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Relation of R Factor and Chromosomal β-Lactamase with the Periplasmic Space

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The release of several R factor and chromosomal β-lactamases by osmotic shock treatment was studied. It was found that those β-lactamases with a molecular weight of about 20,000 were released, but those with a molecular weight of about 30,000 to 44,000 were not released during osmotic shock. This differential release did not depend on whether the structural genes were on the chromosome or on the genome of an R factor. The release or retention of the β-lactamases appeared to be a characteristic of the enzyme rather than the host cell since the same results were obtained when the R factors were harbored by a variety of host bacteria. Studies with bacteria which produced more than one β-lactamase showed that each enzyme reacted independently to the presence of other β-lactamases produced by the host bacterium.

β-Lactamase (penicillinase or penicillin [cephalosporin] β-lactam amidohydrolase, EC 3.5.2.6) is produced by both gram-positive and gram-negative bacteria (2, 40). The structural genes for these enzymes can be situated on the bacterial chromosome or extrachromosomally.

Several enzymes have been shown to be released from bacterial cells during osmotic shock treatment (28-30, 33). The biochemical changes which occur in bacterial cells during this osmotic shock treatment have not been conclusively elucidated. During the process the cells are plasmolyzed with sucrose in the presence of tris(hydroxymethyl)aminomethane (Tris) buffer. Ethylenediaminetetraacetate (EDTA) is then added and seems to cause the main damage to the cell envelope. The evidence tends to suggest that Tris facilitates this damage, which consists mainly of a loss of cell wall lipopolysaccharide together with some release of protein and phospholipid (12, 18, 19, 27, 42, 43). Finally, the cells are exposed to a medium of very low osmotic pressure, i.e., distilled water, thus causing a rapid flow of water into the cytoplasm. This expands the plasma membrane against the mucopeptide layer, and hence some materials present in the previously enlarged periplasm (i.e., the region between the cell wall and the plasma membrane [24]) are forced out through the damaged cell wall (16).

Neu (26), using strains of *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella*, and *Citrobacter*, claimed that episomally mediated β-lactamases were completely released by the osmotic shock technique, whereas β-lactamases of chromosomal origin were bound to the cell more firmly. Curtis, Richmond, and Sykes (4), however, showed that the β-lactamase mediated by R factor TEM in *E. coli* K-12 was "periplasmic" (i.e., released by osmotic shock), regardless of whether its gene was chromosomal or carried on an R factor. This work also shows that the release of β-lactamases by osmotic shock treatment is independent of the location of the structural gene. Moreover, we show in this paper that the criterion which governs the retention or release of β-lactamases from gram-negative bacteria during osmotic shock treatment is the molecular weight of the enzyme. Those β-lactamases with a molecular weight of about 20,000 are released by osmotic shock, but those with molecular weights of 30,000 or more are retained by a variety of gram-negative bacteria during osmotic shock treatment. In addition, when bacteria produce more than one distinct β-lactamase, each enzyme reacts to osmotic shock as if the cells produced that enzyme singly. (Some of the work reported here formed part of a Ph.D. Thesis by J. M. W. accepted by the Univ. of London.)

MATERIALS AND METHODS

Bacterial strains. The organisms used as hosts for various plasmids are shown in Table 1. The R factors studied were R TEM (conferring resistance to ampicillin and streptomycin), R 7268 (conferring resistance to ampicillin, streptomycin, sulfonamide, chlor-

TABLE 1. *Bacterial strains*[a]

Bacteria	β-Lactamase	β-Galactosidase	Reference
Escherichia coli K-12 strains			
J6-2	−	−	3
58.161[b]	−	+	23
D3	+	−	1
G11a1	+	+	10
E. coli 214T	+	+	37
E. coli 419	+	+	15
Klebsiella aerogenes 418	+	+	13
Proteus mirabilis F67	−	−	34

[a] + = Presence of enzymatic activities controlled by genes situated on the bacterial chromosome; − = absence of enzymatic activity.

[b] This strain has been termed K-12 throughout the text.

amphenicol, and kanamycin), and R 1818 (conferring resistance to ampicillin, streptomycin, sulfonamide, and tetracycline) (8). Datta and Kontomichalou (8) showed that in each case the ampicillin resistance was due to a β-lactamase. R TEM has also been referred to as R6K (17), R 7268 has been called R 1 (23) and R 1818 has been referred to as R 46 (23). R 7268 and R 1818 were isolated from strains first characterized by E. S. Anderson. *E. coli* strains K-12 and J6-2 harboring these three R factors from a previous study (38) were used as donors to transfer an R factor to another organism. The P-*lac* element (11) was used when an extrachromosomally mediated β-galactosidase was required. *E. coli* strain J6-2 (P-*lac*) was kindly donated by E. Meynell and was used to construct *Proteus mirabilis* F67 (P-*lac*) which was then used as the P-*lac* donor in this study. H. G. Boman kindly provided strains D3 and G11a1 which produce β-lactamase from genes located on the chromosome of *E. coli* (1, 10).

