there may be some variations in the relative amounts during the development of the petals, it is obvious that lycopene is the main carotene, and that flavoxanthin and chrysanthemaxanthin the main xanthophylls.

DISCUSSION

The examination of *C. officinalis* petals confirms two generalizations which have been made with regard to carotenoid distribution in petals (Goodwin, 1952*c*); (*a*) that 5:8-epoxides (flavochrome, mutatochrome, aurochrome, flavoxanthin and chrysanthemaxanthin) are widely distributed in petals in relatively large amounts and (*b*) that lutein, although present is not, as in green leaves, the major xanthophyll. Furthermore, the investigation adds emphasis to the recent observations made on berries (Goodwin, 1953, and unpublished observations) that when examined with the aid of present knowledge concerning separation and identification of trace carotenoids, the pigment mixture is found to be much more complex than was originally thought. This is mainly due to the fact that the pioneer investigators were mainly interested in isolating the major pigments in order to examine them chemically. The biological significance of these complex mixtures is at present quite obscure.

The present investigation confirms the original observation of Zechmeister & Cholnoky (1932) and Kuhn & Grundmann (1934) on the major pigments present, except that violaxanthin was not detected and although a 'rubixanthin-like' pigment was observed, rubixanthin itself was not detected. These differences may be varietal.

SUMMARY

1. The following hydrocarbon polyenes have been found in the petals of a 'dark' variety of *Calendula officinalis* (pot marigold): phytofluene, β-carotene, γ-carotene, ζ-carotene and lycopene. Pigment *X* closely associated with ζ-carotene, was also present. A poly-*cis*-lycopene was also present; it was probably prolycopene I.

2. The xanthophylls present were flavochrome, mutatochrome, lutein, flavoxanthin and chrysanthemaxanthin; traces of neoxanthin and aurochrome are also probably present. Two pigments were unidentified; pigment 6 was similar to, but not identical with rubixanthin.

3. The relative distribution of these pigments in the petals is recorded.

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Purification and some Properties of Cephalosporin N, a New Penicillin

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A species of *Cephalosporium* isolated by Brotzu (1948) from a sewage outfall off Sardinia can produce at least six different antibiotics (Burton & Abraham, 1951; Crawford *et al.* 1952). One of these substances, named cephalosporin N, has been reported to be a new type of penicillin, distinguished clearly from the penicillins previously encountered by its hydrophilic character and by an activity of the same order of magnitude against a number of Gram-positive and Gram-negative bacteria (Abraham, Newton, Crawford, Burton & Hale, 1953). An account of some of the biological properties of the substance has been given by Heatley & Florey (1953). This paper describes a process whereby cephalosporin N

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can be obtained from culture fluid of the *Cephalosporium* in a form which appears to be at least 75% pure. The production of the active culture fluid is described by Kelly & Miller (1954) and by Hale (1954). The chemical structure of the antibiotic is discussed in the following paper (Newton & Abraham, 1954).

Early experiments established that cephalosporin N was an acidic substance which was rapidly inactivated by dilute acid or alkali, by certain heavy metal ions, and by the enzyme penicillinase. The instability of the substance imposed limitations on the methods available for its purification which were similar to those encountered with the common penicillins. Unlike the latter, however, cephalosporin N could not be extracted from an aqueous solution by organic solvents such as amyl acetate, or butanol, and a different process was therefore required for removing it from the culture fluid.

The process of purification consisted of three main stages: adsorption on a column of charcoal (pH 6) and elution with aqueous acetone; chromatography on acid-washed alumina from aqueous acetone; and countercurrent distribution at 3° in a system composed of water, phenol, carbon tetrachloride and 2:4:6-trimethylpyridine sulphate at pH 6·1. Antibacterial activities were expressed in terms of an arbitrary unit which is described by Kelly & Miller (1954). Good yields were only obtained from charcoal and alumina columns if the amount of adsorbent was not greatly in excess of that required to remove all the active substance. Some irreversible changes occurred and became serious when the adsorbent was present in large excess. The displacement of certain components by others on the columns evidently played an important part in the separation that was achieved.

In a simple phenol–water system the partition coefficient of cephalosporin N was large enough for the convenient use of countercurrent distribution only at a pH lower than that at which the antibiotic was stable. Addition of 2:4:6-trimethylpyridine to the mixture, however, greatly increased the relative solubility of the antibiotic in the organic phase when the pH of the aqueous phase was 6·1. In the system used for countercurrent distribution 2:4:6-trimethylpyridine acted as both a carrier and a buffer, and it could finally be removed under conditions which did not cause inactivation of cephalosporin N. Such systems may well prove to be of value for the separation of other acidic hydrophilic substances.

Cephalosporin N was also subjected to countercurrent distribution in a system composed of ethanol, *sec.*-butanol and a concentrated aqueous solution of ammonium sulphate at pH 6·0, but this system was not used in preparative work, because of the subsequent difficulty of isolating the product in a salt-free form.

Many of the chemical properties of cephalosporin N were analogous to those of other penicillins. Chemical methods of assaying the latter could therefore be applied to the former. Assuming a value for the molecular weight of cephalosporin N these assays provided an estimate of the degree of purity of the preparations obtained.

