins. For the determination of relative rates of enzymic hydrolysis of different cephalosporins in low concentrations an appropriate volume (usually 5 μ l) of the solution of crude β -lactamase from *Ps. pyocyanea* was added to 3 ml of a solution of substrate (50 μ g/ml) in 25 mM-sodium phosphate buffer, pH 7.0, and the initial rate of decrease of E_{260} was measured. Relative rates of hydrolysis at higher substrate concentration (3 mg/ml) were determined in a pH-stat as described by Sabath *et al.* (1965) at pH 7.4. Usually 100–150 μ l of crude enzyme solution was added/1.2 ml of substrate solution.

Hydrolysis of substrate in high concentration (100 mg/ml) was carried out by addition of 1 vol. of enzyme solution to a solution of substrate (200 mg/ml) in 2 M-K₂HPO₄. Small amounts of 5 M-NaOH were added from time to time to maintain the pH between 7.5 and 8.0.

Electrophoresis and chromatography. Electrophoresis on Whatman no. 1 paper at pH 4.5 (pyridine-acetate, 50 mM with respect to acetate), and at pH 9.0 (0.1 M-ammonium carbonate) was carried out at 70 and 45 V/cm respectively in an apparatus similar to that described by Katz, Dreyer & Anfinsen (1959). Electrophoresis at pH 7.0 in collidine-acetate (50 mM with respect to acetate) and at pH 8.7 in *N*-ethylmorpholine acetate (0.33 M) was carried out at 13 V/cm in an apparatus similar to that described by Flynn & de Mayo (1951). Paper chromatography was on Whatman no. 1 paper in butan-1-ol-acetic acid-water (4:1:4, by vol.) or in butan-1-ol-acetic acid-water-

pyridine (15:3:12:10, by vol.). Spots were located visually in u.v. light, or by spraying with ninhydrin, aq. 1% (w/v) AgNO_3 , ammoniacal AgNO_3 [10 ml of aq. 10% (w/v) AgNO_3 + 10 ml of aq. NH_3 (sp.gr. 0.88) + 80 ml of methanol], starch-iodine [10 ml of 10 mm- I_2 in 3 mm-KI, 9 ml of aq. 2% (w/v) soluble starch and 1 ml of $\text{m-Na}_2\text{HPO}_4$ - $\text{m-KH}_2\text{PO}_4$ buffer, pH 7.0], or a platinichloride reagent [27 ml of m-KI , 5 ml of 5% (w/v) K_2PtCl_6 and 100 ml of water]. Radioactive spots were located on paper by radioautography and counted as described by Trown, Abraham, Newton, Hale & Miller (1962).

Thin-layer chromatography. This was carried out on Kieselgel G (300 μm thick) in solvent systems described in the text.

Microbiological assays. These were carried out by the hole-plate method (Brownlee *et al.* 1948). *Alcaligenes faecalis* A.T.C.C. 8750 was used as the test organism for assays of cephalosporin C and its derivatives and *Staphylococcus aureus* N.C.T.C. 6571 for assays of *n*-butylcephalosporin and its derivatives.

Hydroxylamine assays. These were carried out by the method of Boxer & Everett (1949). With cephalosporins the incubation period before addition of Fe^{3+} was decreased to 3 min (Hamilton-Miller, Smith & Knox, 1965). With deacetoxycephalosporins it was necessary to raise the pH of the hydroxylamine reagent to 9.0 to obtain quantitative results.

Reaction of cephalosporins with amino compounds. These reactions were carried out in aqueous solution at 21°C, usually with a cephalosporin concentration of 178 mg/ml (0.35–0.45 M). For reaction with NH_3 the cephalosporin was dissolved in aq. NH_3 (sp.gr. 0.88) or dilutions of the latter. Amino acids and other compounds containing amino groups were used in final concentrations of approx. 1 M. The compound was dissolved in $\text{m-Na}_2\text{CO}_3$ mixed with an appropriate amount of 2 M- NaOH and the cephalosporin was dissolved in the resulting solution. The amount of 2 M- NaOH used was sufficient to give a final pH between 8.0 and 10.5. Changes in u.v.-absorption spectra during the reactions were measured with a Unicam SP. 800 recording spectrophotometer. For this purpose 5 μl samples from the reaction mixture were diluted to 15 ml with water.

Reactions of cephalosporins with amino acid polymers. Cephalosporins were dissolved in water at 100 mg/ml, the resulting solution being used to dissolve polylysine or poly(lysine + serine) at 100 mg/ml. The pH of the mixture was then adjusted to between 9 and 10.5 by the addition of 10 M- NaOH and the course of the reaction followed by diluting 5 μl samples of the solution to 10 ml with water at intervals and measuring the u.v.-absorption spectrum of the diluted samples. Reaction mixtures to be analysed after tryptic digestion were allowed to stand overnight, diluted 1:25 with a solution of crystalline trypsin (1 mg/ml in 25 mM-sodium phosphate buffer, pH 7.4), and incubated at 37°C for 30 min for polylysine reactions or overnight for poly(lysine + serine) reactions. Tryptic digest 'maps' were made by applying 30 μl of the reaction mixture to paper and subjecting it to electrophoresis at pH 4.5 followed by chromatography in the solvent system described by Waley & Watson (1953).

Determination of ammonia. Evolution of NH_3 from the reaction of cephalosporin C with ϵ -aminohexanoic acid (ϵ -aminocaproic acid) and α - and β -alanine was determined

in Conway units (Conway, 1947) at 37°C with 0.5 M- H_2SO_4 in the centre wells. NH_3 was determined by nesslerization, or by titration of the H_2SO_4 with 0.1 M- NaOH . The reaction mixture placed in the annulus of the Conway unit was in 2 M- NaOH - $\text{m-Na}_2\text{CO}_3$ solution, pH 10.5 (0.4 ml). A control experiment showed that at least 90% of a known amount of NH_3 distilled from this solution into the H_2SO_4 within 1 h.

Determination of carbon dioxide. This was done in the apparatus described by Abraham, Baker, Chain & Robinson (1949). A sample (100 μl) of the reaction mixture was washed with 0.9 ml of water into the reaction vessel (containing 1 ml of 2 M-HCl). CO_2 was carried by a stream of CO_2 -free N_2 into two tubes in series, each containing 17.6 mm- Ba(OH)_2 . After 20 min the baryta was titrated against 20 mm-HCl with phenolphthalein as an indicator.

Determination of thiol. Samples (5 μl) of the reaction mixture were assayed for thiol by the method of Ellman (1959).

Ozonolysis. The reaction mixture (2 ml) was diluted tenfold with water. Half of the resulting solution was placed in a pear-shaped flask in an ice bath and ozonized O_2 (containing about 6% of O_3) (40–50 l/h) was passed through it for 30 min. Portions of the resulting solution were added to equal volumes of either freshly prepared aq. 0.4% (w/v) dimedone solution or a saturated solution of 2,4-dinitrophenylhydrazine in 2 M-HCl. Crystalline material that formed was separated by centrifugation and filtration, washed with water on the filter and dried. Formaldehyde was determined by the chromotropic acid assay (Egriwe, 1937).

