REFERENCES

Abraham, E. P. & Newton, G. G. F. (1958). In *Ciba Foundation Symp., Amino Acids and Peptides with Antimetabolic Activity*, p. 205.

Abraham, E. P. & Newton, G. G. F. (1961). *Biochem. J.* **79**, 377.

Bell, P. H. & Roblin, R. O. (1942). *J. Amer. chem. Soc.* **64**, 2905.

Brownlee, K. A., Delves, C. S., Dorman, M., Green, C. A., Grenfell, E., Johnson, J. D. A. & Smith, N. (1948). *J. gen. Microbiol.* **2**, 40.

Eckman, B. (1948). *Acta chem. scand.* **2**, 383.

Felix, A. & Pitt, R. M. (1935). *J. Hyg., Camb.*, **35**, 428.

Goodall, R. R. & Levi, A. A. (1947). *Analyst*, **72**, 277.

Hale, C. W., Abraham, E. P. & Newton, G. G. F. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, no. 1–68, p. 10.

Hodgkin, D. & Maslen, E. N. (1961). *Biochem. J.* **79**, 393.

Jeffery, J. d'A., Abraham, E. P. & Newton, G. G. F. (1960). *Biochem. J.* **75**, 216.

Kodicek, E. & Reddi, K. K. (1951). *Nature, Lond.*, **168**, 475.

Rafter, G. & Colowick, S. P. (1954). *J. biol. Chem.* **209**, 773.

Warburg, O. & Christian, W. (1936). *Biochem. Z.* **285**, 297.

Biochem. J. (1961) **79**, 408

The Cephalosporin C Nucleus (7-Aminocephalosporanic Acid) and some of its Derivatives

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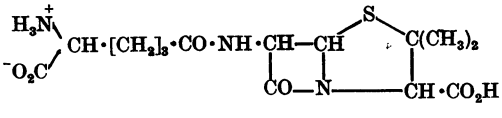
Cephalosporin N (I) (Newton & Abraham, 1954) may be regarded as a derivative of 6-aminopenicillanic acid (II) (Sheehan, Henery-Logan & Johnson, 1953) in which the 6-amino group of the latter is linked to the δ-carboxyl group of D-α-aminoadipic acid. Cephalosporin N has only about 1% of the antibacterial activity of benzylpenicillin against *Staphylococcus aureus* (Abraham, Newton & Hale, 1954).

Cephalosporin C (III) may be regarded as a derivative of 7-aminocephalosporanic acid (IV) in which the 7-amino group of the latter is linked to the δ-carboxyl group of D-α-aminoadipic acid (Abraham & Newton, 1961). Thus cephalosporin C and cephalosporin N have the same side chain, but different nuclei (II and IV respectively). Cephalosporin C has only about 10% of the activity of cephalosporin N against *Staph. aureus* (Newton & Abraham, 1956).

In view of these relationships it seemed probable that an analogue of cephalosporin C, which had a phenylacetyl group instead of a D-(δ-aminoadipoyl) group as a side chain, would be about 100 times as active as cephalosporin C and about one-tenth as active as benzylpenicillin against *Staph. aureus*. The preparation and *N*-acylation of 7-aminocephalosporanic acid provided one possible route to such an analogue.

In the course of work on the chemical structure of cephalosporin C, it became clear that this compound did not undergo a penicillin–penillic acid type of rearrangement and that its β-lactam ring was considerably more stable, under acid conditions, than the β-lactam ring of the penicillins.



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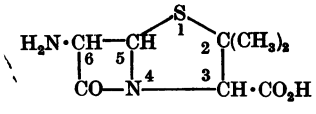

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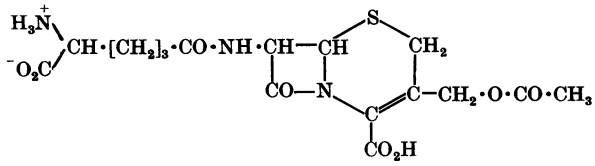

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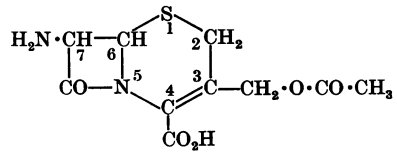
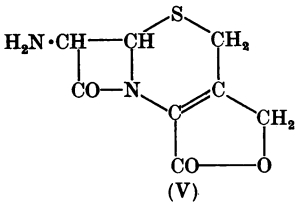


(IV)

This suggested that it might be possible to obtain the cephalosporin C nucleus (7-aminocephalosporanic acid, 7-ACA) by acid-catalysed removal of the side chain of cephalosporin C. The present paper reports evidence for the formation of the cephalosporin C nucleus, in small yield, and describes some of the properties of *N*-phenylacetyl and other *N*-acetyl derivatives of this nucleus. It also reports evidence for the formation of the nucleus of cephalosporin C_c (Abraham & Newton, 1961), which has been assigned the structure (V), and the nucleus of cephalosporin C_A (pyridine) (Hale, Newton & Abraham, 1961) which has been assigned the structure (VI).



