ACTION OF PENICILLIN ON *AEROBACTER CLOACAE*

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ABSTRACT

HUGO, W. B. (University of Nottingham, Nottingham, England), AND A. D. RUSSELL. Action of penicillin on *Aerobacter cloacae*. J. Bacteriol. **82**:411–417. 1961.—A study has been made of the action of penicillin on a penicillinase-producing strain of *Aerobacter cloacae*. It has been found that in culture media rendered hypertonic by the addition of sucrose (0.33 to 0.66 M), provided a source of magnesium ions is present, cell wall lesions occur, giving rise to osmotically fragile spheroplasts. Evidence is also presented of a lethal effect not associated with cell wall damage. Penicillinase production is not a complete challenge to the action of penicillin and in certain experimental situations renders the cells more susceptible to penicillin action.

In a previous paper (Hugo and Russell, 1960*a*), quantitative aspects of the action of penicillin in hypertonic media on a nonpenicillinase-producing strain of *Escherichia coli* have been studied. In this paper we report the action of penicillin on an organism selected from the *Enterobacteriaceae* which is an active penicillinase producer.

The relevant literature was reviewed by Hugo and Russell (1960*a*) but we would draw attention to the findings of Smith, Payne, and Watson (1960) who reported that penicillin does not induce spheroplasts when allowed to act on a strain of *Aerobacter cloacae* in hypertonic medium.

MATERIALS AND METHODS

Organism. A preliminary screening of members of the *Enterobacteriaceae* in our possession showed one organism which was able to destroy penicillin. This was originally received as *Aerobacter aerogenes* NCTC 8197, but its inability to produce gas from glycerol or to ferment inositol led us to examine it more fully.

The organism is a motile, gram-negative rod. On incubation at 37 C in peptone water containing bromocresol purple, the appropriate reagent, and a fermentation tube dispensed in 3-ml screw-capped bottles, acid and gas were produced within 24 hr from arabinose, rhamnose, glucose, sucrose, maltose, raffinose, mannitol, salicin, and xylose. Acid and gas were produced from lactose within 3 to 4 days and slight acid but no gas from glycerol in 7 days. The organism was without action on inulin, adonitol, dulcitol, or inositol. The IMVC characteristics were $--++$. Hydrogen sulphide was produced in a medium containing cysteine as sole sulfur source. Gelatin was liquefied. Urea was not hydrolyzed.

These characteristics are in agreement with the identity of the organism as *Cloaca cloacae* as set out by Brooke (1951) and Cowan (1956) and in Bergey's Manual (Breed, Murray, and Smith, 1957), in which the organism is named *Aerobacter cloacae*. We would point out that the strain of *Aerobacter cloacae* used in this work differs from that used in studies on the actions of 6-aminopenicillanic acid and "Celbenin" (Hugo and Russell, 1960*b*, *c*).

Culture media. Experiments were carried out in a nutrient medium containing in each liter: meat extract (Lab Lemco, Oxoid, England), 10 g; peptone (Oxoid), 10 g; and sodium chloride, 5 g. This medium was supplemented with magnesium sulfate, $MgSO_4 \cdot 7H_2O$, polyethylene glycol, or sugars as required. When a solid medium was required 2%, w/v, agar was incorporated.

Quantitative measurements. Microscopic observations and measurements and spheroplast counts were obtained with an interference microscope (Baker, London, England). Viable counts were made after serial dilution in distilled water. The first dilution tube contained sufficient penicillinase to destroy residual penicillin; this dilution was allowed to stand for 30 min, in which time it was found that all spheroplasts had lysed. The final plating was performed by the double layer agar technique to prevent spreading of the organism. Culture opacity was obtained

in a nephelometer (Evans Electroselenium, Ltd., Harlow, England). Penicillin determinations were made by the cylinder plate method. Membrane filters were used for sterilization of cultures prior to penicillin determinations.

