Biological Assay of Cephalosporin C

By JILLIAN M. BOND,* R. W. BRIMBLECOMBE† AND R. C. CODNER‡

*Medical Research Council Antibiotics Research Station,
4 Elton Road, Clevedon, Somerset*

(Received 29 March 1961)

SUMMARY

A method of assaying the antibiotic cephalosporin C in low concentration in culture fluids by using a strain of *Vibrio cholerae* is described. The formation of spheroplasts of the test organism in subinhibitory concentrations of the antibiotic and chemically related compounds is discussed.

INTRODUCTION

The antibiotic cephalosporin C was first discovered in crude preparations of cephalosporin N (Newton & Abraham, 1954) and it was found that both substances were produced by the same species of *Cephalosporium* (C.M.I. 49187) in fermentations. Cephalosporin N was then being assayed by a plate diffusion method against a strain of *Klebsiella pneumoniae* by using the technique described by Brownlee *et al.* (1948). Cephalosporin C in eluates from carbon adsorption columns was assayed against the same organism, with cephalosporin N as standard, but this test was not sensitive enough to detect the very low concentrations of cephalosporin C present in culture fluids. Therefore an attempt was made to develop a more sensitive assay for cephalosporin C. About 100 strains of Gram-negative organisms were examined by a gutter plate technique to find an organism more sensitive to cephalosporin C than the *K. pneumoniae*. Gram-positive organisms were not considered since these were likely to be sensitive to cephalosporin P, which was also produced by the *Cephalosporium* strain used. A strain of *Vibrio cholerae* 1077 proved to be the most sensitive of the organisms tested. This organism had been obtained from Dr I. N. Asheshov, who had used it as a host organism in the assay of the antiphage activity of various substances (Asheshov, Strelitz & Hall, 1949; Hall, Kavanagh & Asheshov, 1951). It had been maintained in the laboratory for many years and was known to be of attenuated pathogenicity. By dilution assays it was shown to be at least ten times as sensitive to cephalosporin C as *K. pneumoniae* and *Salmonella typhi*.

The usual technique for diffusion assay could not be used since *Vibrio cholerae* 1077 did not grow when deep-seeded in agar medium. By using a surface inoculum there was some growth which was considerably improved by using only 0.75 % (w/v) agar in the medium, which was a papain digest medium as used by Dr Asheshov for growing this organism. Since the conditions of the assay are critical, they are described in detail.

* Present address: National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7.

† Present address: Chemical Defence Experimental Establishment, Porton Down, Salisbury.

‡ Present address: Microbiological Research Establishment, Porton, Salisbury.

METHODS

Papain digest agar. The original method of preparation of papain digest medium (Asheshov, 1941) was modified. Meat meal (No. 1 Meat Protein, K24, Meat and Bone Meal; British Glues and Chemicals Ltd., Imperial House, Kingsway, London, W.C. 2) was used instead of fresh meat, and this was digested with a commercial preparation of papain (British Drug Houses Ltd., Poole, Dorset) at 60° for 8 hr. The following quantities were used: meat meal, 400 g.; papain, 24 g.; glycerol, 20 ml.; distilled water, 8 l.

The pH value was kept at 7.0 by adding 10 % (w/v) ammonia. This concentrated digest was cleared by filtration through kieselguhr on paper, autoclaved at 121° for 20 min. and stored until used to make up the final medium which contained concentrated papain digest, 400 ml.; Bacto yeast extract, 2.5 g.; NaCl, 5.0 g.; trace elements solution, 1.0 ml.; distilled water, 600 ml.; adjusted to pH 7.0 with ammonia; agar, 7.5 g. This was sterilized by autoclaving at 121° for 20 min.

The trace element solution contained: H_3BO_3 , 0.057 g.; $CuSO_4 \cdot 5H_2O$, 0.157 g.; $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$, 1.404 g.; $MnSO_4 \cdot 4H_2O$, 0.081 g.; $ZnSO_4 \cdot 7H_2O$, 0.079 g.; distilled water to 1000 ml.

Nutrient agar. Oxoid bacteriological peptone, 5 g.; Lab-Lemco, 3 g.; New Zealand agar, 20 g.; distilled water, 1000 ml.; pH 7.4.