RESULTS

Purification of cephalosporin N

Adsorption on and elution from charcoal. The active culture fluid was filtered and its pH adjusted to 6·0. It was then percolated down a column packed with granulated charcoal prepared and buffered at pH 6 as described in the Experimental section. Elution was carried out with 60% (v/v) acetone. The total amount of cephalosporin N eluted was 97% of that in the culture fluid. One fraction of the eluate contained 69% of the cephalosporin N in one-fifteenth of the volume of the culture fluid. The activity of the material in this fraction was 4 units/mg. Any cephalosporin P which was present in the culture fluid (Burton & Abraham, 1951) remained adsorbed on the charcoal, but could be removed by elution with an alkaline aqueous solution of acetone.

Chromatography on alumina. Column 1. Acetone was added to selected fractions of the eluate from the charcoal column until its concentration was 70% (v/v) and the pH of the resulting solution was adjusted to 6·0. A small amount of precipitate of low activity came out of solution and was discarded. The supernatant was percolated through a column of acid-washed alumina (pH 4·0), at 2°, until 80% of the alumina was saturated with cephalosporin N (see Experimental). Elution was begun with 20% (v/v) acetone, which removed inactive material, and then continued with 0·02N sodium hydroxide. The cephalosporin N was eluted in a broad band which contained 70% of the active material in one-fifth of the volume of the solution added to the column. The activities of the fractions, based on dry weights obtained by evaporating samples at 100°, varied from 18 to 33 units/mg. (see Experimental and Table 5).

Column 2. Acetone was added to fractions selected from the first alumina column (26·5 units/mg.) until its concentration was 20% (v/v). The pH of the solution was adjusted to 6·0 and it was then chromatographed on a second column of acid-washed alumina (pH 4·6) at 2°. The size of the column was such that 80% of the alumina was saturated with cephalosporin N. Elution was carried out with 0·01N sodium hydroxide and the eluate collected in small fractions. These fractions

contained 80 % of the cephalosporin N which had been adsorbed, with activities varying from 25–40 units/mg. (see Experimental and Table 6). The activities are based on dry weights obtained by evaporating samples at 100°.

Eluates from the second alumina column were combined in groups according to their activity. Acetone was removed and the aqueous solutions were freeze-dried. The following amounts of nearly white powders, containing mainly the sodium salt of cephalosporin N, were obtained from 240 l. of filtered culture fluid: 8·5 g. at 38 units/mg.; 2 g. at 37 units/mg.; 2·5 g. at 27 units/mg.; 1·4 g. at 25 units/mg. Attempts to raise the activity of this material by further chromatography on alumina were unsuccessful.

Table 1. *Recovery of cephalosporin N after chromatography on charcoal and alumina*

Fraction	Total units passed on to next stage	Proportion of activity of original filtrate (%)
Fermentation filtrate	2 060 000	100
From charcoal column	1 410 000	69
From alumina column 1	950 000	46
From alumina column 2	583 000	28
Solid (mainly sodium salt)	494 400	24

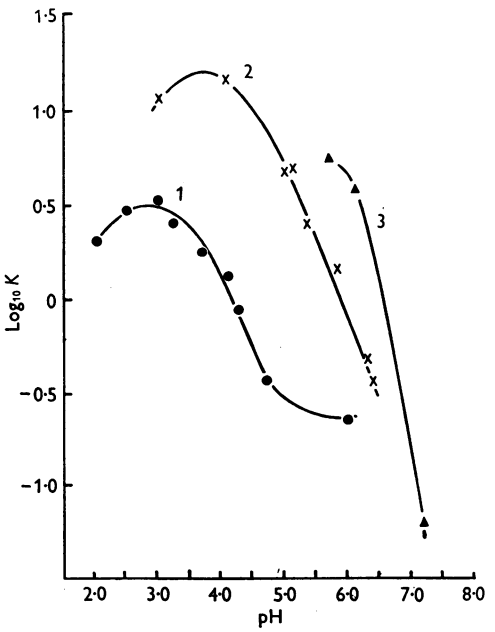

Fig. 1. Partition of cephalosporin N in phenol–water systems at various pH values. (1) Phenol–water. (2) Phenol–*NN*-dimethylaniline–water. (3) Phenol–carbon tetrachloride–2:4:6-trimethylpyridine–water (system 1). K = [activity in phenol phase]/[activity in aqueous phase].

Table 1 summarizes the amounts of cephalosporin N which were recovered at each stage of the purification process.

Partition coefficients in phenol–water systems. The partition coefficient K (activity in phenol phase/activity in aqueous phase) of cephalosporin N between phenol and water was found to vary with the pH of the aqueous phase in the manner shown in Fig. 1 (curve 1). The value of K reached a maximum at pH 3·0. When 12 % (v/v) of *NN*-dimethylaniline was added to the phenol phase of this solvent system, and the pH adjusted with sulphuric acid, K was greatly increased over the range pH 3·0–5·0 and now reached maximum at pH 4·0 (Fig. 1, curve 2). When the stronger base, 2:4:6-trimethylpyridine, was added, the maximum value of K was at about pH 5·3.