Antibiotics. Penicillin was the sodium salt of benzylpenicillin of the British Pharmacopoeia 1958. The preparation used contained no added citrate or surface-active agent. 6-(2,6-Dimethoxybenzamido) penicillinate monohydrate, a synthetic penicillin shown to be resistant to staphylococcal penicillinase (Rolinson et al. 1960), was obtained from Beecham Research Laboratories, Ltd., Brentford, England, and is referred to hereafter as Celbenin. D-Cycloserine (D-4-amino-3-isoxazolidinone) was a gift from Roche Products, Ltd., London, England.

Other chemicals used were of analytical reagent quality.

RESULTS

Inhibition coefficient of penicillin towards Aerobacter cloacae. Bacteriostatic value for four temperatures and bactericidal effect at 37 C are shown in Tables 1 and 2.

TABLE 1. *Inhibition coefficient of penicillin toward Aerobacter cloacae in nutrient broth*

Penicillin concn	Presence or absence of growth after incubation at			
	18 C	25 C	30 C	37 C
units/ml				
1,000	+++[a]	+++	+++	+++
5,000	++	++	+++	+++
7,500	++	++	++	++
10,000	++	++	++	++
12,500	+	++	++	++
15,000	+	+	+	+
17,500	+	+	+	+
20,000	−	−	−	−

[a] +++, growth within 12 hr; ++, growth within 24 hr; +, growth within 48 hr; −, no growth after 48 hr.

TABLE 2. *Effect of penicillin at 37 C on viability of Aerobacter cloacae in nutrient broth*

Penicillin concn	Viable count at 0 hr	Viable count at 5 hr
units/ml	*per ml*	*per ml*
1,000	4.8×10^7	2.95×10^7
5,000	4.8×10^7	2.1×10^5
20,000	4.8×10^7	6.7×10^3

Pattern of penicillinase production. As stated in the introduction, preliminary experiments had shown that this organism produced penicillinase. Factors effecting penicillinase production and action were investigated in more detail.

To ascertain whether the penicillinase was extracellular, culture filtrates from 4 to 5 hr of growth of the organism in 10 ml of 0.33 M or 0.66 M sucrose conversion medium were added to 10-ml broth tubes containing 5 to 50 units/ml of penicillin, which were afterwards seeded with a penicillin-sensitive *Staphylococcus aureus.* Tubes including controls were incubated for 48 hr at 37 C. No growth occurred in any tube, which suggests that no extracellular penicillinase had been produced.

The production of intracellular penicillinase was investigated by growing the organism overnight on the surface of agar or sucrose-magnesium-agar and subjecting the washed cells (0.5 g, wet weight, in 5 ml of water) to disruption (Mickle, 1948). The preparations so obtained were sterilized by passage through a membrane filter, and the filtrate (0.2 ml in each case) tested for its effect on penicillin as described for extracellular penicillinase. Growth of *S. aureus* occurred in the tube containing 50 units/ml of penicillin, thereby giving evidence of an intracellular penicillinase.

Penicillin destruction was further investigated at 18 C, 25 C, and 37 C by sampling from the appropriate culture in a normal conversion cycle (0.5 ml of 17-hr culture inoculated into nutrient broth with 0.66 M sucrose and 0.25%, w/v,

TABLE 3. *Penicillin destruction, %, by growing culture of Aerobacter cloacae in nutrient broth containing 0.66 M sucrose and 0.25%, w/v, $MgSO_4 \cdot 7H_2O$*

Temperature	Time	Initial penicillin concentration	
		5,000 units/ml	20,000 units/ml
	hr	*% destruction*	
18 C	5	42.5	30
	24	56	39
25 C	5	46.5	35.4
	24	ca. 100	43.5
37 C	5	71.5	41.5
	24	ca. 100	61

$MgSO_4 \cdot 7H_2O$), sterilizing by membrane filtration, and assaying the filtrate for residual penicillin. The results, each the mean of two experiments, are summarized in Table 3.

