- James, A. T. & Martin, A. J. P. (1951). *Biochem. J.* **48**, vii.
 Kao, C. H. & Ma, S. (1931). *J. chem. Soc.* p. 2046.
 Kon, G. A. R., Linstead, R. P. & MacLennan, G. W. G. (1932).
J. chem. Soc. p. 2454.
 Lane, J. F. & Wallis, E. S. (1941). *J. Amer. chem. Soc.* **63**,
 1674.
 Oldham, J. W. H. (1950). *J. chem. Soc.* p. 100.
Organic Syntheses (1925). **4**, 9.
 Pontzio, G. (1905). *Gazz. chim. ital.* **35**, II, 569.
 Popják, G., French, T. H., Hunter, G. D. & Martin, A. J. P.
 (1951). *Biochem. J.* **48**, 612.
 Schickltanz, S. T., Steele, W. I. & Blaisdell, A. C. (1940).
Industr. Engng Chem. (Anal. Ed.) **12**, 320.
 Stadtman, E. R. & Barker, H. A. (1949). *J. biol. Chem.* **178**,
 677.
 Varrentrap, F. (1840). *Liebigs Ann.* **35**, 196.
 Whitmore, F. C. & Fleming, C. H. (1933). *J. Amer. chem.*
Soc. **55**, 4161.

Isolation of Antibiotics from a Species of *Cephalosporium*. Cephalosporins P₁, P₂, P₃, P₄ and P₅

BY H. S. BURTON AND E. P. ABRAHAM
Sir William Dunn School of Pathology, University of Oxford

(Received 2 May 1950)

Brotzu (1948) reported that a species of *Cephalosporium* isolated from the sea near Sardinia produced a substance that inhibited the growth of a number of Gram-positive and Gram-negative bacteria. The production of antibacterial substances by the same *Cephalosporium* has recently been studied in this laboratory and in the Antibiotics Research Station of the Medical Research Council at Clevedon (Crawford, Heatley, Boyd, Hale, Kelly, Miller & Smith, 1951). This paper describes the isolation and some of the properties of members of one group of antibiotics obtained from active culture filtrates of the organism.

At an early stage of the present work it was found that some culture fluids contained at least two kinds of antibacterial substance, one of which could readily be extracted into butyl acetate from a weakly acid solution while the other was insoluble in common organic solvents. The active material in the ester-soluble fraction, with which this paper is concerned, was called cephalosporin P. The remaining water-soluble substance, which was discovered independently at Clevedon, had quite different chemical and antibacterial properties. It was named cephalosporin N and will be the subject of a separate publication. Solutions of cephalosporin P inhibit the growth of *Staphylococcus aureus* but not of *Salmonella typhi*, whereas cephalosporin N is active against both organisms. It is probable that the crude extracts prepared by Brotzu (1948) contained mainly cephalosporin N, while the material studied in earlier work at Oxford (Crawford *et al.* 1951), which could be extracted by organic solvents, was cephalosporin P.

It has now been shown that crude cephalosporin P contains five acidic antibiotics. These substances have been named cephalosporins P₁, P₂, P₃, P₄ and P₅. Cephalosporins P₁, P₂ and P₄ have been isolated

in crystalline form. All five substances have been found to have some properties in common with helvolic acid, an antibiotic produced by various strains of *Aspergillus fumigatus* (Chain, Florey, Jennings & Williams, 1943).

Ritchie, Smith & Florey (1951) have shown that cephalosporin P₁ is almost as active as aureomycin and terramycin against the staphylococcus *in vitro*, and that it is less toxic than these substances when given intravenously and can readily be obtained in the blood in bacteriostatic concentration. For reasons that are not yet understood, however, cephalosporin P₁ has proved much less effective than aureomycin or terramycin in curing staphylococcal infections in mice.