Maintenance of test organism. *Vibrio cholerae* 1077 was grown aerobically on papain digest agar in Petri dishes incubated overnight at 37° and then kept at room temperature. Fresh cultures were put up weekly since the organism died out fairly rapidly, especially at 4°. No loss in sensitivity was observed during the course of this study.

Inoculum. A 4–6 hr. culture in papain digest broth was used to inoculate the assay plates. Papain digest broth (100 ml.) in a 250 ml. conical flask was inoculated by a 24 s.w.g. wire loop 2 mm. in diameter filled with growth from a 24-hr. culture on solid medium, and incubated at 37° for at least 4 hr. without shaking.

Assay plates. Each plate consisted of an aluminium frame 32 cm. square and 3 cm. deep, sealed on to a glass sheet with a layer of nutrient agar (110 ml.). A second layer of papain digest agar (110 ml.) was poured on top of this and the plate left to dry at 32° for 1–2 hr. The lid was a second sheet of glass, covered with filter paper, to absorb condensation moisture.

Inoculation. Enough of the 4-hr. *Vibrio cholerae* broth culture was poured on to the plate to flood the surface completely, then the excess liquid was pipetted off. This method gave a more even layer of growth over the whole surface than did the addition of a measured amount of broth, just enough to cover the agar. The inoculated plate was allowed to dry at 32° for a further hour, the samples then added at room temperature, and the plate incubated at 32–37° for 16–20 hr. Since the medium contained only 0.75 % (w/v) agar, holes could not be cut in it, and the samples were placed on the plates by means of Whatman Antibiotic Assay disks, 13 mm. diam., which were dipped in the sample with fine forceps, drained by touching momentarily the rim of the sample container, and laid on the surface of the plate. By this technique each disk soaked up about 0.14 ml. of solution. Plates were incubated at 32° immediately after application of antibiotic.

Standard solutions. When it became available a crystalline preparation of the

hydrated sodium salt of cephalosporin C was used as standard. Solutions of this were made up weekly at 0.4 u./ml. in 0.05 M-phosphate buffer (pH 7.0) and kept at 4°. The concentrations of standard used on the plate were 0.4 and 0.1 u./ml. The cephalosporin C unit was originally based on the cephalosporin N unit assayed against *Salmonella typhi* but was later defined as the activity contained in 0.13 mg. of a Master Standard prepared at Clevedon in June 1958.

Removal of cephalosporin N from samples. Under the conditions at present used for cephalosporin C production, some cephalosporin N was always formed and since *Vibrio cholerae* 1077 was sensitive to cephalosporin N, this had to be removed from culture fluid samples before they could be assayed for cephalosporin C activity. The method adopted depended on the destruction of cephalosporin N by dilute acid in which the cephalosporin C was relatively stable. The conditions were chosen so that the cephalosporin C itself was not inactivated and the formation of cephalosporin N penillic acid was kept as low as possible, since this had some slight activity against *V. cholerae* (G. A. Miller & R. W. Brimblecombe; unpublished observations). Each sample of culture fluid was acidified to pH 2-3 with 2 M-phosphoric acid using a glass electrode, and held at 37° in a water bath for 2 hr. The pH value was then measured, the solution readjusted to pH 7 with N-sodium hydroxide, and the sample incubated at 37° for a further hour to destroy cephalosporin N penillic acid. It was essential that the pH value during this second incubation should not be above 7.5 or the cephalosporin C itself would be destroyed.

Calculation of potency of unknown samples. The arrangement of the samples on the assay plates and the method of calculation was taken from the scheme described in detail by Brownlee *et al.* (1948).

RESULTS

The assay

Inhibition zones. The zones formed by cephalosporin C on *Vibrio cholerae* 1077 plates were unusual. There was a zone of complete inhibition of growth, and outside this a zone of abnormal mucoid growth, more opaque than the normal growth on the rest of the plate. The zones were magnified ($\times 4.5$) and projected on to a flashed white opal glass screen to be measured. On this screen the zone of inhibition was white and the zone of abnormal growth dark brown. By measuring the diameter of this outer zone, rather than the zone of actual inhibition, it was possible to increase the sensitivity of the assay considerably. Concentrations of cephalosporin C down to 0.015 u./ml. then gave measurable zones.