Thiosemicarbazone formation. This was followed spectrophotometrically as described by Rogers, Lomakina & Abraham (1965). A sample (15 μl) of the reaction mixture was mixed with 0.3 ml of 10 mm-sodium acetate buffer, pH 4.5, and an equal volume of 10 mm-thiosemicarbazide in ethanol was added. Samples (50 μl) were taken from the mixture at intervals and diluted to 2 ml with water. The u.v.-absorption spectra of the resulting solutions were measured against a blank consisting of the reaction mixture without the cephalosporin.

2,4-Dinitrophenylhydrazones. These were prepared from the reaction products of cephalosporins with non-polar side chains by addition of an excess of 0.4% 2,4-dinitrophenylhydrazine in 2 M-HCl. The resulting precipitates were filtered off, washed with m-HCl and dried. An authentic sample of the 2,4-dinitrophenylhydrazone of the amide of benzylpenicillate was prepared from benzylpenicilloamide (Mozingo & Folkers, 1949).

Desulphurization with Raney nickel. Cephalosporin C sodium salt (20 mg) was dissolved in 200 μl of aq. NH_3 (sp.gr. 0.88). After 5 min the excess of NH_3 was removed *in vacuo* and the solution diluted with 0.2 M- Na_2HPO_4 to 2 ml, the final pH being 8.6. To this solution was added 200 μl of Raney nickel (Pavlic & Adkins, 1946) and the mixture stirred overnight at room temperature. The insoluble material was then removed by filtration and washed with water, and the combined filtrate was extracted three times with 1 vol. of 16% (w/v) 8-hydroxyquinoline in CHCl_3 and then twice with 1 vol. of CHCl_3 . Then 12 M-HCl (0.11 vol.) was added to the aqueous solution and the mixture heated at 100°C for 5 h in a sealed tube. The resulting solution was divided into two equal parts and each evaporated to dryness *in vacuo* over solid

KOH. For analysis by paper chromatography and electrophoresis one residue was dissolved in water and the other was dissolved in 2M-NH₃ and the solution heated at 100°C for 5 min.

Formation of sulphur-containing lactones. The procedure used to study the formation of the sulphur-containing butenolides ('compounds 1 and 2') described by Abraham & Newton (1961) was as follows. The cephalosporins and/or aminolysis products were treated with 1.25M-HCl at 100°C for 20 and 10 min respectively and the solutions extracted with ethyl acetate. The residues from the extracts were weighed and then analysed by paper electrophoresis and chromatography and by measurements of their u.v.-absorption spectra (Abraham & Newton, 1961).

Polarimetry. Measurements of optical rotation were carried out on solutions contained in 5 cm cells (volume 4 or 1 ml) with a Hilger and Watts M412 polarimeter and a sodium-vapour light-source.

Gel filtration. In two experiments gel filtration was carried out with a column (40 cm × 2 cm) of Sephadex G-10. Elution was with 0.5M-(NH₄)₂CO₃ and 2.5–3 ml fractions were collected.

(1) Cephalosporin C (178 mg/ml) was allowed to react with glucosamine (295 mg/ml of the hydrochloride, in M-Na₂CO₃–2M-NaOH) for 17 min at room temperature, the reaction mixture was diluted tenfold with 0.5M-(NH₄)₂CO₃, and 0.5 ml was applied to the column with Blue Dextran as marker. The spectrum of each fraction was measured between 200 and 300 nm, and analyses of 'bound' and 'free' glucosamine were carried out by using the Elson–Morgan assay (Elson & Morgan, 1933) before and after acid hydrolysis (4M-HCl at 100°C for 4–5 h).

(2) Cephalosporin C (178 mg/ml) was allowed to react with alanine (4 equiv.), and the reaction mixture was kept at room temperature for 3 days, at which time $\lambda_{\max}$ was 275 nm. A sample (50 μ l) of the solution was diluted to 0.5 ml with 0.5M-(NH₄)₂CO₃ and applied to the column. The spectrum of each fraction was measured between 200 and 300 nm.

In a third experiment a column (32 cm × 2 cm) of Sephadex G-15 was used and elution was with 0.1M-(NH₄)₂CO₃; 2.5–3 ml fractions were again collected. [¹⁴C]Glycine (5 μ Ci) was added to 0.2 ml of a solution of glycine (112 mg/ml) in M-Na₂CO₃–2M-NaOH, and cephalosporin C was dissolved in the resulting solution to give a concentration of 178 mg/ml. After 2 min 0.1 ml of the solution, diluted to 0.5 ml with 0.1M-(NH₄)₂CO₃, was applied to the column. The absorption spectrum of each fraction was recorded between 200 and 300 nm, and duplicate 50 μ l samples were taken for determinations of radioactivity. Samples (5 μ l) of each fraction were also chromatographed in butan-1-ol-acetic acid–water. A sharp peak in E_{230} and radioactivity occurred in fraction 4 and a second peak of radioactivity (due to unchanged [¹⁴C]glycine) in fraction 6. Paper chromatography showed that no detectable amount of [¹⁴C]glycine was present in fractions 3 and 4.

RESULTS

Changes in u.v.-absorption spectra of different cephalosporins during hydrolysis and aminolysis

Hydrolysis by β -lactamase from Ps. pyocyanea.

The yields of derivatives with $\lambda_{\max}$ 230 nm that were obtained on enzymic hydrolysis of a number of cephalosporins are expressed in terms of the quotient E_{230}/E_{260} , values for which are given below in parentheses. E_{230} is the maximum value observed after hydrolysis and E_{260} is the value for the parent substance. Under the conditions used the hydrolysis of most substrates, as assessed by the loss of the absorption band with $E_{\max}$ at 260 nm, was virtually complete within 15 min. Since derivatives with $\lambda_{\max}$ 230 nm were unstable (Sabath *et al.* 1965), their production in high yield required rapid hydrolysis of the cephalosporin.

With a substrate concentration of 50 μ g/ml in 10 mM-phosphate buffer, pH 7.4, there was a clear-cut formation of products with $\lambda_{\max}$ 230 nm from cephalosporin C (1.2), deacetylcephalosporin C (0.95), *n*-butylcephalosporin (1.2) and deacetyl-*n*-butylcephalosporin (1.1). In contrast, there was no increase in E_{230} during hydrolysis of deacetoxycephalosporin C and deacetoxy-*n*-butylcephalosporin.