Isolation of cephalosporins P₁, P₂ and P₄

Culture fluids obtained from the Antibiotics Research Station at Clevedon contained much larger amounts of cephalosporin P₁ than of other components of the P complex, and the process of purification was directed towards the isolation of this substance. The minor active constituents occurred in variable amounts in different batches of material. Information concerning cephalosporins P₂, P₃, P₄ and P₅ was accumulated in the course of the work, during which the various fractions obtained were examined by paper chromatography. The cylinder-plate method of assay (Heatley, 1944), with *Staph. aureus* as a test organism, was used to follow the active substances.

The acidic cephalosporin P could be extracted almost completely from an aqueous solution at pH 6.5 into one-fifth of a volume of butyl acetate, and since the extract could be concentrated by distillation without loss of activity this procedure was used for removing the antibiotics from large

batches of culture fluid. Re-extraction of cephalosporin P_1 into aqueous solution in the form of a salt proved unsatisfactory, because the partition coefficient did not greatly favour the aqueous phase until the pH was raised to a value at which inactivation began to occur. A preliminary purification was therefore carried out by precipitations from mixed organic solvents, making use of the fact that the active material was very soluble in acetone, moderately soluble in diisopropyl ether, and insoluble in hexane or light petroleum. The resulting material was then purified further by counter-current distribution between solvents and by chromatography.

Fig. 1. 15-Transfer distribution of partly purified cephalosporin P in a system composed of hexane-diisopropyl ether-acetone-0.5 M-potassium phosphate pH 6.0 (25:8:25:25, by vol.). Lower phase mobile. O-O-O-O, Percentage total activity; x-x-x-x, percentage total weight.

Counter-current distributions were carried out in a solvent system composed of hexane-diisopropyl ether-acetone-M-potassium phosphate, pH 6.0 (1:0.3:1:1, by vol.), the lower phase being mobile. In this system the overall partition coefficient of the active material was close to unity. A distribution curve obtained with one batch of material, which had been subjected to fifteen transfers, is shown in Fig. 1. Most of the activity was present in the central fractions, while most of the impurities were contained in fractions at either end of the series together with minor active components of the mixture.

With a few batches of culture fluid the material recovered from fractions 5-10 of the distribution was almost pure cephalosporin P_1 , which could be crystallized directly from aqueous methanol. Of the relatively small amount of remaining active material

a high proportion of the total quantity of cephalosporin P_3 was contained in fractions 14 and 15, while cephalosporin P_5 was concentrated in fractions 0-5.

With most batches of culture fluid the most active material obtained by counter-current distribution needed further purification before it would yield crystalline cephalosporin P_1 . This material was chromatographed from diisopropyl ether on a column of Florisil (magnesium silicate). By

Fig. 2. 8-Transfer distribution of cephalosporin P_1 in a system composed of hexane-diisopropyl ether-acetone-0.5 M-potassium phosphate pH 6.0 (25:8:25:25, by vol.). Lower phase mobile. O-O-O-O, Percentage total activity; x-x-x-x, percentage total weight.

developing successively with solvents of increasing polarity, such as diisopropyl ether, ethereal acetone, acetone, and acetone containing 1% of water, different constituents of the material passed as bands down the column and were collected in the percolate. The band containing most of the activity normally emerged from the column during elution with diisopropyl ether-acetone. This fraction consisted of cephalosporin P_1 , together with a small amount of cephalosporin P_2 . Elution with acetone

then removed any cephalosporin P_4 . Cephalosporin P_3 was absorbed more strongly than the other antibiotics, and was only removed during the final elution with aqueous acetone.

The purest material in the main active band from the column behaved as though it were a single substance in an eight-transfer counter-current distribution. Fig. 2 shows the result of such a distribution, in which the experimental curve agrees closely with that expected for one substance with a partition coefficient of 1.05. Nevertheless, the material was not always homogeneous. On dissolving its main constituent, cephalosporin P_1 , in diisopropyl ether, a small residue of less soluble material sometimes remained which consisted of cephalosporin P_2 .

The purest preparation of cephalosporin P_1 crystallized readily in needles from aqueous methanol. Cephalosporins P_2 and P_4 crystallized from the same solvent.