A solvent system suitable for countercurrent distributions with a mobile upper aqueous phase, and a ratio of upper phase to lower phase of 3 to 2 by volume, was obtained from the following mixture: phenol saturated with water (40 vol.), carbon tetrachloride (40 vol.), 2:4:6-trimethylpyridine (7·1 vol.), water saturated with phenol (350 vol.), 10 N sulphuric acid (1 vol.). The pH of the aqueous phase (glass electrode) was 6·0–6·2. This was called system 1. Fig. 1 (curve 3) shows the change in K when the pH of this system was varied by the addition of different amounts of sulphuric acid.

Countercurrent distribution. System 1. In a preliminary experiment cephalosporin N (20 units/mg.) was purified by twenty-four fundamental transfers followed by 101 withdrawals of the aqueous phase from tube 24, in system 1, at room temperature. Material which was recovered as a barium salt from the active band in the withdrawn series had an activity of 38 units/mg. However, 35 % of the initial activity was unaccounted for, indicating that breakdown of cephalosporin N had occurred during the experiment. The product was therefore redistributed in system 1 at 3°. After fifteen fundamental transfers, sixty-five withdrawals of the aqueous phase were made from tube 15. Fig. 2 shows the antibacterial activity, ninhydrin colour density and dry weight of selected fractions. The results indicate that the active substance could account for 73 % of the preparation. The appearance of substances A and B confirmed that breakdown of cephalosporin N had taken place during the previous experiment. In the present experiment approximately 85 % of the antibacterial activity of the starting material was accounted for. The material in fractions 28–58 of the first withdrawn series (aqueous phase) was recovered as a barium salt and precipitated by acetone from aqueous solution. The activity of the product was 55 units/mg.

In another experiment a preparation of cephalosporin N obtained from the second alumina column (28 units/mg.) was distributed in system 1 at 3°. After sixteen fundamental transfers, eighty-one withdrawals of the aqueous phase were made from tube 16. Fractions 59–77 of the withdrawn series yielded a barium salt with an activity of 58 units/mg.

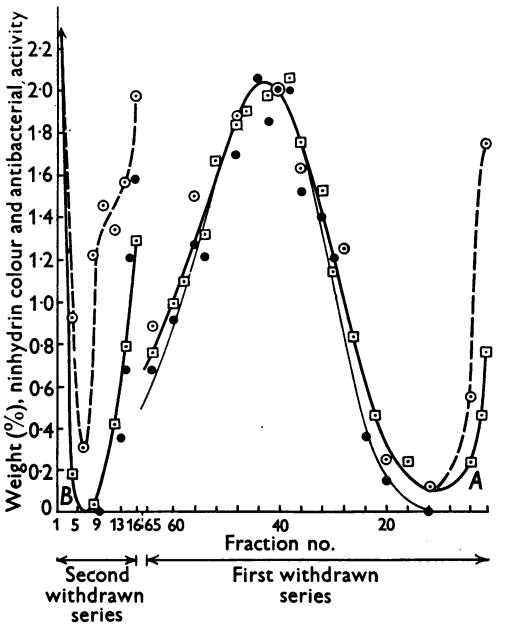

Fig. 2. 80-Transfer distribution of cephalosporin N in system 1 (phenol–carbon tetrachloride–2:4:6-trimethylpyridine–dilute sulphuric acid, pH 6·1). Ratio (upper phase/lower phase) 3:2, by vol. Upper phase mobile. ●—●, relative antibacterial activity; ——, calculated curve ($K = 0{\cdot}24$); ⊙—⊙, % of dry weight; ⊡—⊡, relative ninhydrin colour density.

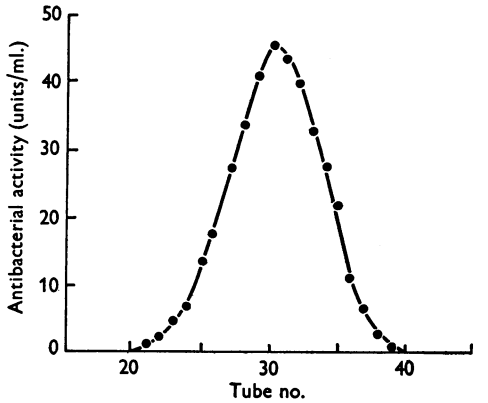

Fig. 3. 49-Transfer distribution of cephalosporin N in system 2 (ethanol–ammonium sulphate–sodium phosphate–citric acid–water, pH 6·0). Upper phase mobile. ●—●, antibacterial activity; ——, calculated curve ($K = 1{\cdot}633$).

Partition coefficients in alcohol–aqueous ammonium sulphate systems. In systems composed of ethanol, or 2-ethoxyethanol, and concentrated ammonium sulphate solutions at pH 5·1, cephalosporin N was appreciably soluble in the alcohol-rich phase. In the system ethanol–water–ammonium sulphate (2·8:6·5:3·0, by weight) the partition coefficient K (activity in alcohol phase/activity in aqueous phase) was 1·57. In the system: 2-ethoxyethanol–water–ammonium sulphate (2·3:5·6:2·0 by weight) K was 2·0. A solvent system suitable for countercurrent distribution of cephalosporin N was obtained from the following mixture: 0·1 M disodium hydrogen phosphate (50·5 ml.); 0·1 M citric acid (14·7 ml.); ammonium sulphate (30 g.); ethanol (30 ml.); *sec.*-butanol (5 ml.). This was called system 2. The pH of the aqueous phase (glass electrode) was 6·0.