In 0.5M-K₂HPO₄ there were similar changes in u.v.-absorption during the hydrolysis of cephalosporin C (1.1) and *n*-butylcephalosporin. On the other hand, there was no increase in E_{230} with deacetylcephalosporin C, despite the fact that E_{260} fell rapidly. Further experiments showed that phosphate concentrations above 0.125 M suppressed the formation of derivatives with $\lambda_{\max}$ 230 nm from deacetylcephalosporin C and deacetyl-*n*-butylcephalosporin respectively. Arsenate showed at least as powerful a suppressive effect as phosphate, whereas pyrophosphate and phosphite showed only relatively small effects at 0.1 M and 0.5 M respectively. High concentrations of sodium chloride or potassium chloride (0.43 M) in 25 mM-phosphate, or 0.5M-tris-HCl were ineffective. High phosphate concentrations appeared to suppress the formation of derivatives with $\lambda_{\max}$ 230 nm and not to accelerate their decomposition, since the stability of the preformed derivative from deacetyl-*n*-butylcephalosporin was not changed significantly when the concentration of phosphate, pH 7.4, was increased from 25 mM to 0.5 M. Higher concentrations of all the buffers tested (0.5 M) inhibited the rate of hydrolysis of both substrates by up to 70%, but substantial suppression of the formation of the compound with $\lambda_{\max}$ 230 nm occurred only with deacetylcephalosporins as substrates and not with the cephalosporins themselves.

When cephalosporins, deacetylcephalosporins, deacetoxycephalosporins or deacetylcephalosporin Clactone were allowed to react with β -lactamase and the solutions then kept at room temperature for periods of up to 1 week, there was no indication in any case of a rise in extinction in the region of 270–280 nm.

The rate of hydrolysis of Δ^2 -cephaloram (3 mg/ml) by the β -lactamase from *Ps. pyocyanea* was about 40% of that of cephalosporin C, and 2 equiv. of acid were produced. The u.v.-absorption spectrum of Δ^2 -cephaloram sodium salt showed inflexions at 235 and 250 nm, some of the absorption at the latter wavelength presumably being due to the phenyl-acetyl side chain. A difference spectrum determined with an equimolar solution of sodium benzylpenicillin as a blank showed $\lambda_{\max}$. 236 nm (ϵ 6700). A corresponding difference spectrum after enzymic hydrolysis of Δ^2 -cephaloram showed $\lambda_{\max}$. 252 nm (ϵ 5400).

Aminolysis. (a) Cephalosporin C and *n*-butylcephalosporin. When a solution of cephalosporin C or *n*-butylcephalosporin sodium salt (about 0.3 M) was made 4 M with respect to ammonia, within 5 min the absorption band with $\lambda_{\max}$. 260 nm disappeared and the extinction at 230 nm reached a maximum (E_{230}/E_{260} ratio 1.3–1.6), as shown in Fig. 1. A similar change occurred more slowly (within 40 min) when cephalosporin C in the same concentration reacted with 1 equiv. of ammonia. Comparable changes in the u.v.-absorption spectra of cephalosporin C and *n*-butylcephalosporin also occurred when aqueous solutions of these compounds (about 0.35 M) were allowed to react at pH 9–10 with between 2 and 6 equiv. of the following: glycine, α -alanine, β -alanine, cysteine, ϵ -aminohexanoate, lysine, glucosamine, ethylenediamine and hydroxylamine. Under the conditions used, and at pH 10, E_{230} with the amino acids reached a maximum within 3 min, and with glucosamine within 15 min. With cephalosporin C and 4 equiv. of hydroxylamine E_{230} reached a maximum very rapidly (within 3 min) at a pH above 8.5.

At pH 7.6 there was relatively little reaction between cephalosporin C and the amino acids tested, or with glycylglycine and glycylglycylglycine, whose amino groups have lower pK_a values than those of α -amino acids.

(b) Deacetyl- and deacetoxy-cephalosporins. Deacetylcephalosporin C and deacetyl-*n*-butylcephalosporin reacted with ammonia and amino acids and lost their absorption band with $\lambda_{\max}$. 260 nm at a rate similar to that observed with the parent compounds. However, no increase in E_{230} occurred on aminolysis of the deacetyl derivatives.

The β -lactam rings of deacetoxycephalosporin C and deacetoxy-*n*-butylcephalosporin were considerably more resistant to alkaline hydrolysis and to aminolysis than those of the deacetyl compounds or the corresponding cephalosporins themselves. Thus in 10 M-sodium hydroxide at room temperature the half-lives of cephalosporin C and deacetylcephalosporin C (50 μ g/ml) were 70 and 100 min respectively as estimated from the fall in E_{260} , whereas the half-life of deacetoxycephalosporin C

Fig. 1. Changes in u.v.-absorption spectra during aminolysis of cephalosporin C (178 mg/ml) in 4 M-NH₃. Spectra were recorded with samples diluted 1:3000. Curve 1, Starting spectrum; curves 2–6, spectra after 6 min, 22 h, 78 h, 6 days and 10 days respectively; curve 7, after 10 days followed by adjustment to pH 2.0 and subsequent adjustment to pH 10.0.

under the same conditions was about 8 h. Corresponding values for the *n*-butyl analogues were 54 min, 76 min and 6 h respectively. No absorption band with $\lambda_{\max}$. 230 nm developed during the alkaline hydrolysis of deacetyl- or deacetoxycephalosporins. The band was observed during alkaline hydrolysis of the cephalosporins, but was much less clear-cut than that produced on enzymic hydrolysis or aminolysis. In the hydroxylamine assay at pH 7.0, as used for penicillins and for cephalosporins with a leaving group at C-10 (Hamilton-Miller, 1967), the reaction with the deacetoxy compounds needed 30 min for completion, although at pH 9.0 it was quantitative within 5 min.

Reaction of deacetoxycephalosporin C and of deacetoxy-*n*-butylcephalosporin with concentrated aqueous ammonia, or amino acids, under conditions used with the parent compounds resulted in a relatively rapid fall in E_{260} , but no increases in E_{230} . In this respect the behaviour of the deacetoxycephalosporins paralleled that of the deacetyl analogues.

It appeared that the first compound produced when the β -lactam ring of a deacetyl- or deacetoxycephalosporin was opened by aminolysis showed $\lambda_{\max}$. in the region of 260 nm but a lower value of $E_{\max}$. than that of the original substance. A plot of the decrease in E_{260} against the decrease in antibacterial activity when deacetoxycephalosporin C sodium salt was dissolved in 3.8 M-ammonia (Fig. 2) indicated that the fall in E_{260} to its final value was only 75% complete when virtually all the activity had been lost. A similar result was obtained with deacetylcephalosporin C and deacetoxy-*n*-butylcephalosporin.

Fig. 2. Relationship between E_{260} and antibacterial activity during aminolysis of deacetoxycephalosporin C (20mg/ml) in 3.8M- NH_3 . Samples were diluted 1:400 at intervals with 0.2M-sodium phosphate buffer, pH 7.0, to stop the reaction. The arrow on the ordinate shows the minimum value to which E_{260} fell.