When the initial penicillin concentration was 1,000 units/ml, approximately 100% had been destroyed after 5 hr at 37 C.

Growth characteristics of organism in various media used. These are illustrated in Fig. 1*a–d*. In Fig. 1*d* it can be seen that optimal growth is obtained with 0.33 M (11.4%, w/v) sucrose because of the ready utilization of sucrose as an energy source; the effect of 0.66 M (22.8%, w/v) sucrose retards the rate of growth, presumably because of the adverse effect of the high osmotic pressure. During the log phase (2nd to 5th hr) the pH falls from 7.2 to about 5.4 (0.33 M sucrose) or 5.9 (0.66 M sucrose). Results for lactose are shown in Fig. 1*b*.

Action of penicillin on Aerobacter cloacae. The summarized results at 37 C, with the use of four stabilising systems at three penicillin concentrations, are given in Table 4. In some instances, the characteristic osmotically fragile spheroplast was obtained to a greater or lesser extent, together with a variety of bizarre morphological variants (Fig. 2*a–c*). There were also present in every instance cells which could not be distinguished microscopically from normal organisms. Optimal conversion to spheroplasts at 37 C was obtained

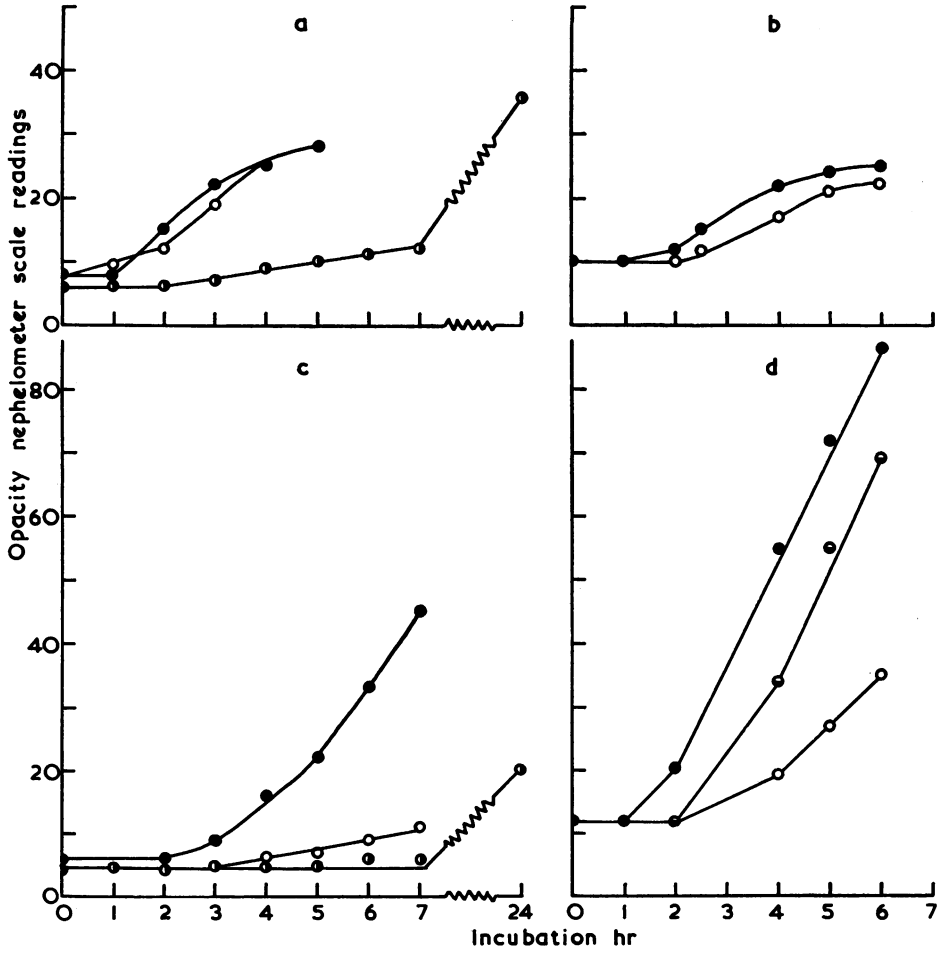

FIG. 1. *Growth of Aerobacter cloacae under various conditions. a—nutrient broth; b—nutrient broth containing 0.25%, w/v, $MgSO_4 \cdot 7H_2O$ and 0.33 M lactose; c—nutrient broth containing 0.25%, w/v $MgSO_4 \cdot 7H_2O$ and 0.66 M sucrose; d—nutrient broth containing 0.25% $MgSO_4 \cdot 7H_2O$ and 0.33 M sucrose.* ●——●, 37 C; ◒——◒, 30 C; ○——○, 25 C; ◑——◑, 18 C.

TABLE 4. *Effect of penicillin on Aerobacter cloacae in nutrient broth and 0.25%, w/v, $MgSO_4 \cdot 7H_2O$ and various stabilizers at 37 C*

Penicillin concn	Stabilizer	Viable count at 0 hr	Viable count at 5 hr	Spheroplast count at 5 hr
units/ml		*per ml*	*per ml*	*per ml*
1,000	Sucrose, 0.33 M	4×10^7	1.4×10^7	—[a]
5,000		4×10^7	2.1×10^4	10^6
20,000		4×10^7		10^6
1,000	Sucrose, 0.66 M	2.5×10^7	2.2×10^6	9.3×10^6 [b]
5,000		3.4×10^7	1.9×10^5	10^6