Properties of cephalosporins P_1 , P_2 , P_3 and P_4

Cephalosporin P_1 . Crystalline cephalosporin P_1 , m.p. 147° (micro block), $[\alpha]_D^{25} + 28^\circ$, analysed as follows: C, 65.8; H, 9.0; $C-CH_3$, 12.1; acetyl 16.8; active hydrogen 0.6%. Nitrogen, halogens, sulphur, and phosphorus were absent. The equivalent weight by titration, using phenolphthalein as an indicator, was 580. On electrometric titration in 60% ethanol the substance behaved as a monobasic acid. It appears to have a polycyclic hydroaromatic structure.

Cephalosporin P_1 is readily soluble in most organic solvents, but sparingly soluble in light petroleum and in water. It is stable in organic solvents, or as the sodium salt at room temperature in aqueous solution at pH 7.0, but is slowly inactivated at pH 8.0 and rapidly at pH 9.6.

When tested by the cylinder-plate method against *Staph. aureus*, cephalosporin P_1 is nearly twice as active as helvolic acid.

Cephalosporin P_2 . Crystalline cephalosporin P_2 , m.p. 151° (micro block), gave the following results on analysis: C, 65.25; H, 8.5; $C-CH_3$, 9.4; OCH_3 , 5.8; acetyl, 18.8; active hydrogen 0.8%.

The substance is an acid readily soluble in ethanol, acetone, and butyl acetate, but much less soluble than cephalosporin P_1 in diisopropyl ether or benzene. When tested by the cylinder-plate method against *Staph. aureus* its activity was similar to that of cephalosporin P_1 .

Cephalosporin P_3 . This antibiotic was less stable than the other cephalosporins and was not isolated in the crystalline state. It is an acidic substance, readily soluble in most organic solvents, but sparingly soluble in water. It formed a white greasy solid with an activity against *Staph. aureus* similar to that of cephalosporin P_1 , but rapidly became in-

active, brown-coloured, and insoluble in acetone, on standing in air.

Cephalosporin P_4 . This acidic antibiotic formed light-fawn coloured crystals, m.p. $220-230^\circ$ (micro block) after one recrystallization from aqueous methanol. It is readily soluble in acetone, but sparingly soluble in water or ether. In ethereal solution it lost no activity in 3 months at room temperature.

Inactivation of cephalosporin P by a preparation of penicillinase

Early in the purification of cephalosporin P crude material extracted from the culture fluid was found to be inactivated by a sample of penicillinase prepared from a strain of *Bacillus subtilis* by the method of Duthie (1944). When purified cephalosporins became available the compounds P_1 , P_2 , P_3 , P_4 and P_5 were tested individually and found to lose activity in the presence of the same enzyme preparation.

Since the cephalosporins have no chemical relationship to penicillin it seemed unlikely that they would be inactivated by penicillinase itself. Crawford (1951) has now found that several strains of aerobic spore-forming organisms produce a substance able to inactivate cephalosporin P_1 , and that the amount of the substance in different culture fluids is not proportional to the amount of penicillinase. It may therefore be concluded that an impurity in the preparation of penicillinase is responsible for the inactivation of the cephalosporins. The inactivating agent, which is heat-labile and non-dialysable, appears to be an enzyme, or group of enzymes, and has provisionally been called P-cephalosporinase.

Behaviour of the cephalosporins on paper chromatograms

Cephalosporin P_5 . The behaviour of the various cephalosporins on paper chromatograms was examined during the course of the purification, and compared with that of helvolic acid. Three solvent systems were used: (1) 0.02N-acetic acid in 25% (v/v) ethanol. (2) Diisopropyl ether saturated with 0.1M-potassium phosphate, pH 7.0. (3) Amyl acetate saturated with 0.1M-potassium phosphate pH 7.0. With systems (2) and (3) the paper was treated with M-potassium phosphate pH 7.0 and dried before use. After development the position of the active spots was determined by placing the paper on plates seeded with *Staph. aureus*. The results given in Table 1 show that the chromatograms sharply differentiate cephalosporin P_3 from P_1 , P_2 and P_4 , and that they provide evidence for the existence of a fifth antibiotic, cephalosporin P_5 . In the countercurrent distribution (Fig. 1) cephalosporin P_5 was found mainly in fractions 0-5.