Fig. 3. Relationship between optical rotation and antibacterial activity during aminolysis of deacetoxy- and deacetyl-cephalosporin C. ●, Deacetoxycephalosporin C (20mg/ml) in 3.8M- NH_3 ; ○, deacetylcephalosporin C in M- NH_3 . Changes in optical rotation were followed with one half of each solution and activities of samples from the other half were assayed after the reaction had been stopped as described in Fig. 2. The values of α are rotations (in degrees) in a tube of 5cm light-path.

When deacetyl- or deacetoxy-cephalosporin C (10mg/ml) was dissolved in aqueous ammonia $[\alpha]_D$ fell rapidly from a positive to a small negative value (Table 1) and there was a linear relationship between this fall and the fall in antibacterial activity (Fig. 3). With deacetylcephalosporin C the fall in E_{260} was only 70% complete when $[\alpha]_D$ had reached its minimum, after 8min. A similar result was obtained with deacetoxycephalosporin C. In contrast, with cephalosporin C the decrease in E_{260} to a minimum value was accompanied by only a very small decrease in $[\alpha]_D$ (Table 1).

(c) Δ^2 -Cephaloram. Aminolysis of Δ^2 -cephaloram (100mg/ml) in 12M-ammonia at 34°C resulted in a decrease in absorption in the range 225–240nm (inflection at 235nm). No increase in E_{230} was

Table 1. Changes in optical rotation of members of the cephalosporin C family and benzylpenicillin as a result of aminolysis

Substance	Original $[\alpha]_D$	$[\alpha]_D$ immediately after aminolysis
Cephalosporin C	+101°	+94°
Deacetylcephalosporin C	+103	-18
Deacetoxycephalosporin C	+132	-23
Cephalosporin C ₆	+146	-58
Benzylpenicillin	+289	+174

observed. The stability of the β -lactam ring in Δ^2 -cephaloram is shown by the fact that even at pH 9.0 hydroxylamine reacted only with the acetoxy moiety of the molecule (Hamilton-Miller, Richards & Abraham, 1970).

Properties of the primary products of aminolysis

Electrophoresis on paper. No significant amount of ammonia or free thiol was liberated during the formation of derivatives with λ_{max} 230nm when cephalosporin C reacted with ϵ -aminohexanoate or α -alanine. The derivative from cephalosporin C and ammonia broke down when the solution containing it was allowed to dry on paper, but survived if the solution (2 μ l, about 50mg/ml) was applied to paper already moistened with buffer at pH 9.0, and could subsequently be eluted with 0.1M-ammonia. When subjected to electrophoresis at pH 9.0 it showed as a single ninhydrin-positive substance with a mobility close to that of cephalosporin C itself. It reduced starch-iodine, iodoplatinate and silver nitrate on paper.

When subjected to electrophoresis at pH 9 the derivative with λ_{max} 230nm from cephalosporin C and ethylenediamine migrated 2.5cm towards the anode, whereas cephalosporin C itself migrated 6cm towards the anode and free ethylenediamine 6.5cm towards the cathode under the same conditions. Glucose was carried 1.0cm towards the cathode by endosmosis. The penicilloamide formed by reaction of benzylpenicillin with ethylenediamine (Schwartz, 1968) migrated 3.0cm towards the anode under similar conditions and benzylpenicillin 7.0cm. Thus the reaction with ethylenediamine apparently resulted in the incorporation into the cephalosporin and the penicillin molecule of a group with a fractional positive charge at pH 9. This was consistent with the expectation that the β -amino group of an amide resulting from an attack of the α -amino group on a β -lactam C=O group would have a pK_a in the region of 9.5.

Gel filtration. The compound with λ_{max} 230nm obtained by the reaction of cephalosporin C with

Fig. 4. Gel filtration on Sephadex G-10 in 0.1 M-(NH₄)₂CO₃ of products of the reaction between cephalosporin C and glucosamine (for details see the text). ○, Acid-labile E_{230} ; ●, E_{260} ; ■, extinction corresponding to free glucosamine (Elson-Morgan method). Bound glucosamine was found in fractions 3 and 4.

glucosamine (see the Materials and Methods section) was partially resolved on a column of Sephadex G-10 (K_d 0.167) from unchanged cephalosporin C (K_d 0.2), and almost completely resolved from unchanged glucosamine (K_d 0.42) (Fig. 4).

A solution of the derivative with $\lambda_{\max}$ 230 nm obtained by reaction of cephalosporin C with [¹⁴C]-glycine and separated from excess of glycine on Sephadex G-15 (see the Materials and Methods section) showed E_{230} 58 and contained 3.66 nCi of ¹⁴C. On the assumption that E_{230} for the derivative was 1.4 times E_{260} for cephalosporin C it followed that the derivative contained 1 residue of glycine/molecule.

Ozonization. A solution of a derivative of cephalosporin C with $\lambda_{\max}$ 230 nm was prepared by dissolving the cephalosporin (178 mg/ml) in aq. ammonia (sp.gr. 0.88) and diluting the mixture tenfold after 5 min. When ozonized oxygen was passed through a portion of this solution for 30 min the absorption maximum at 230 nm disappeared. No significant change occurred in the u.v.-absorption spectrum of a second portion of the solution through which oxygen was passed under similar conditions.

Addition of aqueous dimedone to a sample of the ozonized solution gave a small amount of a white crystalline precipitate (m.p. 183–185°C), which was the dimedone of formaldehyde (mixed m.p. with authentic sample 180–183°C). Addition of 2,4-dinitrophenylhydrazine in 2 M-hydrochloric acid to a further sample gave an orange-yellow precipitate. T.l.c. in benzene-tetrahydrofuran (4:1, v/v) showed that the major component of this precipitate had the same R_F value (0.81) as the 2,4-dinitrophenyl-

hydrazone of formaldehyde. Quantitative assay showed that the yield of formaldehyde was 76% of the theoretical value on the basis of 1 mol of cephalosporin C derivative giving 1 mol of formaldehyde.

Similar qualitative experiments indicated that formaldehyde was formed on ozonization of the corresponding derivative with $\lambda_{\max}$ 230 nm from *n*-butylcephalosporin. No formaldehyde was detected in the solution of this derivative before ozonization.

Desulphurization with Raney nickel. An acid hydrolysate of the material obtained by treatment with Raney nickel of the derivative with $\lambda_{\max}$ at 230 nm from cephalosporin C and ammonia was analysed by paper electrophoresis and chromatography (see the Materials and Methods section). Major spots were obtained in positions corresponding to α -amino adipic acid, glycine, alanine and valine. A fifth spot, due to a substance with no net charge at pH 4.5 and with R_{val} 0.86 in butan-1-ol-acetic acid-water, gave a brilliant yellow colour initially with ninhydrin that turned blue during the course of an hour. There was no indication of the presence of γ -hydroxyvaline or its lactone, or of $\alpha\gamma$ -diamino- β -methylbutyric acid (γ -aminovaline). γ -Hydroxyvaline was not affected by Raney nickel under the conditions used and was detected in hydrolysates of desulphurized deacetylcephalosporin C.