20,000		2.7×10^7	4.8×10^5	ca. 10^6
1,000	Polyethylene glycol, 7.5%, w/v,	7.2×10^7	6.3×10^7	—[c]
5,000		7.2×10^7	1.7×10^6	10^6
20,000		7.2×10^7	3.6×10^4	$<10^6$
1,000	Lactose, 0.33 M	2.2×10^7	8×10^5	2.4×10^6 [d]
5,000		2.2×10^7	3×10^4	ca. 10^6
20,000		2.2×10^7	4.5×10^4	$<10^6$

[a] Some spheroplasts but mainly long rod-like forms, and bizarre forms.
[b] Some bizarre forms observed, but not counted. No long rod forms.
[c] Osmotically stable, rod-like forms, some up to 40 μ in length.
[d] Several bizarre forms were also present, but were not counted.

at 1,000 units/ml of penicillin, with 0.66 M sucrose as stabilizer. This fact was established in six replicate experiments.

It was considered that this optimal conversion might be due to the slower growth rate occasioned by the high osmotic pressure which produces a condition of maximal sensitivity of the cell wall-synthesizing mechanism to penicillin or, alternatively, to a physicochemical phenomenon whereby, for maximal spheroplast formation, an osmotic pressure equivalent to 0.66 M sucrose is required (12 to 14 atmospheres). However, the spheroplast count obtained under these circumstances remains unchanged when the culture is diluted with an equal volume of nutrient broth, thereby reducing the sucrose concentration to 0.33 M; thus, the spheroplasts once formed are stable in an osmotic environment of six to seven atmospheres.

To investigate the effect of a slower growth rate, penicillin action was studied at 25 C and 0.33 M sucrose, which gives a rate of growth similar to that at 37 C and 0.66 M sucrose (Fig. 1*c*, *d*).

Microscopical examination of a 5-hr culture containing 1,000 units/ml of penicillin grown at 25 C showed mainly morphological variants, of the type shown in Fig. 2*e* and *f*, and unchanged cells, which suggests that growth rate alone was not responsible for the increased spheroplast yield.

Effect of temperature. Penicillin was allowed to act on the organism in broth containing 0.66 M sucrose and 0.25%, w/v, Mg^{++} at 18 C and 25 C, which considerably slowed the rate of visible growth (Fig. 1).