Distribution in system 2. A preparation of cephalosporin N (15 units/mg.) was subjected to a forty-nine transfer (fundamental) distribution in system 2 at 16°. The distribution of antibacterial activity is shown in Fig. 3. There was no indication of the presence of more than one active component. No loss of antibacterial activity was detected.

Some properties of cephalosporin N

Cephalosporin N barium salt with an activity of 58 units/mg. was a white powder, readily soluble in water but sparingly soluble in methanol and insoluble in ethanol. It had $[\alpha]_D^{20} + 187°$ in water (c, 0·6). It was not precipitated from concentrated aqueous solution by *NN*-dibenzylethylenediamine, which forms a sparingly soluble salt with benzylpenicillin (Szabo, Edwards & Bruce, 1951). It contained C, H, N and S, but no halogen or phosphorus. After drying *in vacuo* at room temperature, elementary analysis showed: N, 8·7; S, 6·9 %. The atomic ratio of N:S was therefore 3:1. The material showed a strong ninhydrin reaction, and electrometric titration revealed the presence of a basic group of pK 9·8, as well as acidic groups with pK values of 3·2 and less than 2·8. The equivalent weight of the barium salt, estimated from the span of the titration of the basic group, was 495. Assuming that the substance gave the same ninhydrin colour yield per gram molecule as leucine, the molecular weight of the barium salt was estimated to be 450.

Additional evidence for the presence of a basic group in the active molecule was provided by the change with pH of the partition coefficient between phenol and water (Fig. 1). On lowering the pH of the aqueous phase the partition coefficient rose to a maximum at about pH 3·0 and then fell, suggesting that the antibiotic reached an isoelectric point in this region.

Table 2. *Chemical estimations of the antibacterial activity of the pure sodium salt of cephalosporin N*

For methods see text.

Method	Activity of preparation (units/mg.)	Moles of cephalosporin N/381 g.	Estimated activity of pure sodium salt (units/mg.)
Carbon dioxide	13·6	0·181	75
	42·0	0·493	85
	58·0	0·795	73
Hydroxylamine	17·8	0·182	98
	28·0	0·342	82
Iodine	3·5	0·050	70
	17·8	0·238	75
	28·0	0·364	77
Penicillinase	32·0	0·387	82

Table 3. *Estimated relative activities of pure cephalosporin N and benzylpenicillin*

Organism	Activity of cephalosporin N / Activity of benzylpenicillin
Staphylococcus aureus, NCTC 6571	0·01
Staphylococcus aureus (penicillin resistant)	0·04
Pneumococcus, type 1	0·06
Streptococcus viridans, NCTC 3165	0·06
Vibrio cholerae	2
Salmonella typhi	3
Salmonella enteritidis	6
Klebsiella pneumoniae	6

Preparations of cephalosporin N, like benzylpenicillin, evolved carbon dioxide on acid hydrolysis, reacted with hydroxylamine to form a hydroxamic acid, absorbed iodine after alkaline inactivation, and underwent hydrolysis with the liberation of an acidic group on treatment with penicillinase. These reactions were used for the chemical assay of cephalosporin N in terms of benzylpenicillin. Carbon dioxide was determined as described by Abraham, Baker, Chain & Robinson (1949). The reaction with hydroxylamine was carried out by a modification of the method of Ford (1947) and the reaction with iodine by the method of Alicino (1946). The acid liberated on inactivation with penicillinase at pH 7·0 was determined by titration in a manner similar to that described by Wise & Twigg (1950). Assuming that one molecule of cephalosporin N behaved like one molecule of benzylpenicillin in these reactions, and that impurities were inert, the reactions could be used to calculate the number of gram molecules of cephalosporin N in a given weight of an impure preparation. The weight of cephalosporin N in such preparations was then calculated on the assumption that the molecular weight of its barium salt was 427 and its sodium salt 381 (see Newton & Abraham, 1954). This provided an estimate of the degree of purity of the preparations and of the antibacterial activity of the pure substance. The results are shown in Table 2. The values indicate that the antibacterial activity of cephalosporin N sodium salt is about 80 units/mg. and therefore that the preparation of the barium salt obtained in the present work was about 80 % pure. On this basis the data of Heatley & Florey (1953) have been used to estimate the relative activities of pure cephalosporin N and benzylpenicillin against a number of Gram-positive and Gram-negative bacteria (Table 3).

The properties of cephalosporin N described in this paper suggest that it is a penicillin carrying an aminodicarboxylic acid residue in the side chain. A structure of this type would account for the strongly hydrophilic nature of the molecule.