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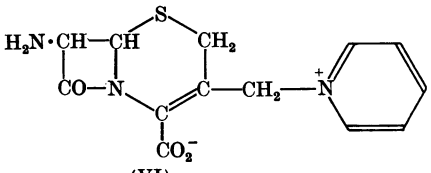


(VI)

After this work was projected, Batchelor, Doyle, Naylor & Rolinson (1959) reported the isolation of 6-aminopenicillanic acid (6-APA) from fermentations of *Penicillium chrysogenum* to which no side-chain precursor had been added. They found that 6-APA had only a weak antibacterial activity, but that it could be readily acylated to yield highly active penicillins. They also found that it had an isoelectric point of about 4·3. This low value for its isoelectric point is not surprising, since the 6-amino group is α to a lactam C=O and β to a thio ether grouping.

The structural relationship of 7-ACA and 6-APA suggested that the former would resemble the latter in having a very low antibacterial activity and a weakly basic amino group. We thus expected that 7-ACA would migrate as an acid on paper electrophoresis at pH 7, but not at pH 4, and that it would be detected on bioautographs only after acylation. The nucleus of cephalosporin C_c (Abraham & Newton, 1961) and that of cephalosporin C_A (pyridine) (Hale *et al.* 1961) were expected to be neutral at pH 7 and to migrate as bases at pH 4, since these two compounds would have one carboxyl group less and one strongly basic group more, respectively, than 7-ACA. The properties of certain products of acid hydrolysis of cephalosporin C, cephalosporin C_c and cephalosporin C_A (pyridine) corresponded so closely with these expectations that we became convinced that these products were, in fact, the desired compounds.

METHODS AND EXPERIMENTAL

Assay and unit of activity. Solutions were assayed by the hole-plate method with *Staph. aureus* (N.C.T.C. 6571) as the test organism. The unit is that used by Abraham *et al.* (1954). Cephalosporin C sodium salt has an activity of 8–10 u./mg.

Paper chromatography. Paper chromatograms were run at 20° on Whatman no. 1 paper in the following solvent systems: (*A*) butan-1-ol–ethanol–water (4:1:5, by vol.) (Batchelor *et al.* 1959); (*B*) ethyl acetate saturated with aqueous sodium acetate buffer (0·1M to Na), pH 5·2, with paper pretreated with the buffer (Hale, Miller & Kelly, 1953). The paper was soaked in the buffer, blotted, hung in a stream of air at room temperature, and used as soon as dry. With this system the solvent front reached the bottom of the paper in about 3 hr. but the solvent was allowed to run for 18 hr.

Paper electrophoresis. Paper electrophoresis on Whatman no. 1 paper (14v/cm., for 3 hr. unless stated otherwise) was carried out as described by Newton & Abraham (1954). The following buffer systems were used: collidine–acetate (0·05M to acetic acid), pH 7·0; pyridine–acetate (0·05 M to acetic acid), pH 4·0 or 4·5; 10% (v/v) acetic acid, pH 2·2; 10% (v/v) formic acid, pH 1·5.

Bioautographs. These were prepared as described by Hale *et al.* (1961).

Treatment with penicillinase on paper. In some cases spots of compounds on paper were sprayed with a solution of penicillinase in 1% (w/v) gelatin before the paper was used for the preparation of a bioautograph. The penicillinase used was a highly purified preparation of the induced enzyme from *Bacillus cereus* 569, kindly provided by Dr M. R. Pollock.

N-*Acylation of compounds on Whatman no.* 1 *paper.* Cephalosporin C, 7-ACA and related compounds were converted into their *N*-phenylacetyl derivatives on paper by a modification of the method of Batchelor *et al.* (1959). After paper chromatography or paper electrophoresis, the papers were dried at room temperature in a current of air. They were sprayed evenly with M-pyridine in 50% (v/v) acetone until they were barely damp. They were then lightly sprayed with 2% (w/v) phenylacetyl chloride in acetone, and again with the pyridine solution until a spot of bromocresol green, which had been placed on the paper, immediately turned blue (pH 5·0). After hanging in an air stream for 3–5 min. the papers were contacted with plates seeded with *S. aureus* (Oxford strain N.C.T.C. 6571). Other *N*-acetyl derivatives were prepared on paper and detected in a similar manner.

N-*Acylation of cephalosporin C and of its derivatives in solution* (*a*). A solution of cephalosporin C or one of its derivatives (1 equiv.) was neutralized (pH 5–6), if necessary, with $NaHCO_3$ solution. Further $NaHCO_3$ solution (4 equiv), which had been gassed with CO_2 to pH 7·0, was added to the solution. At this stage the concentration of the compound to be acylated was about 2% (w/v). Acetone (0·5 vol.) was then added, and the mixture was cooled to 0°.

Phenylacetyl chloride (1·2 equiv.), in 0·5 vol. of acetone, was added to the cooled stirred solution through a capillary over a period of 20 min. The pH of the mixture was maintained at 7·9 (glass electrode) by passing over it a slow stream of CO_2. After the phenylacetyl chloride had been added, the flask was corked, and the mixture was stirred at 0° for 20 min. and at room temperature for a further 30 min. The pH of the solution was checked at 10 min. intervals, and more CO_2 passed into the flask if necessary. Excess of bicarbonate was then neutralized with HCl (pH 5·5–6·0, glass electrode), and the acetone removed *in vacuo*. The pH of the aqueous residue was readjusted to 5–6.

(*b*) Small-scale acylations were carried out in aqueous pyridine. The compound to be acylated (4 mg.) was dissolved in a mixture of water (42 μl.) and pyridine (8 μl.). To a 10 μl. sample of this was added a solution of the acid chloride (1·5 mol.prop.) in acetone (10 μl.). The mixture was kept for 15 min. at room temperature and samples (5–10 μl.) of the mixture (or of the mixture diluted with water if necessary to yield suitable inhibition zones on bioautographs) were applied directly to paper for electrophoresis or chromatography.