Fig. 3. Paper chromatograms of fractions from an 8-transfer distribution of crude cephalosporin P enriched in cephalosporin P_5 . The solvent was 25% ethanol in 0.02N-acetic acid. The position of the antibiotics is marked by clear zones on an agar plate seeded with *Staph. aureus*. All the fractions contain cephalosporin P_1 and fractions 0-3 also contain cephalosporin P_5 .

Table 1. *Chromatography of cephalosporins*

Antibiotic	R_F values in			Other properties
	System 1*	System 2*	System 3*	
P_1	0.45	0.50	1.0	M.p. 147°. Soluble in diisopropyl ether
P_2	0.45	—	—	M.p. 151°. Almost insoluble in diisopropyl ether
P_3	0	0	<0.05	Unstable in the solid state
P_4	0.40	0.50	1.0	M.p. 220-230°
P_5	0.20	—	—	—
Helvolic acid	0	0.6	1.0	M.p. 212-220°

* See text.

Fig. 3 shows chromatograms of the fractions from an eight-transfer counter-current distribution of material already concentrated with respect to cephalosporin P_5 . All the fractions contain cephalosporin P_1 . Cephalosporin P_5 is absent from fractions 5-9 and its concentration relative to cephalosporin P_1 diminishes from fraction 0 to fraction 4.

By comparing the R_F values and other properties of the antibiotics (Table 1), it becomes clear that cephalosporins P_1 , P_2 , P_3 , P_4 and P_5 are all separate compounds, and that they are all different from helvolic acid.

Relationship of cephalosporin P_1 to helvolic acid

During a preliminary examination of crude cephalosporin P it was noticed that some of the physical properties of this material resembled those

of helvolic acid. Later, a resemblance was found between the antibacterial range and also the pharmacological properties of helvolic acid and cephalosporin P_1 (Ritchie *et al.* 1951). This resemblance was heightened by two further observations: (1) helvolic acid, like cephalosporins P_1 , P_2 , P_3 , P_4 and P_5 , was inactivated by incubation with a preparation of penicillinase from a strain of *B. subtilis*; (2) *Staph. aureus* became resistant to cephalosporin P_1 as readily as it did to helvolic acid (Abraham, Callow & Gilliver, 1946). Staphylococci that had become resistant to helvolic acid by growth in the presence of this substance were found to have also become resistant to cephalosporin P_1 . Conversely, organisms that were grown in the presence of cephalosporin P_1 developed resistance to helvolic acid as well as to the cephalosporin. These pro-

perties suggest that the antibacterial activity of helvolic acid and cephalosporin P depends on a similar structural feature.

Cephalosporins P_1 and P_2 , and helvolic acid ($C_{32}H_{44}O_8$), have rather similar molecular formulae. Further information about the chemical nature of these substances will be given in a future publication.

EXPERIMENTAL

Method of isolation

Extraction into butyl acetate. The filtered culture fluid, prepared at the Antibiotics Research Station, Clevedon, (Crawford *et al.* 1951), was adjusted to pH 6.5 by the addition of 10N-HCl and stirred for 5 min. with one-fifth of its volume of butyl acetate. The two phases were separated in a Sharples centrifuge. Over 95 % of the cephalosporin P was extracted into the organic solvent.

Concentration. The butyl acetate extract (40 l.) was concentrated *in vacuo* to 2 l. using a laboratory climbing-film still (Van Heyningen, 1949). Less than 5 % of the activity was lost in this operation. The concentrate was then reduced to 200 ml. by distillation *in vacuo* (bath temperature 55°) from a 5 l. flask.

With some batches of material a white solid separated from the concentrated butyl acetate on standing and was removed. This substance separated from aqueous acetone or ethanol in the form of white crystals, m.p. 236°, and analysed as follows: C, 70.2; H, 9.0; C/CH_3 , 9.4; acetyl, 9.0 %. It was an acidic compound which was sparingly soluble in water and less soluble in acetone than the cephalosporins. When tested by the cylinder-plate method against *Staph. aureus* at pH 7.0 it showed no activity at a concentration of 1 mg./ml.