Formation of sulphur-containing butenolides. After treatment with 1.25 M-hydrochloric acid at 100°C cephalosporin C, deacetylcephalosporin C, the derivatives of these substances formed by aminolysis with ammonia, and deacetoxycephalosporin C yielded material that was soluble in ethyl acetate (Table 2). The material from both deacetylcephalosporin C and the product of its aminolysis contained the sulphur-containing butenolides (compounds 1 and 2) previously obtained from cephalosporin C (Abraham & Newton, 1961). These compounds were not detected after electrophoresis and chromatography on paper in the material from deacetoxycephalosporin C or in the material from the product of aminolysis of cephalosporin C (Table 2).

Stability. Sabath *et al.* (1965) found that the compound with $\lambda_{\max}$ 230 nm that was formed when the β -lactam ring of cephalosporin C was hydrolysed by the β -lactamase from *Ps. pyocyanea* was unstable at pH 7.0. Newton *et al.* (1968) reported that the absorption band with $\lambda_{\max}$ 230 nm that appeared during aminolysis of cephalosporin C subsequently disappeared when the solution was allowed to stand at room temperature. In the present experiments attempts to isolate derivatives with $\lambda_{\max}$ 230 nm by freeze-drying were unsuccessful. For this reason, and because of its potential

Table 2. *Compounds soluble in ethyl acetate on acid hydrolysis of cephalosporins and products of aminolysis*

For details see the text. Spots were detected by their ability to reduce AgNO_3 and/or to absorb u.v. light. Electrophoresis on paper was at pH 7.0 and 14 V/cm for 2.5 h, followed by chromatography in the perpendicular direction in butan-1-ol-acetic acid-water. Compounds 1 and 2 are those described by Abraham & Newton (1961). Pure compound 1 had migration 4 cm, R_F 0.74; compound 2 had migration 2.5 cm, R_F 0.81. Substances with no net charge migrated 1 cm towards the cathode.

Substance hydrolysed with acid	Weight (% of original compound)	Properties of ethyl acetate-soluble material			
		$\lambda_{\text{max.}}$ (nm)		Behaviour of components detected on paper	
		In 1.5 M-HCl	In NaOH (pH 10)	Migration (cm) towards anode	R_F
(1) Cephalosporin C	23	239	278	-1.0 2.5 4.0	0.81 0.83 0.77
(2) Product of aminolysis of (1) ($\lambda_{\text{max.}}$ 230 nm)	11	243	235-280 (plateau)	-1.0 6.0	0.82 0.66
(3) Deacetylcephalosporin C	16	239	278	-1.0 2.5 4.0	0.88 0.87 0.80
(4) Product of aminolysis of (3)	16	239	278	-1.0 3.0 4.5	0.84 0.88 0.78
(5) Deacetoxycephalosporin C	11	246	249, 305	-1.0 6.0	0.89 0.41

immunological significance, the stability of these derivatives was studied further.

At pH 9-10 the derivatives from cephalosporin C showed much higher stability in low than in high concentration. When a solution of a derivative was prepared by dissolving cephalosporin C in aq. ammonia (sp.gr. 0.88) to give a concentration of 150 mg/ml, E_{230} fell to half its maximum value at 20°C in about 2 h. If the solution was diluted immediately to give a concentration of 50 $\mu\text{g}/\text{ml}$, E_{230} did not change significantly during 24 h. The fall of E_{230} with time at the higher concentration indicates that the reaction followed second-order kinetics (Fig. 5). An Arrhenius plot from the second-order rate constants at 0, 26, 37 and 45°C gave a value of 13400 cal/mol for the apparent energy of activation (Fig. 6).

Compounds with $\lambda_{\text{max.}}$ at 230 nm derived from cephalosporin C and *n*-butylcephalosporin were much less stable in dilute acid than in dilute alkali. Fig. 7 shows a plot of the half-lives of these derivatives at 37°C (at concentrations equivalent to 50 μg of the parent compounds/ml) against pH. The derivatives were prepared by dissolving the cephalosporin in 2.4 M-ammonia (100 mg/ml) and diluting samples of the solutions 1:2000 in one-quarter-strength McIlvaine's phosphate-citrate buffer solutions of different pH values. Under these conditions,

Fig. 5. Second-order plot of decay of the compound showing $\lambda_{\text{max.}}$ 230 nm obtained by reaction of cephalosporin C (200 mg/ml) with 2.5 M- NH_3 for 10 min. The resulting solution and dilutions of it in 2.5 M- NH_3 were incubated at 37°C. In the plots of $1/(a-x)$ against time, a is the total fall in E_{230} and x is the fall in E_{230} at time t . Δ , Undiluted solution; $\triangle$, diluted 1:1.33; $\blacksquare$, diluted 1:1.6; $\circ$, diluted 1:2; $\bullet$, diluted 1:4.

Fig. 6. Arrhenius plot for the decay of the compound showing $\lambda_{\max}$. 230nm obtained by the reaction of cephalosporin C with NH_3 .

Fig. 7. Half-lives of compounds showing $\lambda_{\max}$. 230nm in dilute solution. The compounds were obtained by reaction of NH_3 with cephalosporin C (○) and *n*-butylcephalosporin (●) (for details see the text).

the decrease in E_{230} followed first-order kinetics. The half-lives of the two derivatives did not differ significantly.

Changes in the primary products after aminolysis

Ultraviolet-absorption spectra. The disappearance

of the u.v.-absorption band with $\lambda_{\max}$. 230nm that appeared during the aminolysis of cephalosporin C and *n*-butylcephalosporin with ammonia (0.38M) and a variety of amino acids (1.5M) at pH 10–10.5 resulted in an 'eclipse phase' in which there was no selective absorption between 200 and 400nm. This was followed within a few hours by the appearance of a new absorption band with $\lambda_{\max}$. 270nm that was gradually transformed into a band with $\lambda_{\max}$. 280nm (Fig. 1). The E_{280} value reached its maximum between 6 and 12 days with all the amino compounds used except cysteine and mercaptoethylamine, with which it did so in about 26 days at room temperature. Compounds with $\lambda_{\max}$. 270 and 280nm respectively also formed slowly within 7 days at room temperature in solutions obtained after aminolysis of deacetoxycephalosporin C and Δ^2 -cephaloram. The formation of derivatives with $\lambda_{\max}$. 270 and 280nm after aminolysis of cephalosporin C with alanine or ϵ -aminohexanoate was accompanied by the liberation of approx. 1 mol of ammonia/mol.