Extraction of N-*acyl derivatives into butan*-1-*ol or* n-*butyl acetate.* The *N*-phenylacetyl derivatives of cephalosporin C, made by procedure (*a*), were extracted into butan-1-ol at pH 2·2, and re-extracted into aqueous solution at pH 6·5 in the manner described by Newton & Abraham (1954). The *N*-phenylacetyl derivative of 7-ACA made by procedure (*a*), was extracted in a similar manner into *n*-butyl acetate at pH 1·8 and re-extracted into aqueous solution at pH 5·0. The resulting aqueous solutions of the sodium salts were freeze-dried.

Reaction of 7-*aminocephalosporanic acid and its* N-*acyl derivatives with pyridine on paper.* The conversion of 7-ACA into the cephalosporin C_A (pyridine) nucleus and of *N*-acyl derivatives of 7-ACA into compounds of the C_A type could be demonstrated on paper. A strip of Whatman no. 1 paper, containing a spot of the substance studied (in sufficient concentration to give an inhibition zone of convenient size for a final bioautograph) was hung in a closed vessel for 18 hr. at 37° above the surface of aqueous 2M-pyridine adjusted to pH 7·0 with acetic acid. The spot was then subjected to electrophoresis or chromatography on the same paper. For 7-ACA the paper was sprayed with pyridine in 50% (v/v) acetone and then phenylacetyl chloride in acetone before bioautography.

Investigation of conditions for the hydrolysis of cephalosporin C and its derivatives with acid. Cephalosporin C free acid or cephalosporin C_c (50 mg.) (Abraham & Newton, 1961) was dissolved in 1·3 ml. of a solution of the acid used. The mixture was heated in a temperature-controlled bath at 20°, and samples (5 μl.) were removed at selected times for analysis directly by paper chromatography or by paper electrophoresis or both. Hydrolysis of cephalosporin C_A (pyridine) was carried out similarly, but 1·7 μl. samples of the resulting solutions were used for analysis.

Hydrolysis of cephalosporin C free acid (50 mg. in 1·3 ml. of water) was also carried out with various amounts (20–500 mg.) of Dowex 50X8 (H form; 200–400 mesh). At equilibrium the pH of the solution varied from 2·8 to 2·4. After the mixture had been kept at 20° for a given time (without stirring), its pH was brought to 5·0 with 8% (w/v) $NaHCO_3$ solution, and then to 6·5 with N-NaOH. The solution was freed from the resin and a sample (5 μl.) was analysed by paper electrophoresis.

Production of crude 7-*aminocephalosporanic acid.* Cephalosporin C sodium salt (2 g.) was dissolved in 30 ml. of water, the pH adjusted to 2·5 by addition of Dowex 50X8 (H form; 200–400 mesh) (about 2 g. of damp resin), and the resin filtered and washed with 10 ml. water. To the combined filtrate and washings, containing cephalosporin C free acid, was added 10·2 ml. of N-HCl. The solution was kept at 20° for 3 days and then added to a column (2.1 cm. diam. × 7 cm.) of Dowex 1X8 (200–400 mesh; acetate form). The eluate was collected in 5 ml. fractions (1–12) and the column was eluted with water until a total of 34 fractions had been collected. Elution was then begun with 0·5N-acetic acid and a further 66 fractions were collected. The extinction at 260 mμ was measured for each fraction.

Fractions 2–16 were pooled and concentrated *in vacuo*, when cephalosporin C_c (312 mg.) (Abraham & Newton, 1961) separated in crystalline form. Fractions 36–45 contained most of the 7-ACA as revealed by the formation of an active derivative when ten 10 μl. spots from each fraction were phenylacetylated on paper. These fractions were pooled and freeze-dried to yield a white powder (40 mg.). On phenylacetylation by procedure (*a*) 1 mg. of this material yielded 250 units of activity against *Staph. aureus*.

Purification of crude 7-*aminocephalosporanic acid by paper electrophoresis.* The crude 7-ACA obtained from the Dowex 1 (acetate) column (35 mg.) was further purified by electrophoresis in a Beckman Spinco Model CP continuous-flow paper-electrophoresis cell. The buffer used was made by adding pyridine to 0·05 N-acetic acid until the pH rose to 4·0. The cell was run at constant current (40 mA), the potential being 880 V. The sample, in 20 ml. of buffer, was fed to the curtain during 24 hr. and 32 fractions were collected, the fractions being numbered from cathode (1) to anode (32). The volume of each fraction was about 12 ml. At the end of the experiment the paper curtain was sprayed with ninhydrin. This revealed a strong band of material with very little mobility which flowed off the curtain in fractions 13–15, a band of strongly acidic material, which had flowed into the anode wick, and a faint band of a product (corresponding to α-aminoadipic acid) which had migrated towards the anode and flowed off the curtain mainly in fraction 24.

Fractions 22 and 23 contained the 7-ACA as revealed by the formation of an active derivative when ten 10 μl. spots were phenylacetylated on paper.