The results in Table 5 indicate that, after 5 hr at 18 C, no spheroplasts could be observed at any penicillin concentration, presumably because of lack of growth over this period, although there was a decrease in viable population. Prolonged incubation (24 hr) led to spheroplast induction; morphological variants (of type in Fig. 2*c*) were present only at 1,000 units/ml.

The use of 25 C, which allows growth to proceed rather more rapidly than at 18 C, induced some spheroplasts after 5 hr. After 24 hr, growth (presumably caused by destruction of penicillin by penicillinase) had occurred at 1,000 units/ml, but spheres were present at both the higher penicillin levels, although the yield with 20,000 units/ml was still low.

Effect of reduced inoculum size. The fact that inoculum size affects the susceptibility to penicillin of penicillinase-producing organisms was investigated in relation to spheroplast formation by adding a reduced volume of inoculum (0.1 ml of a 17-hr culture grown at 37 C) to 10 ml of

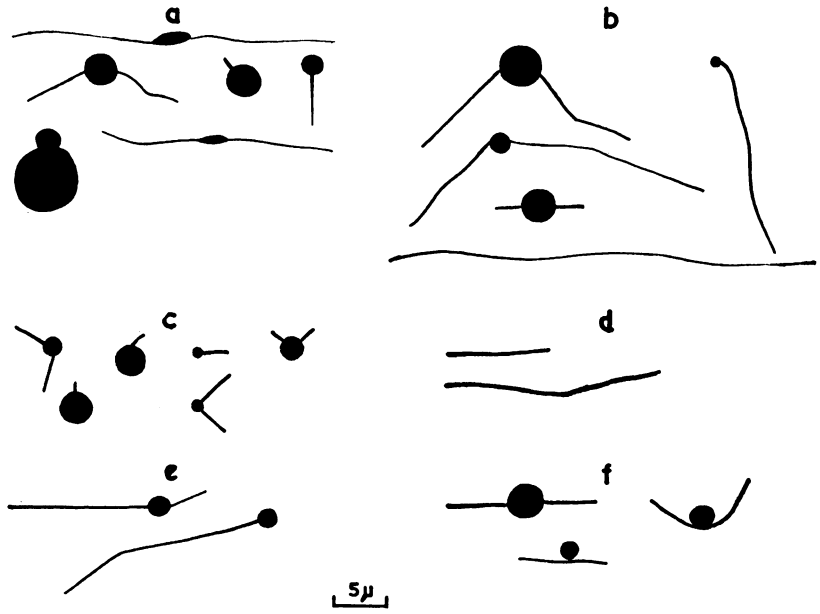

FIG. 2. *Diagrams of morphological variants induced in Aerobacter cloacae by antibiotics in nutrient broth containing 0.25%, w/v, $MgSO_4 \cdot 7H_2O$ with: a—1,000 units/ml of penicillin and 0.33 M sucrose; b—1,000 units/ml of penicillin and 7.5% w/v, polyethylene glycol; c—1,000 units/ml of penicillin and 0.66 M sucrose; d—720 μg/ml of Celbenin and 0.66 M sucrose; e—14.4 mg/ml of Celbenin and 0.66 M sucrose; f—21.6 mg/ml of Celbenin and 0.66 M sucrose.*

TABLE 5. *Effect of penicillin concentration and temperature on Aerobacter cloacae in nutrient broth containing 0.66 M sucrose and 0.25%, w/v, $MgSO_4 \cdot 7H_2O$*