Morphology of organisms in the outer zone. Microscopical examination of wet preparations of organisms, suspended in papain digest broth, from a typical zone of abnormal growth showed that the normal comma form of the vibrio had disappeared and the growth consisted of spherical forms. These spheroplasts appeared to consist of an outer membrane within which was dense granular material and frequently large clear vacuoles, often crescent shaped. Similar spherical forms produced by *Escherichia coli*, *Proteus vulgaris*, *Aerobacter aerogenes*, and other Gram-negative bacteria in the presence of penicillin have been described by McQuillen (1958, 1960), Lederberg (1956), Hahn & Ciak (1957), Gebicki & James (1960) and others, and in the presence of 6-aminopenicillanic acid by Hugo & Russell (1960). Normal organisms of the strain we used of *Vibrio cholerae* varied in size

from 1.5 to 4.0 μ long $\times$ 0.2 to 0.4 μ wide. The spheroplasts varied in diameter from 3.0 to 7.0 μ with occasional large forms up to 10–11 μ in diameter. They were always perfectly spherical in shape. The proportion of spheroplasts to normal bacteria decreased towards the outer edge of the zone.

When suspended in a 10–20 % (w/v) sucrose solution, the spheroplasts remained intact for many hours, but when suspended in distilled water they lysed rapidly, leaving much less optically dense ghost forms and amorphous masses of debris. The spheroplasts were shown by microscopical examination to be formed in the outer zones as soon as growth on the assay plate was visible to the naked eye, i.e.

Fig. 1

Fig. 1. Zone diameter/log concentration relationship on *Vibrio cholerae* assay plates, of the inner and outer zones of cephalosporin C. All zone diameters as measured on projector in mm. (i.e. $\times 4.5$ magnification), totals of eight zones. $\blacktriangle$ — $\blacktriangle$, outer zone diameter squared/1000; $\times$ — $\times$, outer zone diameter. $\circ$ — $\circ$, inner zone diameter squared/1000; $\bullet$ — $\bullet$, inner zone diameter.

Fig. 2

Fig. 2. Outer zone diameter/log concentration curves from *Vibrio cholera* plates. All zone diameters are measured on projector in mm. (i.e. $\times 4.5$ magn.), totals of eight zones. Cephalosporin C: $\circ$ — $\circ$, zone diameter; $\bullet$ — $\bullet$, zone diameter squared/1000. Fermentation broth: $\times$ — $\times$, zone diameter; $\blacktriangle$ — $\blacktriangle$, zone diameter squared/1000.

after about 3–4 hr. at incubation temperature. When there was no growth of the organism, e.g. at 4°, no spheroplasts were formed. When subcultured on to fresh medium, spheroplasts from assay plates produced colonies of normal *Vibrio cholera* organisms.

Outer and inner zones. On certain plates, both the edge of the outer zone and the edge of the inner zone of complete inhibition were measured and from this the width of the zone of spheroplasts determined. The width of this zone, in relation to the total zone diameter, increased as the concentration of the cephalosporin C decreased. Thus at 5 u./ml. cephalosporin C, the width of the spheroplasts zone was 17.8 % of the total zone diameter, but at 0.156 u./ml. it was 20 %. The plot of log con-

centration against the square of the zone diameter for both types of zone was linear, but the two lines were not parallel, as shown in Fig. 1.

Dose response curve. When the assay technique was well established and a solid standard became available, accurate assays were carried out with cephalosporin C standard solutions at eight concentrations from 4.0 to 0.03 u./ml. (eight replicates of each concentration were set up on eight assay plates). The relationship between log concentration and the square of the zone diameter was linear, whereas the plot of log concentration against the zone diameter showed second degree curvature, as is shown in Fig. 2. Culture fluid, from which cephalosporin N had been removed, and which had been shown by chromatography and electrophoresis to contain no acid-stable antibiotic other than cephalosporin C, was tested at several concentrations and the same linear relationship was found between the square of the zone diameter and log concentration (see Fig. 2).