Freeze-drying of fraction 22 yielded a residue which was too small to be weighed accurately. In aqueous solution the ultraviolet-absorption spectrum of the product showed a plateau at 260 mμ, and its total weight (43 μg.) was estimated from the extinction at this wavelength on the assumption that the molecular weight of the substance was that of 7-ACA and that its molecular extinction was the same as that of cephalosporin C.

The product was dissolved in a mixture of 1 ml. of water and 0·5 ml. of 0·25 M-sodium phosphate buffer, pH 6·5. The solution was cooled to 0° and 1 ml. of a 4% (w/v) solution of phenylacetyl chloride in acetone was added slowly, with stirring, during 20 min. The mixture was kept for a further 20 min. at 0° and subsequently for 30 min. at room temperature. It was then diluted to 5 ml. with water and assayed against *Staph. aureus*. The assay indicated that 1 mg. of the product yielded 1450 units of activity on phenylacetylation.

Table 1. *Results from bioautographs prepared after electrophoresis*

Electrophoresis was at 14v/cm. (except formic acid 10v/cm.) for 3 hr. Distance travelled from origin is expressed as + or − to indicate movements to anode or cathode. Glucose was revealed by spraying with 2% (w/v) aniline hydrogen phthalate in butanol-saturated water, and leucine, α-aminoadipic acid and glycine by ninhydrin.

Compound	Distance travelled (cm.) from origin in buffer: Collidine–acetate pH 7·0	Pyridine–acetate pH 4·5	Pyridine–acetate pH 4·0	Formic acid pH 1·5	Acetic acid pH 2·2
Cephalosporin C	+5·2	+7·5	+7·2	−3·2	−0·4
N-Phenylacetylcephalosporin C	+8·2	+12·0	—	—	—
Cephalosporin C_c	−1·5	−2·0	—	−4·0	−5·0
N-Phenylacetylcephalosporin C_c	+3·4	—	—	—	—
Cephalosporin C_A (pyridine)	−1·5	—	−2·9	—	−4·7
Cephalosporin C_A (pyridine) nucleus	−1·5	—	−9·8	—	−9·9
Cephalosporin C_c nucleus	−1·5	−4·5	—	—	−13·1
Penicillin nucleus (6-APA)	+7·5	+2·1	0·0	−6·5	−10·0
Cephalosporin C nucleus (7-ACA)	+7·5	+3·4	+1·0	−4·5	−4·0
N-Phenylacetyl derivative of 6-APA (benzylpenicillin)	+5·2	+7·5	—	—	—
N-Phenylacetyl derivative of 7-ACA	+5·1	+7·5	—	—	—
Glycine	−1·5	—	—	−10·2	−14·1
Leucine	−1·5	−2·0	—	−7·4	−10·7
α-Aminoadipic acid	—	+4·0	—	−6·0	−7·8
Glucose	−1·5	−2·0	—	—	−1·0

(Cephalosporin C sodium salt shows an activity of 8–10u./mg.) On the basis of this value the overall yield of 7-ACA obtained after chromatography on Dowex 1 was 0·6% of that theoretically possible. However, the overall yield of 7-ACA isolated after continuous paper electrophoresis was only about 0·006%. Hence, a large proportion of the product was lost during the latter process.

Preparations of 7-ACA obtained in this way still contained a small amount of α-aminoadipic acid. They could be freed from α-aminoadipic acid by electrophoresis on paper in 10% (v/v) acetic acid, pH 2·2, and elution from the paper with 0·1N-acetic acid. Under these conditions of electrophoresis 7-ACA migrated towards the cathode about half as fast as α-aminoadipic acid.

RESULTS

Products of hydrolysis of cephalosporin C and cephalosporin C_c. Bioautographs, made after paper electrophoresis (Table 1) and paper chromatography (Table 2), of solutions obtained by keeping cephalosporin C free acid in dilute HCl at 20° showed the presence of two active compounds. When the paper was sprayed with phenylacetyl chloride two further active compounds were revealed. The first two compounds corresponded with unchanged cephalosporin C and cephalosporin C_c. The third had the properties expected of 7-ACA and the fourth had the properties expected of the nucleus of cephalosporin C_c. The fourth compound was indistinguishable from a compound found to be present in hydrolysates of cephalosporin C_c.

Products of hydrolysis of cephalosporin C_A (pyridine). Hydrolysates obtained by treating cephalosporin C_A (pyridine) with dilute HCl at 20° contained unchanged cephalosporin C_A and a second compound whose activity was greatly increased by phenylacetylation. The second compound had the properties expected of the nucleus of cephalosporin C_A (pyridine).

Table 2. *Results from bioautographs prepared from paper chromatograms*

Paper chromatograms were run with butan-1-ol–ethanol–water (4:1:5, by vol.).

Compound	R_F
Cephalosporin C	0·04
N-Phenylacetylcephalosporin C	0·13
Cephalosporin C_c	0·09
N-Phenylacetylcephalosporin C_c	0·23
Cephalosporin C_A (pyridine)	0·00
Cephalosporin C_A (pyridine) nucleus	0·07
Cephalosporin C_c nucleus	0·26
Penicillin nucleus (6-APA)	0·14
Cephalosporin C nucleus (7-ACA)	0·14
N-Phenylacetyl derivative of 6-APA (benzylpenicillin)	0·45
N-Phenylacetyl derivative of 7-ACA	0·40