The molecular weights of the β-lactamases produced by these bacteria and by bacteria harboring R factors are shown in Table 2.

Media and cultural conditions. Bacteria were routinely grown overnight at 37 C in 65-ml amounts of Oxoid no. 2 nutrient broth, and viable counts were usually carried out on MacConkey agar (Oxoid Ltd.). Viable counts were also made on media containing 5.0 μg of ampicillin per ml to estimate the number of R^+ bacteria with the following exceptions: *E. coli* strains 214T(7268) and 214T(1818)—120 μg of ampicillin per ml; *Klebsiella aerogenes* strains 418(7268) and 418(1818)—5 μg of streptomycin per ml; *E. coli* strains K-12(TEM/1818) and K-12(1818/TEM)—10 μg of tetracycline and 200 μg of ampicillin per ml; K-12(7268/1818)—10 μg of tetracycline and 10 μg of chloramphenicol per ml; and K-12 (TEM/7268)—10 μg of chloramphenicol and 200 μg of ampicillin per ml. Ampicillin was kindly donated by Beecham Research Laboratories; streptomycin was donated by Glaxo Research Ltd.; tetracycline was donated by Cyanamid of Great Britain Ltd.; and chloramphenicol was given by Parke, Davis & Co. Minimal salts medium when required was that of Davis and Mingioli (9) made and supplemented as described by Smith (38). This was solidified by Davis standard agar (Davis Gelatin Ltd.) to a final concentration of 1.5%. All cultures were grown without aeration. Cultures in nutrient broth or on MacConkey agar were incubated overnight, whereas those on minimal salts medium were incubated for 2 to 3 days.

In vitro transfer experiments. The transfer of extrachromosomal elements was carried out in liquid cultures, and the organisms were then plated out on suitably supplemented minimal salts medium as described by Smith (38). R^+ bacteria were routinely selected on media containing 2.5 μg of ampicillin per ml. However, 10 μg of streptomycin per ml was used for the selection of *E. coli* strains 214T(7268) and 214T(1818), and for the double R factor strains of *E. coli* K-12 the following antibiotic concentrations were used to select for the second R factor being introduced: K-12(7268/1818) and K-12(TEM/1818)—10 μg of tetracycline per ml; K-12(1818/TEM)—400 μg of ampicillin per ml; and K-12(TEM/7268)—10 μg of chloramphenicol per ml. Possible transfers were confirmed by extensive streakouts on selective media. For P-*lac* transfer, an exponential donor culture of *P. mirabilis* F67(P-*lac*) was used, the cells were spun together at the start of the mating period to promote cell contact, and 5.6 mg of lactose per ml in minimal salts medium without citrate was used for selection.

Osmotic shock treatment. The method used for osmotic shock treatment was based on that of Neu and Chou (28). The cells from 300 ml of an overnight nutrient broth culture were precipitated by centrifuging at 5,000 × *g* for 20 min at 2 C. The pellet was resuspended in 12 ml of 10 mM Tris-hydrochloride buffer (pH 7.3) containing 30 mM NaCl at 4 C; i.e., this suspension was 25 times more concentrated than the overnight culture and contained about 10^{10} cells/

TABLE 2. *Molecular weights of the β-lactamases*

β-Lactamase	Location of gene[a]	Mol wt	Reference
Klebsiella aerogenes 418	C	20,080	7
Escherichia coli 214T	C	31,150	7
E. coli 419	C	31,530	7
E. coli G11a1	C	29,000	21
E. coli D3	C	29,000	21
R-TEM	R	21,110	7
R-7268	R	20,600	7
		22,000	20
R-1818	R	44,600	5, 6

[a] C, Chromosome; R, R factor-mediated β-lactamase when *E. coli* K-12, which otherwise lacks a significant level of β-lactamase, was the bacterial host.

ml. The suspension was halved to give control and test samples, and each was centrifuged at 5,000 × g for 20 min at 2 C. The control pellet was resuspended to 6 ml with 25 mM sodium phosphate buffer (pH 7.4) at 4 C and then ultrasonically disintegrated to obviate crypticity as described by Smith (38).