Acidification (pH < 2) of solutions of derivatives with $\lambda_{\max}$. 230nm resulted in very rapid loss of the absorption band (Fig. 8). Subsequent adjustment to pH 10.0 resulted in the immediate appearance of an absorption band with $\lambda_{\max}$. 270nm. Acidification of a solution of the derivative with $\lambda_{\max}$. 280nm also caused a rapid loss of this absorption band; on adjustment of the pH of the resulting solution to 10.0 a band with $\lambda_{\max}$. 270nm appeared immediately. This change was demonstrated with derivatives with $\lambda_{\max}$. at 280nm obtained from cephalosporin C and a variety of different amino acids, and the mean value obtained for (final E_{270})/(original E_{280}) was 0.73 (s.d. 0.04). The expected value for a penamaldate $\rightarrow$ penaldate conversion is $15000/17000 = 0.83$ (Woodward, Neuberger & Trenner, 1949; Brown, 1949).

Properties of derivatives with $\lambda_{\max}$. 270nm and $\lambda_{\max}$. 280nm. At pH 4.5 solutions of compounds with $\lambda_{\max}$. 270nm lost most of their absorption, but a new absorption band with $\lambda_{\max}$. 265–270nm, corresponding to that of a thiosemicarbazone, appeared on reaction at this pH with thiosemicarbazide. In one experiment E_{270} fell from 0.85 at pH 10.0 to 0.11 at pH 4.5 and $E_{265-270}$ rose by 0.4 after reaction for 2h with 5mM-thiosemicarbazide.

Addition of 2,4-dinitrophenylhydrazine in 2M-hydrochloric acid to solutions of the products of reactions between ammonia and *n*-butylcephalosporin, deacetyl-*n*-butylcephalosporin, deacetoxy-*n*-butylcephalosporin, cephaloram and Δ^2 -cephaloram resulted in the precipitation of 2,4-dinitrophenylhydrazones. The 2,4-dinitrophenylhydrazones from cephaloram and Δ^2 -cephaloram were indistinguishable by t.l.c. in benzene–tetrahydrofuran and ethyl acetate–light petroleum from that formed from the

Table 3. *Thin-layer chromatography of 2,4-dinitrophenylhydrazones from degradation products of cephalosporin*

2,4-Dinitrophenylhydrazones were prepared from solutions of derivatives with $\lambda_{\max}$. 270nm obtained after aminolysis of cephalosporins with ammonia. T.l.c. was on Kieselgel G. The petroleum used had b.p. 80–100°C.

Compound	R_f			
	In benzene-tetrahydrofuran (3:2, v/v)	In benzene-tetrahydrofuran (3:7, v/v)	Ethyl acetate-petroleum (3:7, v/v)	Ethyl acetate-petroleum (7:3, v/v)
2,4-Dinitrophenylhydrazone of derivative from:				
Cephaloram	0.31	0.71	0.045	0.25
Δ^2 -Cephaloram	0.31	0.70	0.045	0.26
2,4-Dinitrophenylhydrazone of benzylpenaldamide	0.31	0.71	0.05	0.26
2,4-Dinitrophenylhydrazine	0.71	0.85	0.22	0.75
2,4-Dinitrophenol	0.76	—	—	0.49

Fig. 8. Conversion of compound with $\lambda_{\max}$. 230nm into compound with $\lambda_{\max}$. 270nm. Curve 1, Cephalosporin C; curve 2, after treatment of solution giving curve 1 with NH_3 (for details see Fig. 1); curve 3, after adjustment of solution giving curve 2 to pH 2.0; curve 4, after adjustment of solution giving curve 3 to pH 10.0.

amide of benzylpenaldic acid (Table 3). When a reaction mixture of cephalosporin C and alanine with $\lambda_{\max}$. 275nm (see the Materials and Methods section) was subjected to gel filtration on Sephadex G-10, the first two fractions with u.v. absorption to emerge had $\lambda_{\max}$. 280nm and the following three fractions had $\lambda_{\max}$. between 270 and 273nm.

Compounds showing $\lambda_{\max}$. at 270nm were made in two ways [by acidification either of solutions showing $\lambda_{\max}$. at 280nm (see Fig. 1), or of solutions showing $\lambda_{\max}$. at 230nm (see Fig. 8), followed by adjustment to pH 10.0], and the two solutions were mixed. The compounds could not be separated either on Sephadex G-10 or on electrophoresis at pH 9.0 followed by chromatography. It thus seems likely that the same compound is formed by the action of acid on either precursor.

When subjected to electrophoresis on paper at pH 9.0 the derivative with $\lambda_{\max}$. 280nm migrated towards the anode at approximately the same rate as cephalosporin C. After subsequent chromatography in a perpendicular direction in butan-1-ol-acetic acid-water it gave two ninhydrin-positive spots, one with $R_{A1a} < 0.1$, corresponding to that of the derivative with $\lambda_{\max}$. 270nm, and one corresponding to alanine. It had thus been converted into the derivative with $\lambda_{\max}$. 270nm and alanine by the acidic solvent system.

For a similar experiment the derivative with $\lambda_{\max}$. 280nm was obtained from a solution at pH 10.0 of cephalosporin C (0.38M) in glycine (1.9M) containing $11\mu\text{Ci}$ of $[1-^{14}\text{C}]$ glycine/ml. After 66h, its yield was about 70% of the theoretical value (based on the molar extinction of penamaldates and of cephalosporin C). Duplicate samples ($5\mu\text{l}$) of the solution were subjected to electrophoresis on paper at pH 9.0 followed by chromatography in butan-1-ol-acetic acid-water in the perpendicular direction. The R_f value of a radioactive ninhydrin-positive spot that had remained near the origin during electrophoresis showed that it was unchanged glycine. Two further radioactive ninhydrin-positive spots were derived from material that migrated as a single substance towards the anode (Fig. 9). One showed an R_f value corresponding to that of glycine. The second had a very low R_f value, and the corresponding material eluted with 0.1M-ammonia from the duplicate paper showed $\lambda_{\max}$. 270nm.