Reproducibility of the assay. Under routine working conditions three samples of cephalosporin C (0.8, 0.4 and 0.1 u./ml.) were assayed against cephalosporin C standard by the normal technique. Twelve replicate plates were filled on 1 day, three by each of four operators. As there was some evidence that this assay showed day-to-day variation, the experiment was repeated, again with twelve plates, one operator filling three plates/day for 4 consecutive days, the samples being kept at 4°. The results are shown in Table 1.

Table 1. *Reproducibility of the cephalosporin C assay for plates filled on one day by four operators and for plates filled on four consecutive days by one operator*

Experiment		Known potency of cephalosporin C sample		
		0.8 u./ml.	0.4 u./ml.	0.1 u./ml.
Twelve plates filled on 1 day by four operators	Estimated potency (mean of twelve results)	0.74	0.40	0.10
	Standard deviation	0.0415	0.0247	0.0095
	Coefficient of variation	5.6 %	6.2 %	9.5 %
Three plates filled on 4 consecutive days— one operator	Estimated potency (mean of twelve results)	0.81	0.40	0.10
	Standard deviation	0.0742	0.0183	0.0080
	Coefficient of variation	9.2 %	4.6 %	7.9 %

Activity of some related antibiotics against Vibrio cholerae

Cephalosporin P at concentrations considerably higher than those produced in either cephalosporin C fermentation or the special cephalosporin P fermentation (Crawford *et al.* 1952) were completely inactive against *Vibrio cholerae*. Cephalosporin N was active on *V. cholerae* plates, down to 1.0 u./ml. and formed zones identical in appearance, macroscopically and microscopically, to cephalosporin C zones.

Penicillin (above 5 u./ml.) formed zones on *Vibrio cholerae* plates similar to those formed by cephalosporin C, but the zone of abnormal growth was narrower as compared with the total zone diameter. For example, penicillin at 125 u./ml. formed about the same size zone as 0.4 u./ml. cephalosporin C; with penicillin the

width of the zone of abnormal growth was 8.4 % of the total, whereas with cephalosporin C the width of the 'spheroplast' zone was 18.1 % of the total zone diameter. However, the abnormal growth of the penicillin zone consisted of spheroplasts identical in appearance with those formed by cephalosporins C and N. 6-Aminopenicillanic acid at concentrations above 0.4 mg./ml. gave inhibition zones of the cephalosporin C type with outer zones containing typical spheroplasts, while 0.2 mg./ml. gave only spheroplast zones. With 6-aminopenicillanic acid, the width of the spheroplast zone varied from 13.3 % of the total zone diameter at 3.2 mg./ml.

Fig. 3. Outer zone diameter squared/1000/log concentration relationship on *Vibrio cholerae* plates for cephalosporin C, $\times$ — $\times$; cephalosporin N, $\bigcirc$ — $\bigcirc$; and penicillin, $\bullet$ — $\bullet$; 6-aminopenicillanic acid, Δ — Δ . All zone diameters as measured on projector in mm. (i.e. $\times 4.5$ magnification), totals of eight zones.

to 16.1 % at 0.4 mg./ml. When assayed against cephalosporin C standard, 6-aminopenicillanic acid at 3.2 mg./ml. is equivalent to cephalosporin C 0.48 u./ml. The relationships of the square of the zone diameter to log concentration of cephalosporin N, penicillin and 6-aminopenicillanic acid against *V. cholerae* are compared with that of cephalosporin C in Fig. 3.

The quaternary pyridine compound of cephalosporin C, cephalosporin C_A (Hale, Abraham & Newton, 1958, 1961), produced zones on *Vibrio cholerae* plates identical with cephalosporin C zones, although the compound was relatively inactive against *V. cholerae*.

An attempt was made to compare the activity of cephalosporin C and related compounds against *Vibrio cholerae*, *Staphylococcus aureus* H and *Salmonella typhi*. As these compounds are similar in structure and molecular weight, their diffusion constants will be similar. By testing all the compounds on a single assay plate for each organism, measurement of the zones of inhibition gives a valid comparison of their relative activities. Average results from a number of plates of this type are given in Table 2.