Precipitation with light petroleum. To the concentrate (200 ml.) was added 2 l. of light petroleum (b.p. 100–120°) with stirring. A precipitate formed which contained 80 % of the active material but only 60 % of the total solids.

Precipitation with diisopropyl ether. The active precipitate was separated by centrifuging and dissolved in acetone (100 ml.). Diisopropyl ether (1 l.) was added with stirring to the acetone. An almost inactive precipitate formed which contained nearly 20 % of the total material. This was removed at the centrifuge. About 97 % of the active material remained in solution.

Precipitation by concentration in diisopropyl ether-light petroleum. The crude cephalosporin in diisopropyl ether (1 l.) was mixed with an equal volume of light petroleum (b.p. 100–120°). An almost inactive precipitate was removed. The ratio of light petroleum to diisopropyl ether was then increased by concentrating the solution *in vacuo* to 750 ml., when most of the cephalosporin P was precipitated. The precipitate at this stage contained about 50 % of the total activity in the culture fluid and was about half as active against *Staph. aureus* as pure cephalosporin P_1 .

Transfer between solvents. With some batches of material, containing large amounts of inactive material, further purification was carried out in the following manner before undertaking a counter-current distribution between solvents. If this were not done large volumes of solvent were necessary in the distribution to avoid the separation of the system into three phases.

The crude precipitate was dissolved in acetone (50 ml.),

diisopropyl ether (1.3 l.) and hexane (900 ml.) were added, and the mixture was shaken twice with one-tenth of its volume of m-potassium phosphate, pH 7.0, the pH being kept constant by the addition of a few drops of conc. NaOH. About 85 % of the activity remained in the less polar phase. The aqueous phase, containing much inactive material, was rejected.

The solvent was removed from the less polar phase *in vacuo*, the residue dissolved in acetone (1100 ml.) and the solution mixed with an equal volume of m-potassium phosphate, pH 7.0. The acetone-phosphate buffer was shaken with 1/20 vol. of a mixture of hexane-diisopropyl ether (3:2). About 90 % of the activity remained in the aqueous acetone. The latter was concentrated *in vacuo* to 1500 ml., centrifuged clear, and acidified to pH 2.0, when the cephalosporin P was precipitated.

Counter-current distribution between solvents. The crude cephalosporin (11 g.) was subjected to fifteen transfers in a system composed of hexane-diisopropyl ether-acetone-0.5M-potassium phosphate, pH 6.0 (25:8:25:25, by vol.). The transfers were carried out in separating funnels containing 240 ml. of each phase, and the lower phase was mobile. At the end of the experiment the active material in each lower phase was removed by three extractions with a mixture of ether (46 ml.) and light petroleum (b.p. 60–80°; 23 ml.) and the extracts were added to the corresponding upper phases. Samples of the resulting solutions were used to determine the amount of material in each tube. The samples (1–5 ml.) were placed in small weighed test tubes held in a metal block. The solvent was evaporated in a stream of dry air, the block being finally heated in a water bath to 80°, and the tubes were then reweighed. The results of one experiment are shown in Fig. 1.

Crystallization of cephalosporin P_1 . The main quantity of material in fractions 5–10 was recovered by evaporating the appropriate solutions *in vacuo* to dryness. The resulting white solid could be crystallized from aqueous methanol and after three crystallizations yielded pure cephalosporin P_1 , m.p. 145° (4 g.).

Chromatography on Florisil. With some batches of culture fluid the most active material obtained from the counter-current distribution did not yield crystalline cephalosporin P_1 . In these cases the material was purified further by chromatography on Florisil (magnesium silicate, provided by the Floridin Company, Warren, Pa., U.S.A.) or on alumina (pH 5). The active constituents of the various fractions were analysed by paper chromatography. The precise behaviour of the active material on the Florisil chromatograms varied with the loading of the column and the impurities present. The following is a typical experiment.