Variation in the composition of hydrolysates of cephalosporin C and cephalosporin C_A with experimental conditions. Table 3 indicates that, for a given concentration of aqueous acid, the concentration of cephalosporin C gradually declines, and that 7-ACA, cephalosporin C_c, and cephalosporin C_c nucleus successively reach a maximum concentration. The amounts of 7-ACA and cephalosporin C_c then decline in turn. Table 4 indicates that, when hydrolysis was catalysed by Dowex 50 (H form),

Table 3. *Products of hydrolysis of cephalosporin C and cephalosporin C_c*

Hydrolysis was carried out at 20° with various amounts of HCl or, in one case, toluene-*p*-sulphonic acid (for details see Methods). The amount of acid used is stated in terms of equiv./mole of cephalosporin C. Samples of the hydrolysate were subjected to paper electrophoresis or chromatography to separate products of hydrolysis, and the papers then sprayed with a solution of phenylacetyl chloride (for details see Methods). Bioautographs were prepared (after phenylacetylation) with *Staph. aureus* as the test organism. The number of crosses gives an indication of the relative sizes of inhibition zones.

	Conditions of hydrolysis		Size of inhibition zone on bioautograph attributed to phenylacetyl derivative of			
Substance	HCl (equiv./mole)	Time (hr.)	Cephalosporin C	7-ACA	Cephalosporin C_c	Cephalosporin C_c nucleus
Cephalosporin C	1·1	24	+ + + +	+	+	−
		48	+ + +	+ +	+	−
		72	+ + +	+ +	+ +	−
		96	+ +	+ +	+ +	−
		120	+	+	+ + +	−
	2·1	16	+ + + +	+ +	+	−
		42	+ + +	+ + +	+	+
		72	+ +	+ + + +	+ +	+
		90	+	+ +	+ + +	+ +
	3·1	42	+	+ +	+ + +	+ +
		72	−	+	+ + +	+ +
		90	−	±	+ + +	+ +
	4·1	42	±	+	+ + +	+ + +
		72	−	±	+ + +	+ + +
		90	−	−	+ +	+ + +
	5·5	16	+ +	+ + +	+ +	+ +
		40	±	+	+ + +	+ + +
		64	−	±	+ +	+ + +
	11·0	18	+ +	+ +	+ + +	+ + +
		24	+	+ +	+ +	+ + +
		40	−	±	+	+ + + +
		72	−	−	+	+ + + +
	1·2*	15	+ + + +	+	+	−
		87	+ + +	+ + +	+ +	−
Cephalosporin C_c	1·1	42	.	.	+ + + + +	−
	6·0	18	.	.	+ + +	+ + +
		90	.	.	+ +	+ + +
	11·0	18	.	.	+ + +	+ + + +

* Toluene-*p*-sulphonic acid.

Table 4. *Products of hydrolysis of cephalosporin C*

Hydrolysis was carried out at 20° with Dowex 50X8 (200–400 mesh; H form) (for details see Methods). The amount of Dowex 50 used is stated in terms of mg. of damp resin/50 mg. of cephalosporin C. The pH values of the solutions at equilibrium varied from 2·8 to 2·4. Hydrolysates were analysed as described for Table 3.

Conditions of hydrolysis		Size of inhibition zone on bioautograph attributed to phenylacetyl derivative of			
Amount of resin (mg.)	Time (hr.)	Cephalosporin C	7-ACA	Cephalosporin C_c	Cephalosporin C_c nucleus
20	46	+ + + +	+	+	+
40	46	+ + +	+ +	+ +	+ +
100	15	+ +	−	+ +	+ + +
	46	+	−	−	+ + +
500	36	−	−	−	+ + +

Table 5. *Products of hydrolysis of cephalosporin C_A (pyridine)*

Hydrolysis was carried out at 20° with various amounts of HCl (for details see Methods). The amount of acid used is stated in terms of equiv./mole of cephalosporin C_A. Hydrolysates were analysed as described for Table 3.

Conditions of hydrolysis		Size of inhibition zone on bioautograph attributed to phenylacetyl derivatives of	
HCl (equiv.)	Time (hr.)	Cephalosporin C_A (pyridine)	Cephalosporin C_A (pyridine) nucleus
1·2	72	+ + + + + + + +	+ + + + + + + +
	400	+ + + + + +	+ + + + + +
11	16	+ + + + + +	+ + + + + +
	72	+ + + + + +	+ + + + + +
	400	+ + +	+ + + + +

cephalosporin C_c nucleus was formed much more rapidly than in a solution of HCl having a similar pH. Table 5, when compared with Table 3, indicates that cephalosporin C_A (pyridine) and cephalosporin C_A (pyridine) nucleus were considerably more stable in aqueous acid than cephalosporin C and 7-ACA respectively.

Estimations of the activity of solutions obtained by hydrolysing cephalosporin C for 72 hr. with 2·1 equiv. of acid (see Table 3) were made by the hole-plate method before and after phenylacetylation of the solutions. Before phenylacetylation 20 % of the original activity remained. Bioautographs made after paper electrophoresis indicated that about half of this activity was due to unchanged cephalosporin C and half to cephalosporin C_c. After phenylacetylation the activity of the hydrolysate was about 125 % of that of the original solution of cephalosporin C. Phenylacetylation of cephalosporin C and cephalosporin C_c under similar conditions resulted in an approximately twofold increase in activity. Hence the total activity of the phenylacetyl derivative of the 7-ACA formed on hydrolysis was about 85 % of that of the original cephalosporin C. On the assumption that 1 mg. of 7-ACA gives 1450 units of activity on phenylacetylation (see Experimental section) the amount of 7-ACA formed under the conditions used was 0·93 % of that theoretically possible.