EXPERIMENTAL

Antibacterial assay. Solutions were assayed by the method of Brownlee *et al.* (1948), using *Klebsiella pneumoniae* (Departmental strain) as a test organism, or by a similar method using *Salmonella typhi*, strain 'Mrs S.' (Felix & Pitt, 1935).

Filtration of culture fluid. The culture fluid (280 l.) was cooled to 20° by circulating cold water through the jacket of the fermenter. The pH of the culture fluid (8·0) was adjusted to 6·0 by adding 1 l. of 11 N-HCl. The fluid was then pumped through a cloth bag filter and the filtrate was passed through a filter press containing Fords Sterimats (F.C.B. size PA/20 cm.). The final filtrate (240 l.) was perfectly clear.

Charcoal column

Preparation of the charcoal. Granular charcoal (20 to 60-mesh/in., 270 kg., grade BO, manufactured by Farnell Carbons Ltd., London) was suspended in tap water and transferred to a glass column made from two 3 ft. sections of 9 in. industrial glass pipe. The dimensions of the packed charcoal were 23 × 120 cm. About 30–50 l. of N-HCl were percolated down the column until the effluent had a strongly acid reaction. The evening before use the column was washed with 50 l. of distilled water and then with phosphate

Table 4. *Charcoal column for adsorption of cephalosporin N from culture fluid*

For details see text.

Fraction	Solvent	pH	Vol. in litres	Total units $\times 10^{-3}$
Filtrate	Water	6·0	240	2060
Percolate 1	Water	6·8	70	—
Percolate 2		6·9	70	35
Percolate 3		6·4	75	112
Percolate 4 and water wash		6·2	35	84
Eluate 1	Acetone (60%, v/v, in water)	6·2	35	126
Eluate 2		6·6	2	100
Eluate 3		6·9	16	1410
Eluate 4		7·0	4	112
Total recovered				1979

buffer, pH 6·0 (132 g. KH_2PO_4/l. + 15 ml. 40% (w/v) NaOH/l.), until the pH of the percolate rose to 6·0. Usually 40–50 l. of buffer were required. The following morning percolation with a few litres of the buffer was continued to check that the pH of the percolate had not changed appreciably. The phosphate buffer was then washed out of the column with 50 l. of distilled water, when the pH of the percolate usually rose to 6·4.

Adsorption and elution of cephalosporin N. The clarified culture fluid (240 l.), acidified to pH 5·8–6·0, was percolated down the column at a rate of 2 l./min. The pH of the effluent (which tended to rise) was checked frequently and kept at about 6·0 by adjusting the pH of the culture fluid being added to the column. The percolate was collected in three fractions of about 70 l. and one of 35 l. The column was then washed with 10 l. of distilled water and elution was begun with a 60% (v/v) solution of acetone in water. The eluate was collected in one fraction until a marked increase in the brown colour of the effluent was noted. The main band of cephalosporin N was closely associated with this pigment. The activity of material in eluate 3 (Table 4) was 4 units/mg. The details of the remainder of the elution are shown in Table 4.

Regeneration of the charcoal. After elution of the cephalosporin N, 0·5 N-NaOH was percolated down the column until the effluent became alkaline. Any cephalosporin P which was adsorbed on the charcoal was eluted during this process. The column was then washed with a mixture of 0·2 N-NaOH and acetone (3:2, by vol.) until the effluent, which at first contained much dark brown pigment, was only a pale-brown colour. Free alkali was removed from the column with distilled water (50 l.) and then N-HCl was passed down it until acid broke through. The column was left in contact with acid until the next batch of culture fluid was ready. It was regenerated many times without serious loss of efficiency.

Alumina columns

Preparation of alumina for column 1. Aluminium oxide (British Drug Houses Ltd., 'For chromatographic analysis') which had previously been used for chromatography, was washed with 0·05 N-NaOH until almost colourless, washed with tap water, and then stirred with an excess of 0·05 N-HCl. Finally it was thoroughly washed with distilled water, filtered and dried in a current of air at 30°. The dry powder was heated to 750° in a muffle furnace for 4 hr., cooled, and sieved to provide a fraction of 60–120 mesh. This material was suspended in distilled water and stirred mechanically, while 11 N-HCl was added from time to time until a glass electrode suspended in the water gave a permanent pH reading of 4·0. It was then dried at 30°.

Preparation of alumina for column 2. New aluminium oxide (British Drug Houses Ltd., 'For chromatographic analysis') was suspended in water and stirred. 11 N-HCl was added from time to time until the pH remained stationary at 4·6. The alumina was dried at 30° and sieved to provide a fraction of 60 to 120-mesh.