A solution of impure cephalosporin (1.44 g.) in diisopropyl ether was run on to a column of Florisil (100–200 mesh) 29 cm. long and 2 cm. in diameter. The chromatogram was developed with a series of solvents of increasing polarity and the eluate was collected in 100 ml. fractions, samples of which were used to determine activity and dry weight. Development with diisopropyl ether (300 ml.) resulted in the elution of inactive material (300 mg.). Further development with diethyl ether (750 ml.) removed most of the cephalosporin P_1 and P_2 (380 mg.). Slightly less active material, containing cephalosporin P_4 , was then eluted with acetone (50 ml.), having moved down the column as a sharp yellow band. Finally, material containing cephalosporin P_3 was eluted with acetone containing 1 % (by vol.) of water.

The major active fraction (380 mg.) was rechromatographed on a column of Florisil 20 cm. long and 1.7 cm. in diameter. Elution with diethyl ether (735 ml.) removed 125 mg. of inactive material. Subsequent elution with acetone (600 ml.) removed 132 mg. of almost homogeneous cephalosporin P_1 containing a little cephalosporin P_2 . A final elution with acetone containing 1% of water by vol. removed 120 mg. of almost inactive material. Cephalosporin P_1 was dissolved in diisopropyl ether (50 ml.) and the solution centrifuged from a small amount of cephalosporin P_2 . After evaporation of the ethereal solution the remaining cephalosporin P_1 was crystallized from aqueous methanol.

The fraction from the first column which contained cephalosporin P_3 was rechromatographed, the same series of solvents as before being used for development. The cephalosporin P_3 was again eluted by aqueous acetone. On the other hand, when active material containing no cephalosporin P_3 was rechromatographed no activity remained on the column at this stage.

The fraction containing cephalosporin P_4 was concentrated to about 5 ml. Ether (20 ml.) was then added and the mixture allowed to stand at 0°. Cephalosporin P_4 separated in crystalline form and was recrystallized from aqueous methanol.

Paper chromatography. Chromatograms on Whatman no. 1 paper (42 × 23 cm.) were carried out in large accumulator jars (42 cm. high), the tops of which (26 × 23 cm.) had been ground flat and sealed with glass lids. The jars carried a stainless steel trough supported on a stainless steel frame. Three systems were used for development: (1) ethanol-0.1N-acetic acid-water (2.5:2.0:5.5, by vol.), (2) diisopropyl ether saturated with 0.1M-potassium phosphate, pH 7.0, (3) amyl acetate saturated with 0.1M-potassium phosphate, pH 7.0.

The paper was treated with M-potassium phosphate buffer, pH 7.0 before use with systems (2) and (3). After being dipped in the buffer solution it was pressed between sheets of filter paper and then dried in air.

Samples for analysis, containing 10–20 µg. of antibiotic in 5 µl. of acetone or diisopropyl ether, were placed near the top of the paper sheet in a series of spots 2.5 cm. apart. The chromatograms were run for about 5 hr. at 24°, after which the paper was dried in air. The paper was then cut into strips, 1 cm. wide, containing the material from the different samples, and the strips were placed for 30 min. on large plates of agar bulk-seeded with *Staph. aureus* (National Collection of Type Cultures (N.C.T.C.) No. 6571). The strips were then removed and the plates incubated for 18 hr. at 37°, when clear zones in which no growth had occurred marked the positions of the active spots.

Acquired resistance of *Staph. aureus* to helvolic acid and cephalosporin P_1 . (a) An aqueous solution of the Na salt of helvolic acid (0.04 mg. in 0.04 ml.) was added to 10 ml. of sterile broth in an Erlenmeyer flask, giving a concentration of 4 µg./ml. The broth was inoculated with three drops of an 18 hr. culture of *Staph. aureus*, N.C.T.C. No. 6571 (about 30×10^6 cells), and the flask placed on a shaker at 37°. There was no visible growth in 6 hr., but heavy growth in 24 hr. Two drops of this culture were used to inoculate 10 ml. of broth in a second flask containing helvolic acid at a concentration of 50 µg./ml. There was heavy growth after overnight incubation. Assay plates were seeded with an appropriate dilution of this culture, and with a subculture obtained from it that had grown in normal broth. When tested on the plates by the cylinder-plate method, neither helvolic acid nor

cephalosporin P_1 gave a measurable zone of inhibition at a concentration of 40 µg./ml. When tested on a comparable plate seeded with a culture of the original staphylococcus, cephalosporin P_1 gave zones of 32 and 21 mm. diameter at concentrations of 40 and 10 µg./ml. respectively, while helvolic acid gave zones of 29 and 18 mm. at the same concentrations.