Properties of 7-aminocephalosporanic acid in relation to those of 6-aminopenicillanic acid

A sample (50 μg.) of 7-ACA that had been finally purified by electrophoresis on paper at pH 2·2 yielded no detectable amount of α-aminoadipic acid on hydrolysis with N-HCl at 105° for 16 hr. Paper chromatography of the hydrolysate showed a weak ninhydrin-positive spot, corresponding to glycine, which was similar in intensity to a spot in the same position obtained from a hydrolysate of 50 μg. of cephalosporin C.

With ninhydrin 7-ACA gave a brownish-yellow colour on paper, similar to that given by 6-APA. In both cases the colour later changed to a grey–blue. Unlike 6-APA, however, 7-ACA could be detected on paper as a dark (light-absorbing) spot by placing the paper before a source of ultraviolet light (230–400 mμ). The inhibition zone obtained on a bioautograph after 7-ACA had been phenylacetylated on paper was undiminished when the spot of 7-ACA was first sprayed with a solution of purified penicillinase in ten times the concentration required to inactivate a similar amount of benzylpenicillin.

7-ACA migrated towards the anode at almost the same rate as 6-APA, but faster than cephalosporin C, on paper electrophoresis at pH 7·0. At pH 4·5 it migrated about half as fast as cephalosporin C and somewhat faster than 6-APA. At pH 4·0 both 7-ACA and 6-APA showed only a small mobility towards the anode, but the former moved significantly further than the latter. On paper electrophoresis at pH 2·2, 7-ACA moved towards the cathode at about half the rate of 6-APA or α-aminoadipic acid, and less rapidly than aspartic acid. At pH 1·5, however, 7-ACA migrated 0·7 times as fast towards the cathode as 6-APA.

On paper chromatography in butan-1-ol–ethanol–water, 7-ACA, 6-APA, and *N*-phenylacetyl cephalosporin C showed very similar R_F values.

7-ACA, unlike 6-APA, was partly transformed, when exposed on paper to the vapour of 2M-pyridine–acetate, pH 7, to a compound that showed no net charge on electrophoresis at pH 7·0. The new compound was detected by its activity after phenylacetylation on paper. It was indistinguishable, on paper electrophoresis at pH values between 2·2 and 7·0 and on paper chromatography in butan-1-ol–ethanol–water, from the product of acid hydrolysis of cephalosporin C_A (pyridine) that was assumed to be the cephalosporin C_A (pyridine) nucleus.

N-Acyl derivatives of 7-aminocephalosporanic acid

Spots of 7-ACA on paper were converted by treatment with a number of different acid chlorides into derivatives which showed activity against

Staph. aureus. The sizes of inhibition zones on bioautographs suggested that the activity of the phenoxyacetyl derivative was similar to that of the phenylacetyl derivative but that the activities of the benzoyl, *iso*butyryl, propionyl and acetyl derivatives were of a lower order. All these derivatives migrated towards the anode at a rate similar to that of cephalosporin C when subjected to electrophoresis on paper at pH 4·5 or pH 7·0. The phenylacetyl derivative was readily extractable into butyl acetate from an aqueous solution at pH 1·8.

The phenylacetyl, phenoxyacetyl, and propionyl derivatives of 7-ACA were separated from each other, and from the corresponding derivatives of 6-APA, when chromatographed on paper in an ethyl acetate–sodium acetate buffer system. The R_F values of the different compounds relative to that of benzylpenicillin (the *N*-phenylacetyl derivative of 6-APA) are given in Table 6. The R_F values of both series of derivatives increase in the order propionyl, phenylacetyl, phenoxyacetyl to a similar relative extent, but the R_F of each derivative of 7-ACA is considerably lower than that of the corresponding derivative of 6-APA.

Spots on paper of the phenylacetyl and phenoxyacetyl derivatives of 7-ACA were exposed to the vapour of aqueous pyridine–acetate at 37° for 24 hr. Electrophoresis and bioautography (with *Staph. aureus* as the test organism) then revealed the presence of two compounds which showed no net charge at pH 7·0, in addition to the original derivatives. A similar result was obtained when the phenylacetyl derivative of 7-ACA was kept in 2M-pyridine (pH 7) for 48 hr. at 37° and then subjected to electrophoresis. It thus appeared that the derivatives had been partly transformed to pyridinium compounds of the cephalosporin C_A type. However, the areas of the inhibition zones produced by the new compounds of the C_A type were about 0·3 times as large as those produced by the remaining phenylacetyl and phenoxyacetyl derivatives of 7-ACA. In comparable experiments with cephalosporin C the inhibition zone produced by cephalosporin C_A was about twice as large as that produced by the remaining parent compound.

Under the conditions used no inhibition zones were produced by the phenylacetyl derivative of 7-ACA, or by the C_A compound formed from it, when attempts were made to obtain bioautographs with *Salmonella typhi* as the test organism. With *Staph. aureus* as the test organism the size of the inhibition zone produced by a spot on paper of about 10 μg. of the phenylacetyl derivative of 7-ACA was not significantly changed when the paper was sprayed, before testing, with a solution of penicillinase which completely inactivated a 50 μg. spot of benzylpenicillin.