Chromatography on column 1. A sample (100 ml.) of eluate 3 from the carbon column was extracted with three lots of butyl acetate (100 ml. each) saturated with water; 79 ml. of aqueous solution remained. Eluate 3 therefore contained approximately 21% (v/v) of acetone. Acetone (26 l.) was added to bring the acetone concentration to 70% (v/v) and some material of low activity, which came out of solution, was allowed to settle and was removed by filtration. The pH of the clear filtrate was adjusted to give a reading of 6·0 (glass electrode) and a sample of this solution was percolated down an alumina column (0·5 × 15 cm.). After the passage of 25 ml. of solution, antibacterial activity was detected in the percolate. From these data a column was constructed containing enough alumina to retain cephalosporin N from 1·25 times the volume of the main bulk of the solution. The column (16 cm. diameter), containing 6 kg. of the alumina suspended in 70% (v/v) acetone, was prepared in a glass tube around which cold ethanol (2°) was circulated. The bulk of the clear filtrate (42 l.), cooled to 2°, was percolated through the alumina at about 10 l./hr. Elution was begun with a cold aqueous solution of acetone (20%, v/v) and continued with cold 0·02 N-NaOH. Eluates were adjusted to pH 6·0 immediately they were obtained. Details of the column are given in Table 5.

Chromatography on column 2. Eluates 12–20 (see Table 5) from the first alumina column were combined and 2·25 l. of acetone were added to bring the acetone concentration to 20% (v/v). A small pilot column was used to determine (as for column 1) the amount of alumina required for chromatography of this solution. Table 6 shows the results obtained when 5 l. of solution were chromatographed on 400 g. of alumina. The column (5 cm. diameter) was surrounded by a glass jacket and cooled with ethanol at 2°. The remainder of the eluate selected from column 1 was chromatographed separately.

Table 5. *Chromatography of cephalosporin N on alumina. Column* 1

For details see text.

Fraction	Solvent	pH	Vol. in litres	Units/mg.	Total units ($\times 10^{-3}$)
Charcoal eluate 3	Acetone (70%, v/v)	6·0	42	—	1360
Percolate		5·8	42	—	—
Eluate: 1	Acetone (20%, v/v)	5·6	2	—	—
2		5·7	2	—	36
3		4·8	2	—	38
4		4·3	2	—	22
5		4·2	2	—	11
6		4·0	1	—	3·6
7	0·02 N-NaOH	4·1	1	—	6·7
8		4·1	1	—	17
9		4·1	1	—	27
10		4·2	1	—	33
11		4·3	1	16	37
12		4·2	1	17·7	45
13		4·4	1	24·4	74
14		4·5	1	23·0	84
15		4·5	1	31·0	135
16		4·4	1	33·5	158
17		4·3	1	32·0	148
18		4·5	1	21·5	112
19		4·8	1	18·8	95
20		5·0	1	20	99
21		5·2	1	13·3	67
22		5·4	1	—	39
Total recovered					1286

Table 6. *Chromatography of cephalosporin N on alumina. Column* 2

For details see text.

Fraction	Solvent	pH	Vol. in litres	Units/mg.	Total units ($\times 10^{-3}$)
Eluates 12–20 from previous column	Acetone (20%, v/v)	6·0	5	—	370
Percolate		5·1	5	—	—
Eluate: 1	Water	4·7	0·5	—	—
2		4·6	0·5	—	7·5
3	0·01 N-NaOH	4·6	0·5	25·1	16
4		4·7	0·5	36·0	23
5		4·9	0·5	34·5	25
6		5·2	0·5	31·5	23
7		5·0	0·5	39·5	33
8		5·2	0·5	36·5	32
9		5·1	0·5	25·4	27
10		5·6	0·5	24·5	25
11		5·6	0·5	—	25
12		6·4	0·5	—	22
13		6·9	0·5	—	20
14		7·8	0·5	—	16
Total recovered					298

Approximate determination of the dry weight of eluate fractions. Samples of the eluates were measured into weighed glass tubes and dried in a stream of air while the tubes were maintained at 100°. Owing to decomposition, dry weights estimated by this method were liable to be lower than those obtained by drying *in vacuo* at 20°.

Countercurrent distributions

Solvents. 'AnalaR' phenol and 'AnalaR' CCl_4 were used throughout.

Determination of antibacterial activity. System 1. Samples (0·2 ml.) were taken from even-numbered fractions of the first withdrawn series (aqueous phase) and from the second withdrawn series (phenol phase), and added to 2 ml. of 0·1 M sodium phosphate buffer, pH 7·4. The solutions were extracted twice with CCl_4 (4 ml.) and the final volume of the aqueous phase was noted. Further dilutions to give solutions containing 1–4 units/ml. were made with 0·05 M sodium phosphate buffer, pH 6·8. These solutions were assayed against *Salm. typhi.*

System 2. Water (40 ml.) was added to the combined phases of each fraction (20 ml. each), when a single phase resulted. Samples of these solutions were diluted until they contained 1–4 units/ml. and assayed against *Kleb. pneumoniae.*

Determination of dry weight. System 1. (1) *First withdrawn series (aqueous phase).* CCl_4 (10 ml.) and 0·3 N-$Ba(OH)_2$ (0·175 ml.) were added to samples (5 ml.) taken from every fourth member of the first withdrawn series. The mixture was shaken to establish equilibrium and the pH of the aqueous phase was adjusted to 8·2–8·6 with $Ba(OH)_2$ if necessary. The layers were separated and the aqueous phase was again extracted with CCl_4 (10 ml.). Sufficient 0·1 N-H_2SO_4 (usually 0·12 ml.) was added to the aqueous phase to bring its pH to 6·0–6·4 and a third extraction with CCl_4 (10 ml.) was made. The final volume was measured and the aqueous solution centrifuged to free it from precipitated $BaSO_4$. Samples (3 ml. each) from the clear supernatant were evaporated to dryness *in vacuo* at 30–35° in weighed tubes. Blank determinations on 3 ml. of the aqueous phase gave a dry weight of less than 0·1 mg.