Reactions of cephalosporins with lysine polymers

When cephalosporin C reacted with polylysine or poly(lysine+serine) at pH 9.5 there was a fall in E_{260} over a period of 15min to about half the original value and a rise in E_{230} to about 0.7 times the original

Fig. 9. Two-way electrophoresis followed by chromatography of compound showing $\lambda_{\max}$ 280nm derived from reaction of [^{14}C]glycine with cephalosporin C applied at Y (for details see the text). Values in parentheses show radioactivity (c.p.m.) on paper. Spot 1, glycine marker applied at X after electrophoresis; spot 2, unchanged glycine; spot 3, glycine formed from precursor of compound in spot 4; spot 4, penaldete-type compound. Electrophoresis was in *N*-ethylmorpholine-acetate buffer, pH 8.7, at 14 V/cm for 2 h.

nal value of E_{260} . The increased E_{230} disappeared on acidification. A sample (0.2ml) of a reaction mixture of cephalosporin C and poly(lysine+serine) was passed through a column (23cm $\times$ 1cm) of Sephadex G-25 equilibrated with 10mM-tris buffer, pH 9.0, 2–3ml fractions being collected. Only fractions 1 and 2 showed a decrease in E_{230} after acidification, and unchanged cephalosporin C was eluted mainly in fractions 4 and 5. When the pH of acidified fractions 1 and 2 was adjusted to 8–10 there was no significant increase in E_{270} , nor was any such increase observed when reaction mixtures were kept until E_{230} had disappeared (24h). The derivative with $\lambda_{\max}$ 230nm obtained by the reaction of cephalosporin C (50mg/ml) with polylysine showed, undiluted, a half-life of about 100min (comparable with those for the derivatives formed by reactions with amino acids), but that obtained from poly(lysine+serine) was considerably more stable (half-life > 3h). A striking finding was that these half-lives were little altered by dilution of the reaction mixtures 1:1000.

The tryptic digest 'map' of polylysine (see the Materials and Methods section) showed three spots due to substances with a net positive charge at pH 4.5, which were identified as lysine, dilysine and a higher oligomer (see Waley & Watson, 1953; Miller, 1964). A similar 'map' of the reaction mixture between cephalosporin C and polylysine contained, in addition to these three spots, a spot due to

a substance with a net negative charge that was identified as unchanged cephalosporin C and a spot due to a substance (A) with virtually no net charge and with a very low R_F (<0.05). Substance A occupied a similar position after electrophoresis and chromatography as a sample of the derivative with $\lambda_{\max}$ 270nm obtained from the reaction mixture of cephalosporin C and lysine. The tryptic digest 'map' of poly(lysine+serine) showed spots due to serine, lysine and three peptides with a net positive charge. The 'map' of the reaction mixture between cephalosporin C and poly(lysine+serine) showed these five spots and also a spot that coincided with that given by substance A.

Deacetyl- and deacetoxy-cephalosporin C reacted with polylysine more slowly than did cephalosporin C (times for maximum fall in E_{260} were about 1 h and 3 h respectively) and there was no increase in E_{230} in either case. When the reaction mixtures were allowed to stand for 4 days at room temperature, acid-labile peaks with $\lambda_{\max}$ between 270 and 280nm appeared, with E about 0.6 times the original E_{260} value. This corresponded to a yield of penamaldete of about 30% of the theoretical value. After 4 days the reaction mixture gelled.

DISCUSSION

The behaviour on paper electrophoresis and Sephadex G-10 of the labile products with $\lambda_{\max}$ 230nm indicated that their formation when the β -lactam ring of a cephalosporin (II) was split by a β -lactamase or by aminolysis in aqueous solution involved no extensive fragmentation of the molecule. The production of formaldehyde on ozonolysis of these products suggested that they contained the group $=\text{CH}_2$ in place of the substituent $-\text{CH}_2\text{Y}$ (Y being a leaving group such as acetoxy or pyridinium) at C-3 in the cephalosporins. Hence a possible structure for the compounds with $\lambda_{\max}$ 230nm was (VII), this structure being formed by a concerted reaction as shown in Scheme 1. A structure of type (VII) was proposed by Eggers, Kane & Lowe (1965) as a hypothetical intermediate in the conversion of cephaloram into a 6H-1,3-thiazine derivative by treatment with sodium benzyloxide in benzyl alcohol.

The u.v.-absorption with $E_{\max}$ at 230nm did not obviously distinguish structure (VII) from alternative possibilities. However, enzymic hydrolysis of Δ^2 -cephaloram gave no support to a structure with a single double bond in the Δ^2 -position and thus a vinyl sulphide grouping, which had been considered by Newton *et al.* (1968). A structure with an exocyclic methylene and a saturated tetrahydrothiazine ring would contain an allyl sulphide grouping, which may show significant absorption at 230nm (Koch, 1949). But the formation of such a structure

Scheme 1.

on aminolysis would have involved a simultaneous nucleophilic attack at both C-8 and C-4 and was not consistent with the evidence that only one molecule of the amino compound took part in the reaction.

Structure (VII) accounted for the failure to detect the formation of compounds with $\lambda_{\max}$ 230nm on aminolysis of deacetyl- and deacetoxy-cephalosporins, since these substances have no leaving group at C-10 (R' in II). Measurements of u.v.-absorption spectra suggested that in both cases the immediate products of the reaction resembled the cephalosporins themselves in showing $\lambda_{\max}$ in the region of 260nm, but differed from them in having molecular extinctions that were only one-third to one-quarter as high. A similar behaviour, though with a change to $\lambda_{\max}$ 265nm, had previously been found with deacetylcephalosporin C lactone and had been attributed to the opening of the β -lactam ring without other changes in the molecule (Abraham & Newton, 1961). Subsequent work confirmed that the fused dihydrothiazine- γ -lactone ring system showed this type of u.v.-absorption spectrum (E. M. Wilson & A. G. Long, personal communication). Hence structures (VIII) and (IX) could be postulated for the first substances formed on aminolysis of deacetyl- and deacetoxy-cephalosporins respectively. Structure (VIII) is consistent with the formation of compounds 1 and 2 (Abraham & Newton, 1961) on acid hydrolysis of the compound concerned. Although the evidence presented here for structures (VII), (VIII) and (IX) is not conclusive, strong support for these structures has been

obtained from studies of proton-magnetic-resonance spectra (Hamilton-Miller *et al.* 1970).

The formation of compounds with structure (X), instead of (XI), when deacetylcephalosporins were hydrolysed by a β -lactamase and the inhibition of the formation of compounds of structure (X) by inorganic phosphate remain to be explained. One possibility is that the enzyme is instrumental in converting the hydroxyl group at C-10 into a leaving group. The failure of the products of enzymic hydrolysis to give rise to the penaldate and penamaldate type of compounds with $\lambda_{\max}$ 270 and 280nm respectively may be attributed to the loss of C-8 as carbon dioxide from structure (X), in a manner analogous to the conversion of a penicilloate into penilloate (Mozingo & Folkers, 1949).

Acidification of compounds assigned the structure (VII) led to the immediate fragmentation of the molecule and the formation of penaldates (V), which showed a characteristic $\lambda_{\max}$ 270nm when the solution was adjusted to pH 10.0 (Brown, 1949). When the solutions of the first products of aminolysis were not acidified penaldate formation occurred relatively slowly and after an interval ('eclipse phase') in which there was no selective absorption in the u.v. region. The nature of the compounds present in the eclipse phase has not been established. With structures of type (VII), at least, the reaction involved in their formation in concentrated solution appears to be a bimolecular one, like that leading to the formation of compounds 1 and 2 from cephalosporin C on acid hydrolysis (Abraham & Newton, 1961).