(b) A culture of *Staph. aureus* resistant to cephalosporin P_1 was prepared in a manner similar to that described for helvolic acid. When tested on plates seeded with this culture, neither cephalosporin P_1 nor helvolic acid gave measurable zones of inhibition at a concentration of 40 µg./ml.

Inactivation by a preparation of penicillinase. (a) Four 0.5 ml. samples of a solution of cephalosporin P_1 (10 µg.) in 0.1M-potassium phosphate, pH 7.0, were mixed with 0.5 ml. of solutions of penicillinase in water containing 125, 31, 7.8 and 1.9 µg., respectively, of the enzyme preparation. The penicillinase was a preparation made by Duthie (1944). The mixtures were incubated for 2 hr. at 37° and then assayed by the cylinder-plate method using *Staph. aureus* as a test organism. The results were as follows:

Penicillinase preparation (µg./ml.)	125	31	7.8	1.9
Loss of activity (%)	100	65	20	10

Similar results were obtained with cephalosporins P_2 , P_3 , P_4 , and P_5 .

(b) One 0.5 ml. sample of a solution of crude cephalosporin P_1 (20 µg.) in 0.1M-potassium phosphate, pH 7.0, was mixed with 0.5 ml. of a solution of the penicillinase preparation (500 µg.) in water, and a second sample with a similar solution of 'penicillinase' that had been heated for 10 min. at 100°. Both mixtures were incubated at 37° for 2 hr. The cephalosporin in the first solution was completely inactivated, while that in the second solution showed no loss of activity.

(c) A solution of the penicillinase preparation (10 mg.) in distilled water (10 ml.) was placed in a cellophan tube and dialysed for 18 hr. at 2° against distilled water (10 ml.). The tube was slowly rotated throughout the dialysis to stir the liquid inside and outside the membrane. The dialysate and the solution inside the tube were then tested separately against cephalosporin P_1 . When 0.5 ml. of the dialysate was incubated at 37° with 0.5 ml. of a solution of cephalosporin P_1 (5 µg.) the antibiotic lost no activity. When 0.25 ml. of the solution remaining inside the cellophan tube was incubated at 37° with 0.75 ml. of a solution of cephalosporin P_1 (5 µg.) there was complete loss of activity.

(d) A solution of helvolic acid (40 µg./ml.) in 0.1M-potassium phosphate buffer, pH 7.0, containing the penicillinase preparation (500 µg./ml.) was incubated at 37° for 2 hr. No activity against *Staph. aureus* could be detected by the cylinder-plate method. A control solution incubated in the absence of penicillinase lost no activity.

Effect of metal ions and cysteine. Solutions (1 ml.) of the Na salt of cephalosporin P_1 (10 µg./ml.) were incubated for 2 hr. at 37° in the presence of 1 mg. each of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, ZnSO_4 , AgNO_3 , $\text{Pb}(\text{NO}_3)_2$, HgCl_2 , and cysteine. There was no loss of activity.

SUMMARY

1. A species of *Cephalosporium* produces a group of antibiotics that have been called collectively cephalosporin P, and an antibiotic with entirely

different properties which has been named cephalosporin N. Cephalosporin P consists of acidic substances that are soluble in organic solvents, and are active mainly against Gram-positive organisms. Cephalosporin N cannot be extracted from aqueous solution by common organic solvents and is active against both Gram-positive and Gram-negative organisms.