Table 6. *Comparison of derivatives of 7-aminocephalosporanic acid and 6-aminopenicillanic acid on paper chromatography*

Chromatograms were run in an ethyl acetate–sodium acetate buffer system, pH 5·2 (for details see Methods). Spots were revealed on bioautographs, *Staph. aureus* being the test organism.

Derivative	$R_{benzylpenicillin}$ for derivative of 7-ACA	$R_{benzylpenicillin}$ for derivative of 6-APA
Propionyl	0·18	0·43
Phenylacetyl	0·46	1·00
Phenoxyacetyl	0·51	1·07
α-Phenoxypropionyl	0·78	1·28

Properties of the cephalosporin C_c nucleus and cephalosporin C_A (pyridine) nucleus

The cephalosporin C_c nucleus and the cephalosporin C_A (pyridine) nucleus behaved as though they had no net charge when subjected to electrophoresis on paper at pH 7·0, but migrated towards the cathode at pH 4·5, pH 4·0 and pH 2.2. At pH 7·0 they were not resolved from each other, but at pH 2·2 the cephalosporin C_c nucleus migrated further towards the cathode than the cephalosporin C_A nucleus. The compounds were readily distinguished on chromatograms run with butan-1-ol–ethanol–water.

The activity of the cephalosporin C_c nucleus and the cephalosporin C_A (pyridine) nucleus (formed from 400 μg. and 40 μg. respectively of cephalosporin C) was not affected significantly, on paper, by a solution of penicillinase which completely destroyed 50 μg. of benzylpenicillin under similar conditions.

The cephalosporin C_c nucleus did not appear to react with pyridine to give a derivative of the C_A type.

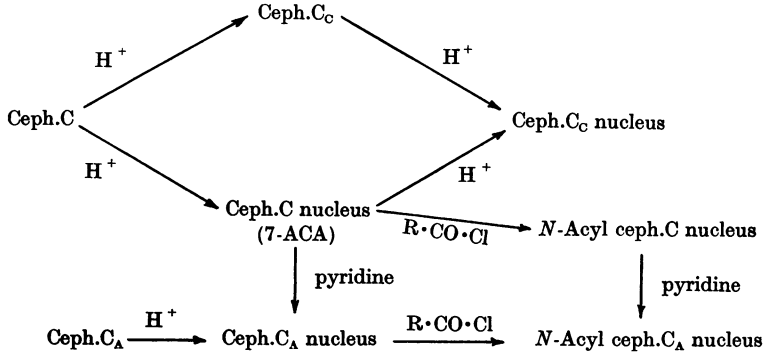
DISCUSSION

Some of the products that are formed when cephalosporin C or cephalosporin C_A (pyridine) is treated with dilute acid at room temperature appear to be related according to Scheme I (p. 415). The cephalosporin C_c nucleus is a relatively stable end product than can be formed either from 7-ACA or from cephalosporin C_c. The cephalosporin C_A nucleus can be formed from 7-ACA by reaction with pyridine or from cephalosporin C_A by hydrolysis.

The yield of 7-ACA, at least, was very small under the conditions used and the formation of this compound would have been difficult to detect, had it not given a highly active *N*-phenylacetyl derivative. The susceptibility of the acetoxy group in both cephalosporin C and 7-ACA to acid-cata-

lysed displacement places a serious obstacle in the way of attempts, by methods of the type described in this paper, to obtain substantial amounts of 7-ACA from cephalosporin C. It is possible that these methods would be satisfactory, however, for the preparation of the nucleus of cephalosporin C_c and that of cephalosporin C_A.

which increased in the order, propionyl, phenylacetyl and phenoxyacetyl, and the size of inhibition zones indicated that the activity of the *N*-propionyl and *N*-benzoyl derivatives against *Staph. aureus* was much less than that of the *N*-phenylacetyl and *N*-phenoxyacetyl derivatives. Similar relationships were obtained with the corresponding



Scheme I

Preliminary experiments on the purification of 7-ACA gave a product whose *N*-phenylacetyl derivative was about 100 times as active as cephalosporin C against *Staph. aureus*. This product yielded no α-aminoadipic acid on hydrolysis and showed, like 6-APA, an atypical ninhydrin reaction on paper. Neither 7-ACA, nor the nucleus of cephalosporin C_c or of cephalosporin C_A, was isolated in crystalline form. Nevertheless, the properties of these products and their acyl derivatives, as revealed by paper electrophoresis, paper chromatography and bioautography, formed a self-consistent picture which could not be accounted for in terms of alternative structures. The hypothesis was considered that the substance now assumed to be 7-ACA was the corresponding deacetyl compound, but this was rejected on two counts. First, the substance reacted with pyridine to form a derivative of the cephalosporin C_A type; cephalosporin C reacts with pyridine in this way (Hale *et al.* 1961), but deacetylcephalosporin C does not (J. d'A. Jeffery, G. G. F. Newton & E. P. Abraham, unpublished work). Secondly, the substance was formed under acid conditions in which, by analogy with deacetylcephalosporin C, deacetyl-7-ACA would be expected to undergo rapid lactonization to the nucleus of cephalosporin C_c (J. d'A. Jeffery, G. G. F. Newton & E. P. Abraham, unpublished work).