In the reaction mixture resulting from the aminolysis of cephalosporins with amino acids the formation of penaldates is accompanied by the liberation of ammonia, presumably from N-5. The formation of penaldates is followed by their conversion into penamaldyl derivatives (VI) with $\lambda_{\max}$ 280nm. Bradshaw, Eardley & Long (1968) reported that treatment of sodium cephaloram with

aniline at 80°C in an aqueous solution kept at pH 7.5 yielded a penamaldate (VI) in which $R = C_6H_5 \cdot CH_2$ and $R'' = H$, and in which the amino group was presumably derived from the nitrogen atom of the dihydrothiazine ring. Analysis of the penamaldyl derivatives formed in the presence of an excess of amino acid under the conditions used here showed that it was mainly the amino acid that entered into Schiff base formation with the aldehyde groups of the penaldate.

The reaction between cephalosporin C and lysine polymers followed the same general pattern as that between cephalosporin C and amino compounds of small molecular weight. However, the derivatives with λ_{max} 230nm obtained from ammonia or amino acids were more stable in dilute than in concentrated solutions, whereas the stability of those from the polymers was virtually unaffected by dilution. This may be attributed to steric factors that make it difficult for groups attached to the polymer molecules to engage in bimolecular reactions. Schwartz (1969) observed that polylysine catalyses the aminolysis of benzylpenicillin by tris, and suggested that the polymer can 'activate' the β -lactam ring.

The findings described here indicate that haptens resulting from the primary reaction of cephalosporins, deacetylcephalosporins and deacetoxycephalosporins with the amino groups of proteins will differ from each other and from the penicilloyl haptens derived from the penicillins in several respects other than in the nature of their *N*-acyl side chains. Deacetoxycephalosporins are likely to react less readily than cephalosporins or their deacetyl analogues. Conjugates formed from protein with cephalosporins would be expected to contain a Δ^4 -dihydrothiazine ring with an exocyclic methylene group at C-3 and those formed with deacetoxy- and deacetyl-cephalosporins to contain a Δ^3 -dihydrothiazine ring and a hydrogen and hydroxyl group respectively substituent at C-10.

The carboxylate ion at C-4 in conjugates from the cephalosporins, being adjacent to a double bond, might differ significantly in relative orientation from the corresponding carboxylate at C-3 in the penicilloyl haptens. The primary cephalosporin conjugates would be expected to have labile haptens whose fragmentation would eventually leave only the side chain and the carbon atoms of the β -lactam ring attached through C-8 to the protein.

J. M. T. H.-M. held a Medical Research Council Junior Research Fellowship during this work. We are indebted to Miss Anne Fairbairn for assistance and to the Medical Research Council and the National Research Development Corporation for financial support.

REFERENCES

- Abraham, E. P., Baker, W., Chain, E. B. & Robinson, R. (1949). In *The Chemistry of Penicillin*, p. 10. Ed. by Clark, H. T., Johnson, J. R. & Robinson, R. Princeton University Press.
- Abraham, E. P. & Newton, G. G. F. (1961). *Biochem. J.* **79**, 377.
- Batchelor, F. R., Dewdney, J. M. & Gazzard, D. (1965). *Nature, Lond.*, **206**, 362.
- Boxer, G. E. & Everett, P. M. (1949). *Analyt. Chem.* **21**, 670.
- Bradshaw, J., Eardley, S. & Long, A. G. (1968). *J. chem. Soc. C*, p. 801.
- Brown, E. V. (1949). In *The Chemistry of Penicillin*, p. 473. Ed. by Clarke, H. T., Johnson, J. R. & Robinson, R. Princeton University Press.
- Brownlee, K. A., Delves, C. S., Dorman, M., Green, C., Grenfell, E. & Smith, N. (1948). *J. gen. Microbiol.* **2**, 40.
- Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, 2nd ed. London: Crosby Lockwood.
- Dawson, R. M. C. & Elliott, W. H. (1959). In *Data for Biochemical Research*, p. 200. Ed. by Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. Oxford University Press.
- Egriwe, E. (1937). *Z. analyt. Chem.* **110**, 22.
- Eggers, S. H., Kane, V. V. & Lowe, G. G. (1965). *J. chem. Soc.* p. 1262.

- Ellman, G. L. (1959). *Archs Biochem. Biophys.* **82**, 70.
- Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
- Flynn, F. V. & de Mayo, P. (1951). *Lancet*, ii, 235.
- Hamilton-Miller, J. M. T. (1967). *Nature, Lond.*, **214**, 1333.
- Hamilton-Miller, J. M. T., Richards, E. & Abraham, E. P. (1970). *Biochem. J.* **116**, 385.
- Hamilton-Miller, J. M. T., Smith, J. T. & Knox, R. (1965). *Nature, Lond.*, **208**, 235.
- Jeffrey, J. d'A., Abraham, E. P. & Newton, G. G. F. (1961). *Biochem. J.* **81**, 591.
- Katz, A. M., Dreyer, W. J. & Anfinsen, C. B. (1959). *J. biol. Chem.* **234**, 2897.
- Koch, H. P. (1949). *J. chem. Soc.* p. 387.
- Loder, B., Abraham, E. P. & Newton, G. G. F. (1961). *Biochem. J.* **79**, 408.
- Miller, W. G. (1964). *J. Am. chem. Soc.* **86**, 3913.
- Mozingo, R. & Folkers, K. (1949). In *The Chemistry of Penicillin*, p. 535. Ed. by Clarke, H. T., Johnson, J. R. & Robinson, R. Princeton University Press.
- Newton, G. G. F. & Abraham, E. P. (1956). *Biochem. J.* **62**, 651.
- Newton, G. G. F., Abraham, E. P. & Kuwabara, S. (1968). *Antimicrobial Agents & Chemotherapy 1967*, p. 449.
- Pavlic, A. A. & Adkins, H. (1946). *J. Am. chem. Soc.* **68**, 1471.
- Rogers, H. J., Lomakina, N. & Abraham, E. P. (1965). *Biochem. J.* **97**, 579.
- Sabath, L. D., Jago, M. & Abraham, E. P. (1965). *Biochem. J.* **96**, 739.
- Schneider, C. H. & deWeck, A. L. (1965). *Nature, Lond.*, **208**, 57.
- Schwartz, M. A. (1968). *J. pharm. Sci.* **57**, 1209.
- Schwartz, M. A. (1969). *J. med. Chem.* **12**, 36.
- Trown, P. W., Abraham, E. P., Newton, G. G. F., Hale, C. W. & Miller, G. A. (1962). *Biochem. J.* **84**, 157.
- Waley, S. G. & Watson, J. (1953). *Biochem. J.* **55**, 328.
- Woodward, R. B., Neuberger, A. & Trenner, N. R. (1949). In *The Chemistry of Penicillin*, p. 415. Ed. by Clarke, H. T., Johnson, J. R. & Robinson, R. Princeton University Press.