(2) *Second withdrawn series (phenol phase).* From every second fraction of the second withdrawn series a 5 ml. sample was added to a mixture of water (5 ml.) and CCl_4 (20 ml.). 0·3 N-$Ba(OH)_2$ (1–2 ml.) was added until the pH of the aqueous phase at equilibrium was 8·2–8·6. The mixture was centrifuged and the upper (aqueous) layer removed. The lower layer (phenol–CCl_4) was shaken with water (2 ml.) and centrifuged. The upper layer was added to the first aqueous extract and the bottom layer was discarded. The combined aqueous extracts, still at pH 8·2–8·6 were extracted with CCl_4 (10 ml.). 0·1 N-H_2SO_4 was then added to bring the pH to 6·0–6·5 and a final extract made with CCl_4 (10 ml.). The final volume was measured (usually 8·2–8·5 ml.) and the solution centrifuged to remove the precipitated $BaSO_4$. Samples (3 ml. each) were then evaporated to dryness *in vacuo* at 30–35° in weighed tubes.

Ninhydrin determinations. System 1. Samples (0·2 ml. each) of the solutions prepared for estimations of dry weight were added to 0·5 ml. ninhydrin solution (Moore & Stein, 1948). The colour density at 570 mμ. was measured after heating the mixture at 100° for 20 min. (Newton & Abraham, 1950). The same colour densities were obtained from samples of the first withdrawn series whether the solvent was removed or not. No attempt was made to relate the colour density to the dry weight of the samples.

Recovery of cephalosporin N from countercurrent distribution fractions. System 1. The following operations were carried out in a cold room at 2–3°. The selected fractions from the first withdrawn series were pooled, the pH of the solution was brought to 7·0–7·2 by the addition of 0·3 N-$Ba(OH)_2$, one vol. of CCl_4 added, and the mixture shaken. After the phases had separated, the CCl_4 containing most of the phenol originally present in the aqueous phase was drawn off. The pH of the aqueous phase was adjusted to 8·3 and it was extracted twice with one vol. of CCl_4 to remove the 2:4:6-trimethylpyridine. Finally the aqueous phase was acidified to pH 6·5 and extracted once more with one vol. of CCl_4. The solution was then filtered to remove any $BaSO_4$ which remained and any residual CCl_4 was removed *in vacuo*. The solution was then freeze-dried. The product was dissolved in water (1–5 ml.) and acetone was added until its concentration was 95% (v/v). The mixture was centrifuged and the supernatant removed. The sticky precipitate of cephalosporin N was ground under dry acetone, when it formed a fine white powder. It was then centrifuged and dried *in vacuo*.

Distribution in system 1 *at* 3°. (*a*) The starting material was a preparation of cephalosporin N barium salt (38 units/mg.) obtained by distributing material from an alumina column in system 1 at room temperature. This material (approx. 400 mg.) was dissolved in 30 ml. of upper layer and 20 ml. of lower layer of the solvent system. 2:4:6-Trimethylpyridine (0·5 ml.) was added to bring the pH of the upper layer to 6·1 at equilibrium. The mixture was transferred to a glass countercurrent distribution machine (Craig, Hausmann, Ahrens & Harfenist, 1951), and eighty transfers of the upper layer were carried out. Withdrawals of the aqueous phase were made from tube 15 and after sixty-four transfers fresh upper layer was no longer added to tube 0. In this way two withdrawn series remained at the end of the distribution, sixty-five fractions (30 ml.) of the aqueous phase forming the first withdrawn series and sixteen (0–15) fractions (20 ml.) of phenol phase forming the second withdrawn series. Immediately the distribution was finished, ninhydrin determinations were carried out on samples of the first withdrawn series. Fractions 28–58 from this series were pooled after 5 ml. samples had been removed from the even-numbered fractions. They yielded 120 mg. of barium salt assaying at 55 units/mg.

(*b*) The starting material in this experiment was 5·7 g. cephalosporin N sodium salt (28 units/mg.), prepared by chromatography on alumina. To convert it into the barium salt it was dissolved in a mixture of 75 ml. of each layer of a solvent system made up from phenol saturated with water (250 ml.), water saturated with phenol (250 ml.), 2:4:6-trimethylpyridine (20 ml.) and 10 N-H_2SO_4 (14 ml.). 10 N-H_2SO_4 (0·5 ml.) was added to bring the pH of the aqueous layer to 5·6 at equilibrium. The two layers were separated and the aqueous phase was discarded. The phenol phase was shaken with 25 ml. of fresh aqueous phase, which was again discarded after separation of the layers. The phenol phase was shaken with water (25 ml.) and 0·3 N-$Ba(OH)_2$ was added until the pH of the aqueous phase was 8·0. CCl_4 (225 ml.) was added in 75 ml. portions, the equilibrium reaction of the aqueous phase being adjusted back to pH 7·5 with 0·3 N-$Ba(OH)_2$ (in all 190 ml.) after each addition. The lower layer was discarded and the aqueous layer, after its pH had been adjusted to 8·3 with $Ba(OH)_2$, was extracted twice with CCl_4 (250 ml.). The aqueous phase was then brought to pH 6·4 with H_2SO_4 and extracted again with CCl_4 (250 ml.). It was filtered, freed from traces of CCl_4 *in vacuo*, and freeze-dried to yield 5 g. of cephalosporin N barium salt (30 units/mg.).