In a number of its properties 7-ACA showed a close similarity to 6-APA. This resemblance extended to the *N*-acetyl derivative of the two compounds. When chromatographed on paper in an ethyl acetate–sodium acetate buffer system the *N*-acyl derivatives of 7-ACA showed R_F values *N*-acyl derivatives of 6-APA. However, the behaviour of 7-ACA and 6-APA on paper electrophoresis at pH values between 4·5 and 2·2 indicated that the carboxyl group of the former was significantly stronger than that of the latter. This difference in the pK_a of the carboxyl group may be partly responsible for the fact that the R_F values of *N*-acyl derivatives of 7-ACA in ethyl acetate–sodium acetate buffer were considerably lower than the R_F values of the corresponding derivatives of 6-APA. The *N*-phenylacetyl derivative of 7-ACA also differed from the corresponding derivative of 6-APA (benzylpenicillin) in being relatively insensitive to purified penicillinase from *B. cereus*. Stability to penicillinase is therefore not destroyed when the δ-(α-aminoadipoyl) side chain of cephalosporin C is exchanged for a non-polar grouping.

SUMMARY

1. Treatment of cephalosporin C with dilute acid, at room temperature, has produced, in small yield, a compound whose properties correspond with those of the cephalosporin C nucleus (7-aminocephalosporanic acid). This compound is formed by the hydrolytic removal of the D-(δ-aminoadipoyl) side chain of cephalosporin C.

2. 7-Aminocephalosporanic acid showed no antibacterial activity under the test conditions used, but formed active *N*-acyl derivatives. A purified sample of 7-aminocephalosporanic acid yielded an *N*-phenylacetyl derivative whose activity against *Staphylococcus aureus* was about 100 times that of cephalosporin C.

3. Treatment of cephalosporin C with dilute acid also yields the nucleus of cephalosporin C_c. The formation of this compound involves hydrolysis of an acetoxy group and subsequent lactonization, as well as removal of the side chain. The same compound is formed on hydrolysis of cephalosporin C_c.

4. Hydrolysis of cephalosporin C_A (pyridine) in dilute acid yields the nucleus of cephalosporin C_A (pyridine). This nucleus is also formed when 7-aminocephalosporanic acid reacts with aqueous pyridine.

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REFERENCES

Abraham, E. P. & Newton, G. G. F. (1961). *Biochem. J.* **79**, 377.
Abraham, E. P., Newton, G. G. F. & Hale, C. W. (1954). *Biochem. J.* **58**, 94.
Batchelor, F. R., Doyle, F. P., Naylor, J. H. C. & Rolinson, G. N. (1959). *Nature, Lond.*, **183**, 257.
Hale, C. W., Miller, G. A. & Kelly, B. K. (1953). *Nature, Lond.*, **172**, 545.
Hale, C. W., Newton, G. G. F. & Abraham, E. P. (1961). *Biochem. J.* **79**, 403.
Newton, G. G. F. & Abraham, E. P. (1954). *Biochem. J.* **58**, 103.
Newton, G. G. F. & Abraham, E. P. (1956). *Biochem. J.* **62**, 651.
Sheehan, J. H., Henery-Logan, K. R. & Johnson, D. A. (1953). *J. Amer. chem. Soc.* **75**, 3292.

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Studies on Vitamin A Esterase

4. THE HYDROLYSIS AND SYNTHESIS OF VITAMIN A ESTERS BY RAT INTESTINAL MUCOSAE*

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Most of the work on vitamin A esterase has been on the hydrolysis of the esters; studies on the esterification are very few. In a preliminary communication High, Bright & Powell (1956) have reported esterification of vitamin A by small intestine and kidneys of rats. Krinsky (1958) has made a rather detailed investigation of esterification of vitamin A by cattle retina. While the present work was in progress, Pollard & Bieri (1960) reported their experiments on the esterification of vitamin A by acetone-dried pancreas of rat and chick.

Since the work of Gray & Cawley (1942) and of Clausen (1943) it has been generally assumed that vitamin A esters undergo hydrolysis and re-esterification during the process of absorption from the small intestine. More recently we have demonstrated that irrespective of whether vitamin A is given orally to rats as the free alcohol, as its acetate or as its palmitate, the esters found in the intestinal muscles, mesenteric lymph, blood and liver are invariably those of higher fatty acids (Mahadevan, Krishnamurthy & Ganguly, 1959), thus supporting the possibility of hydrolysis and re-esterification in the intestine during absorption.

It is demonstrated in this report that the mucosae of the small intestine of rats show hydrolytic and esterifying activities towards the vitamin. Some of the general properties of these activities are described and it is indicated that the esterification of vitamin A is apparently independent of coenzyme A and adenosine triphosphate requirements.

* Part 3. Krishnamurthy, Seshadri Sastry & Ganguly (1958).

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MATERIALS AND METHODS

Materials. Sodium taurocholate and palmitic acid were obtained from British Drug Houses Ltd. The palmitic acid was recrystallized from ethanol before use. Coenzyme A (CoA), *p*-chloromercuribenzoate and the disodium-trihydrate salt of adenosine 5′-triphosphate (ATP) were from Sigma Chemical Co. Tween 20 (polyoxyethylene sorbitan monolaurate) was from the Atlas Powder Co., Wilmington, Delaware, U.S.A., and diethylamine acetarsol (Acetylarsan) from May and Baker Ltd., Bombay, India. Di*iso*propyl phosphorofluoridate (DFP), tetraethyl pyrophosphate (TEPP) and diethyl *p*-nitrophenyl phosphate (E–600) were kindly procured for us by Dr S. Y. Thompson of the