Temperature	Penicillin concn	Viable count at 0 hr	Viable count at 5 hr	Spheroplast count at 5 hr	Viable count at 24 hr	Spheroplast count at 24 hr
	units/ml	*per ml*	*per ml*	*per ml*	*per ml*	*per ml*
18 C	1,000	3.9×10^7	1.1×10^7	—[a]		4.9×10^6
	5,000	3.9×10^7	2.7×10^6	—[a]		3.1×10^6
	20,000	2.7×10^7	4.5×10^6	—[a]	8.3×10^5	1.7×10^6
25 C	1,000	3.9×10^7	7.6×10^6	2.8×10^6[b]		(Growth)
	5,000	3.9×10^7	3.9×10^5	2×10^6	$>10^7$	4×10^6
	20,000	2.7×10^7	5.1×10^5		2.9×10^5	ca. 10^6
37 C	20,000	2.7×10^7	$4.8 \quad 10^5$	$<10^6$	6.2×10^4	$<10^6$

[a] Forms too small to differentiate spheres (if any) from rods.
[b] Low count due to high no. of bizarre forms.

broth containing 0.66 M sucrose, 0.25%, w/v, Mg^{++}, and varying amounts of penicillin. Tubes were examined after 5 and 24 hr of incubation at 18, 25, or 37 C. Results are shown in Table 6.

The interesting fact emerges that at slower growth rates (18 C and 25 C) no spheroplasts were seen at 5 hr but were formed within 24 hr at penicillin levels of 1,000 and 5,000 units/ml. At 37 C, however, a 54% yield of spheroplast was obtained after 5 hr by 1,000 units/ml, but at 24 hr this culture was microscopically indistinguishable from a 24-hr penicillin-untreated broth culture.

No spheroplasts were induced at any temperature by 20,000 units/ml, and few by 5,000 units/ml at 37 C.

Effect of Celbenin and cycloserine. In all studies of the action of penicillin on this organism, the

TABLE 6. *Effect of temperature on penicillin action on Aerobacter cloacae with reduced inoculum size in nutrient broth containing 0.66 M sucrose and 0.25%, w/v, $MgSO_4 \cdot 7H_2O$*

Temperature	Penicillin concn	Conversion after incubation for	
		5 hr	24 hr
	units/ml	%	
18 C	1,000	0	26[a]
	5,000	0	36
	20,000	0	< 10
25 C	1,000	0	46
	5,000	0	28
	20,000	0	< 10
37 C	1,000	54	(Growth)[b]
	5,000	0	ca. 10
	20,000	0	< 10

[a] Morphological variants present but not counted.

[b] Penicillinase production.

former is subject to decomposition by a penicillinase. To study the characteristic attack on cell wall synthesis without this complication, a brief experimental investigation was made with (i) Celbenin, a synthetic penicillin resistant to staphylococcal penicillinase (Rolinson et al., 1960), and (ii) cycloserine. The former has been shown (Hugo and Russell, 1960*b*) to induce cell wall lesions in gram-negative bacteria similar to those produced by benzylpenicillin, and the latter in *Escherichia coli* by Ciak and Hahn (1959) and by ourselves. Results obtained with Celbenin are shown in Table 7.

Microscopical examination of Celbenin-treated cultures revealed, at 720 μg/ml in 0.33 and 0.66 M sucrose media, mostly rod-shaped organisms 10 to 20 μ long or even longer (Fig. 2*d*). Even with Celbenin concentrations of 14.4 and 21.6 mg/ml, mostly aberrant forms (Fig. 2*e*, *f*), with very few long rod-shaped organisms and spheroplasts, were observed.

Viable counts at 5 hr indicate that the drug, even in high concentrations, has little effect on the viability of the organism.