The barium salt of cephalosporin N (4·4 g.) was added to 60 ml. of upper layer and 40 ml. of lower layer of system 1; 0·025 ml. of 10 N-H_2SO_4 was required to restore the equilibrium reaction of the aqueous phase to pH 6·1. The aqueous phase (30 ml.) and 20 ml. of the phenol phase were put into tubes 0 and 1 of the countercurrent distribution apparatus. Additions of 2:4:6-trimethylpyridine or H_2SO_4 had to be made to the fractions during the first four transfers, but thereafter the pH of the aqueous phases remained constant. Ninety-seven transfers were carried out, withdrawals of the aqueous phase being made from tube 16. Colorimetric ninhydrin determinations on samples of fractions 1–31 and 33–57 of the withdrawn series were made while the distribution was in progress and on fractions 59–77 immediately after the distribution was finished. It was thus possible to decide which fractions to pool for recovery of cephalosporin N, within 30 min. of the end of the distribution. Fractions 20–52 were pooled, freed from solvent and concentrated as described above. They yielded 700 mg. of barium salt assaying at 58 units/mg. The whole experiment was completed in 36 hr.

Evolution of CO_2. This was measured in the apparatus described by Abraham *et al.* (1949). Preparations of cephalosporin N (20–40 mg.) were dissolved in water (1 ml.), enough N-H_2SO_4 was added to lower the pH to 2·0, and the solution was diluted with 0·5 N-H_2SO_4 to 2·5 ml. In control experiments 0·8 mole of CO_2 was obtained from 1 mole of sodium benzylpenicillin under these conditions.

Inactivation with penicillinase. Cephalosporin (40 mg., 32 units/mg.) was dissolved in water (10 ml.) at 25°. A glass electrode was immersed in the solution. A dialysed solution of penicillinase, provided by the Distilers Company (Biochemicals) Ltd., Liverpool (1 ml., containing 2·8 mg. of solid), was added. 0·01 N-NaOH was run into the stirred solution at a rate such that the pH was maintained at 7·0. The rate was constant (1 ml./25 min.) during much of the reaction. After 2·5 hr., when 3·7 ml. 0·01 N-NaOH had been added, there was no further change in pH.

A solution (10 ml.) of sodium benzylpenicillin (10 mg.) absorbed 0·01 N-NaOH under these conditions at an initial rate which was 1·7 times the rate with cephalosporin N.

Iodometric assay. This was done by a method similar to that of Alicino (1946), except that the inactivation by alkali was carried out in 0·4 N-NaOH for 30 min. at 17° and the titration with sodium thiosulphate was carried out 15 min. after the addition of iodine; 3 mg. of sodium benzylpenicillin and 150–300 units of cephalosporin N were used in each titration. For five titrations on a single sample of cephalosporin N (mean = 5·59) the S.D. was 0·108. For four different samples varying in activity from 3·5 to 28 units/mg. the coefficient of correlation between antibacterial activity and differential iodine absorption was 0·83.

Hydroxylamine assay. This was carried out by a method similar to that of Ford (1947), except that ethanol (0·33 vol.) was added to the solution 10 min. after the addition of $FeCl_3$ solution, and the colour density was then read in a Spekker photoelectric absorptiometer, against the corresponding penicillinase-treated control, using a blue-green (Ilford 603, transmitting 470–520 mμ.) filter. For six assays of a single sample (mean = 0·444) the S.D. was 0·007. For four different samples varying in activity from 3·5 to 28 units/mg. the coefficient of correlation between antibacterial activity and the differential Spekker reading was 0·71. Interfering substances appeared to be present in some samples of cephalosporin N, however, since the variation between samples was much greater than the variation in repeated assays of a single sample.

The pigment formed in this procedure from cephalosporin N, unlike that from benzylpenicillin (Baker, Dobson & Martin, 1950) could not be extracted from aqueous solution by *iso*amyl alcohol or butanol.

SUMMARY

1. Cephalosporin N is an acidic antibiotic produced by a strain of *Cephalosporium*. It is inactivated by the enzyme penicillinase but differs strikingly from the common penicillins in its antibacterial activity and hydrophilic character.

2. Cephalosporin N has been purified by chromatography on charcoal and alumina and by countercurrent distribution in a phenol–water system containing 2:4:6-trimethylpyridine as a carrier. The resulting product appears to be at least 75% pure.

3. The acid-base properties and solubility of cephalosporin N, together with its chemical reactivity, suggest that it is a penicillin containing an aminodicarboxylic acid in the side chain.

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