2. Crude cephalosporin P can contain at least five antibiotics, which have been named cephalosporin P₁, P₂, P₃, P₄ and P₅. Cephalosporin P₁ was the major active substance in extracts of the culture fluids that were used in the present work. By counter-current distribution between solvents and chromatography on Florisil, cephalosporins P₁, P₂ and P₄ have been isolated in crystalline form.

3. Cephalosporin P₁ is almost twice as active as helvolic acid against *Staph. aureus*, but the two antibiotics resemble each other in certain chemical and biological properties. Staphylococci that acquire resistance to cephalosporin P₁ by growing in

its presence become, at the same time, resistant to helvolic acid. Similarly, resistance acquired to helvolic acid is accompanied by an increase in resistance to the cephalosporin. Cephalosporin P₁ is inactivated by a substance that is present in a preparation of penicillinase. This substance has been provisionally named P-cephalosporinase. The same preparation of penicillinase also causes the inactivation of cephalosporins P₂, P₃, P₄, P₅, and helvolic acid.

We are grateful to Miss J. Moss and Miss A. Pill for technical assistance, to Dr K. Crawford and to the Medical Research Council's Antibiotic Research Station at Clevedon for the production of culture fluids and extracts containing the cephalosporins, and to the Floridin Company of Warren, Pa., U.S.A., for a gift of Florisil.

One of us (H.S.B.) is indebted to the Medical Research Council for a personal grant. The work has been supported by grants from the Medical Research Council and the Rockefeller Foundation.

REFERENCES

- Abraham, E. P., Callow, D. & Gilliver, K. (1946). *Nature, Lond.*, **158**, 818.
 Brotzu, G. (1948). *Lav. Ist. Ig. Cagliari*.
 Chain, E., Florey, H. W., Jennings, M. A. & Williams, T. I. (1943). *Brit. J. exp. Path.* **24**, 108.
 Crawford, K. (1951). Private communication.
 Crawford, K., Heatley, N. G., Boyd, P. F., Hale, C. W., Kelly, B. K., Miller, G. A. & Smith, N. (1951). *J. gen. Microbiol.* (in the Press).
 Duthie, E. S. (1944). *Brit. J. exp. Path.* **25**, 96.
 Heatley, N. G. (1944). *Biochem. J.* **38**, 61.
 Ritchie, A. C., Smith, N. & Florey, H. W. (1951). *Brit. J. Pharmacol.* **6**, 430.
 Van Heyningen, W. E. (1949). *Brit. J. exp. Path.* **30**, 302.

Tissue Fractionation Studies

1. THE EXISTENCE OF A MITOCHONDRIA-LINKED, ENZYMICALLY INACTIVE FORM OF ACID PHOSPHATASE IN RAT-LIVER TISSUE

BY J. BERTHET AND C. DE DUVE

Department of Physiological Chemistry, University of Louvain, Belgium

(Received 28 March 1951)

In the course of an investigation on the specific glucose-6-phosphatase of liver, the results of which have been published elsewhere (Duve, Berthet, Hers & Dupret, 1949; Hers, Berthet, Berthet & Duve, 1951), some peculiar observations were made on the unspecific acid phosphatase which is also present in hepatic tissue. Evidence was obtained, indicating that this enzyme is associated with mitochondrial granules by some kind of labile linkage, which renders it inactive towards added glycerophosphate at pH 5.

Experiments which substantiate this conclusion are reported in the present paper. A short account of these findings has already been published in pre-

liminary form (Duve, Berthet, Berthet & Appelmans, 1951).

METHODS

Fractionation of tissue constituents. Rats, fasted for 12 hr. were killed by a blow on the head and bled, and the liver taken out immediately and immersed in an ice-cold isotonic solution. In most cases, the chilled organ was pressed through a wire mesh (Claude, 1946), in order to remove the main vascular and connective network, and the resulting pulp was weighed and homogenized in cold 0.25 M-sucrose, using a Pyrex glass homogenizer of the Potter-Elvehjem type (Potter & Elvehjem, 1936).

In the subsequent fractionation, the technique of Claude (1946), as modified by Schneider (1948), was followed in all