Table 2. Activity of cephalosporin C and related antibiotics

Test organism	Antibiotic	µg./ml.	Average zone diameters (mm.)
<i>Vibrio cholerae</i> 1077 (outer zone of abnormal growth measured)	Cephalosporin C	44.7	28.6
	Cephalosporin N*	621.0	28.4
	Penicillin	60.0	26.8
	Cephalosporin P	588.0	0
	Cephalosporin C _A (pyridine)	275.0	25.5
<i>Salmonella typhi</i> (Oxford) (zone of inhibition)	Cephalosporin C	1117.3	19.5
	Penicillin	6.0	14.0
	Cephalosporin P	588.0	0
	Cephalosporin C _A (pyridine)	343.0	19.3
<i>Staphylococcus aureus</i> H6571 (zone of inhibition)	Cephalosporin C	1117.3	20.4
	Penicillin	0.8	17.1
	Cephalosporin P	58.8	20.1
	Cephalosporin C _A (pyridine)	137.6	20.3

* Impure sample.

A number of other antibiotics were found to be active against *Vibrio cholerae* but did not produce the cephalosporin C type of zone. Streptomycin, aureomycin, terramycin, chloramphenicol and erythromycin all formed zones of complete inhibition around which there was no abnormal growth, and no spheroplasts were found.

DISCUSSION

The *Vibrio cholerae* assay for cephalosporin C proved extremely useful when only very low concentrations of cephalosporin C were being produced in the culture fluids. Previously, culture fluid samples had to be concentrated by freeze-drying and reconstituted in a small volume after the destruction of cephalosporin N, then assayed against *Klebsiella pneumoniae*. With the *V. cholerae* assay, culture fluids could be assayed without concentration; after the destruction of cephalosporin N, any cephalosporin P present could be ignored. Removal of cephalosporin N by acid inactivation was essential in samples from these early fermentations, where the amount of cephalosporin C was very low as compared with cephalosporin N. As fermentation conditions were improved and the yield of cephalosporin C considerably increased, this step was not so necessary, since the amount of cephalosporin N present was not enough to affect significantly the higher cephalosporin C titres.

However, any change in fermentation conditions was likely to alter the balance between cephalosporin C and cephalosporin N, and a frequent check on this would be needed if the cephalosporin N were not routinely destroyed before assay of cephalosporin C.

Newton & Abraham (1956) stated that cephalosporin C was insensitive to some preparations of penicillinase, and therefore the destruction of cephalosporin N by incorporation of penicillinase in the assay medium was possible. However, this technique cannot be recommended until more is known of the relative stability of cephalosporin C to different penicillinase preparations under varying conditions.

The best linear fit for log dose response curves was obtained with the square of the zone diameter, when a large number of replicate zones were measured. It has been shown (Gridgeman, 1943; Wood, 1944) that where a four-point assay technique is employed, second-degree curvature does not affect the estimate of potency ratio. In most instances, the approximate titre of the samples to be assayed was known, and they could be diluted to within the range of the standards. Humphrey & Lightbown (1952) showed that under these conditions the use of the square of the zone diameter in calculation was justified only if very accurate assays were required.

The action of cephalosporin C against *Vibrio cholerae* was similar to that of 6-aminopenicillanic acid and penicillin, apparently affecting cell wall synthesis and causing the formation of spherical bodies. The osmotic sensitivity of these spherical forms and their ability to develop into normal bacillary organisms showed that these were spheroplasts with an incomplete cell wall, but not true protoplasts devoid of all cell wall material, as described by Brenner *et al.* (1958). In view of the similarities which exist between the chemical structures of cephalosporin N (Newton & Abraham, 1954), cephalosporin C (Abraham & Newton, 1961), the cephalosporin C₄ derivatives (Hale, Abraham & Newton, 1961), and penicillin, and of the common relationship to 6-aminopenicillanic acid (Batchelor, Doyle, Nayler & Rolinson, 1959), it was of interest to find that these compounds all show spheroplast-forming action similar to that of 6-aminopenicillanic acid against this strain of *V. cholerae*. No abnormal growth forms other than spheroplasts were found in these zones, at any concentration of these antibiotics. Cephalosporins C and N caused the formation of spheroplasts over a wider range of concentrations than penicillin and therefore formed larger zones of abnormal growth on *V. cholerae* assay plates.