D-Cycloserine, 200 μg/ml, was found to induce spheroplast formation in *Aerobacter cloacae* in broth containing 0.25%, w/v, $MgSO_4 \cdot 7H_2O$ and either 0.33 M or 0.66 M sucrose or lactose. The spheroplasts burst on dilution into water, and

TABLE 7. *Effect of Celbenin on Aerobacter cloacae in nutrient broth containing 0.25%, w/v, $MgSO_4 \cdot 7H_2O$ and 0.66 or 0.33 M sucrose*

Sucrose concn	Celbenin concn	Corresponding dose of penicillin G	Viable count at 0 hr	Viable count at 5 hr
M	*μg/ml*	*units/ml*	*per ml*	*per ml*
0.66	720	1,000	9×10^7	6.5×10^7
	3,600	5,000	9×10^7	2.1×10^7
	14,400	20,000	9×10^7	1.2×10^7
	21,600	30,000	9×10^7	1.1×10^7
0.33	720	1,000	9×10^7	9.8×10^7
	21,600	30,000	9×10^7	1.7×10^6

were not formed when the organism was treated with D-cycloserine in nutrient broth from which both Mg^{++} and sucrose had been omitted.

DISCUSSION

When penicillin acts upon *Aerobacter cloacae* it can be seen from experimental evidence presented in this paper that at least three biochemical events must be occurring.

1) The cell wall-synthesizing mechanism is affected, giving rise, in a hypertonic medium, to the characteristic osmotically fragile spheroplast and also to many other types of cell malformation.

2) A penicillin-destroying system is operative.

3) There is a killing effect other than a lysis due to defective cell wall synthesis.

The results obtained in this paper can, at least in part, be ascribed to the sequential or simultaneous operation of these three events. The killing effect is demonstrated by the inability of high (20,000 units/ml) penicillin levels to induce a 1-for-1 conversion of rods to spheres under a variety of conditions (Tables 4 to 6); furthermore, preliminary experiments with exponentially-dividing cells at 25 C and 37 C, in which low spheroplast yields were induced at this high penicillin concentration, suggested that its lethal effect was not solely concerned with active growth of the organism. By deliberately slowing the rate of cell division by using 0.66 M sucrose in place of 0.33 M sucrose (Table 4), or by using lower temperatures (Table 5), it was again possible to demonstrate killing of the cells without production of spheroplasts at this penicillin concentration.

Our results suggest that the ability of rods to

be transformed into spheroplasts depends to some extent on whether they are able to produce sufficient penicillinase to remove penicillin to a level at which cell division can commence. If this destruction is carried out at such a rate that the survivors of the initial lethal effect can divide rapidly, the resulting forms will be either morphological variants (Fig. 2) or "normal" rods. Retarding the growth rate will enable survivors of the initial lethal effect to be transformed into spheroplasts. Thus, in certain circumstances we have the paradoxical event that the production of penicillinase can render this organism more vulnerable to the lytic action of penicillin.

The low spheroplast levels obtained at 20,000 units/ml may be explained if bacteria are killed by a mechanism other than that initiating lysis (as found previously with *E. coli* (Hugo and Russell, 1960*a*)), since penicillin concentrations are still high even after 24 hr of incubation (Table 3). Celbenin, shown to have a similar cytotoxic action to benzylpenicillin (Hugo and Russell, 1960*b*), a fact confirmed by Gooder and Maxted (1961), and shown by Rolinson et al. (1960) to be resistant to staphylococcal penicillinase, but not to penicillinase produced by *Bacillus cereus*, induced mainly aberrant forms (Fig. 2*d-f*) even in high concentration. The ability of the organism to produce an enzyme "Celbeninase" (Barber, 1960), capable of inactivating the antibiotic, was not investigated.

By means of cycloserine, an antibiotic not destroyed by penicillinase, and by dilution experiments on spheroplasts induced by 1,000 units/ml of penicillin, the internal osmotic pressure of the spheroplasts was calculated as lying between five and six atmospheres.

It is of interest to note that Smith et al. (1960) were unable to induce spheroplasts by penicillin in their strain of *A. cloacae*, but did not state whether there was any loss of viability and whether there was any penicillinase production.

It is not possible to overlook the fact that the effect of penicillin on protein synthesis, permease activity, and amino acid incorporation may be contributing to the lethal effect other than lysis observed in this work (Gale and Folkes, 1953*a*, *b*).

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