REFERENCES

- ABRAHAM, E. P. & NEWTON, G. G. F. (1961). The structure of cephalosporin C. *Biochem. J.* **79**, 377.
- ASHESHOV, I. N. (1941). Papain digest media and standardisation of media in general. *Canad. J. publ. Hlth*, **32**, 468.
- ASHESHOV, I. N., STRELITZ, F. & HALL, E. (1949). Antibiotics active against bacterial viruses. *Brit. J. exp. Path.* **30**, 175.
- BATCHELOR, F. R., DOYLE, F. P., NAYLER, J. H. C. & ROLINSON, G. N. (1959). Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. *Nature, Lond.* **183**, 257.
- BRENNER, S., DARK, F. A., GERHARDT, P., JEYNES, M. H., KANDLER, O., KELLENBERGER, E., KLIENEBERGER-NOBEL, E., MCQUILLEN, K., RUBIO-HUERTOS, M., SALTON, M. R. J., STRANGE, R. E., TOMCSIK, J. & WEIBULL, C. (1958). Bacterial Protoplasts. *Nature, Lond.* **181**, 1713.
- BROWNLEE, K. A., DELVES, C. S., DORMAN, M., GREEN, C. A., GREENFALL, E., JOHNSON, J. D. A. & SMITH, N. (1948). The biological assay of streptomycin by a modified cylinder plate method. *J. gen. Microbiol.* **2**, 40.
- CRAWFORD, K., HEATLEY, N. G., BOYD, P. F., HALE, C. W., KELLY, B. K., MILLER, G. A. & SMITH, N. (1952). Antibiotic production by a species of *Cephalosporium*. *J. gen. Microbiol.* **6**, 47.

- GEBICKI, J. M. & JAMES, A. M. (1960). Spheroplasts of *Aerobacter aerogenes*. *J. gen. Microbiol.* **23**, 9.
- GRIDGEMAN, N. T. (1943). The technique of the biological vitamin A assay. *Biochem. J.* **37**, 127.
- HAHN, F. E. & CIAK, J. (1957). Penicillin-induced lysis of *Escherichia coli*. *Science*, **125**, 119.
- HALE, C. W., ABRAHAM, E. P. & NEWTON, G. G. F. (1958). Properties of some derivatives of cephalosporin C and heterocyclic tertiary bases. *Int. Congr. Biochem. Vienna*, **1**, 68.
- HALE, C. W., ABRAHAM, E. P. & NEWTON, G. G. F. (1961). Derivatives of cephalosporin C formed with certain heterocyclic tertiary bases—the cephalosporin C_A family. *Biochem. J.* **79**, 403.
- HALL, E., KAVANAGH, F. & ASHESHOV, I. N. (1951). Action of forty-five antibacterial substances on bacterial viruses. *Antibiot. & Chemother.* **2**, 369.
- HUGO, W. B. & RUSSELL, A. D. (1960). Action of 6-aminopenicillanic acid on Gram-negative bacteria. *Nature, Lond.* **188**, 875.
- HUMPHREY, J. H. & LIGHTBOWN, J. W. (1952). A general theory for plate assay of antibiotics—with some practical applications. *J. gen. Microbiol.* **7**, 129.
- LEDERBERG, J. (1956). Bacterial protoplasts induced by penicillin. *Proc. nat. Acad. Sci., Wash.* **42**, 574.
- MCQUILLEN, K. (1958). Lysis resulting from metabolic disturbance. *J. gen. Microbiol.* **18**, 498.
- MCQUILLEN, K. (1960). Bacterial Protoplasts. In *The Bacteria*, Vol. 1, p. 249. Ed. I. C. Gunsalus and R. Y. Stanier. New York: Academic Press.
- NEWTON, G. G. F. & ABRAHAM, E. P. (1954). Degradation, structure and some derivatives of cephalosporin N. *Biochem. J.* **58**, 103.
- NEWTON, G. G. F. & ABRAHAM, E. P. (1956). Isolation of cephalosporin C, a penicillin-like antibiotic containing D- α -aminoadipic acid. *Biochem. J.* **62**, 651.
- WOOD, E. C. (1944). Mathematics of biological assay. *Nature, Lond.* **153**, 681.