The test pellet was resuspended in 20% sucrose in 30 mM Tris-hydrochloride buffer (pH 7.3) at room temperature, and then sufficient EDTA (disodium salt) was added to a final concentration of 1 mM and a final volume of 6 ml. The mixture was stirred for 5 min, and the cell suspension was centrifuged at 5,000 × g for 20 min at 2 C. The cells in the pellet were then osmotically shocked by resuspension to 6 ml with distilled water at 4 C, the tube containing the cells being kept in crushed ice water. The cell suspension was stirred for 5 min with a glass rod. The mixture was centrifuged at 5,000 × g for 20 min at 2 C, and both the pellet and the supernatant were retained. The supernatant formed the osmotic shock supernatant (OSS) and contained any enzymes which may have been released by the osmotic shock treatment. For enzyme assays on the supernatant, 0.5 M sodium phosphate buffer (pH 7.4) was added to give a final concentration of 25 mM, i.e., comparable with the phosphate concentration in the control sample. The pellet formed the osmotic shock cells (OSC). To release those enzymes retained by these cells during the osmotic shock treatment, they were resuspended to 6 ml with 25 mM phosphate buffer (pH 7.4) at 4 C and ultrasonically disintegrated. Both the control and the OSC ultrasonically disintegrated extracts were centrifuged at 32,000 × g for 1 h at 2 C to precipitate inert cell debris. The supernatant fluids were reserved as crude enzyme preparations which were used for enzyme estimations together with the OSS.

Throughout the osmotic shock technique, the pellets of *E. coli* were resuspended by use of a glass rod and bench Vortex mixer, but this method was unsatisfactory with *K. aerogenes* since clumps could not be dispersed satisfactorily. Control experiments showed that 35-s treatment with a laboratory mixer emulsifier (Vortex Mixers) dispersed the clumps without damaging the cells as judged by viable counts. Hence, in all osmotic shock experiments with *K. aerogenes*, the test and control pellets were resuspended in this way.

Assay of β-lactamase activity. β-lactamase was assayed by use of the hydroxylamine method as described by Dale and Smith (6). Routinely, benzylpenicillin (kindly donated by Glaxo Laboratories Ltd.) was used as the substrate at a final concentration of 1.8 mg/ml in each reaction mixture. When activity against sodium oxacillin (kindly donated by Bristol Laboratories) or sodium ampicillin ("Penbriten," kindly donated by Beecham Research Laboratories) were estimated, a final concentration of 2 mg/ml was used. The assay was carried out at 30 C. Enzyme activities were expressed as micromoles of substrate hydrolyzed per min per 10^9 viable cells, i.e., standard international units (IU) (41) per 10^9 viable cells. In practice, milliunits were used where 1 mU = 0.001 U.

Assay of β-galactosidase activity. β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) was assayed by measuring at room temperature the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) at a concentration of 1 μmol/ml in 0.25 M sodium phosphate buffer (pH 7.4). The release of *o*-nitrophenol (ONP) was followed colorimetrically at 420 nm, and enzyme activities were expressed as micromoles of ONP released per 10^9 viable cells, i.e., standard international units (41) per 10^9 viable cells. Again, in practice milliunits were used. This enzyme is not normally released from the cells during osmotic shock treatment (28–30, 33) and was therefore included in the experiments to check that lysis of the cells had not occurred.

RESULTS

Osmotic shock of bacterial strains possessing chromosomally mediated β-lactamases. *E. coli* strains 214T, 419, G11a1, and D3, and *K. aerogenes* 418 were subjected to the osmotic shock procedure. In all experiments the percentage of recoveries and percentage of releases of both β-lactamase and β-galactosidase were estimated. To calculate the percentage release of the enzymes, the activity of the osmotic shock supernatant was expressed as a percentage of the total activity in the OSS plus that recovered from the OSC. The percentage of recovery was calculated from the total activity in the OSS and OSC divided by the activity recovered from the control cells. The results (Table 3) show that, whereas most of the β-lactamase of *K. aerogenes* 418 was released by osmotic shock, none of the β-lactamase from *E. coli* strains 214T, 419, G11a1, and D3 was released by this treatment. It was found that all these bacteria synthesized β-galactosidase even though strain D3 is classified as a non-lactose fermenter on MacConkey agar. It would seem that the β-galactosidase permease system is either defective or lacking in this strain. Consequently, β-galactosidase was used in all these bacteria to check for bacterial lysis, and it was found that β-galactosidase was not released in any experiment.

Osmotic shock of bacterial strains possessing R factors mediating β-lactamases. Since the previous experiment showed that one β-lactamase mediated by a chromosomal gene could be released by osmotic shock, in contrast to the hypothesis of Neu (26), the behavior of R factor-mediated β-lactamase during osmotic shock was next investigated. *E. coli* K-12 strains carrying each of the R factors TEM, 7268, and 1818 were subjected to the osmotic shock procedure. In addition, strains of J6-2 harboring these three R factors were also tested for release of β-lactamase by osmotic shock. Since this strain is a non-lactose fermenter, the P-*lac* element was introduced by mating with *P.*

TABLE 3. *Absolute activities: Percentage of release and recovery of β-lactamase and β-galactosidase during osmotic shock treatment of bacteria producing one β-lactamase*

Organism	Extract[a]	β-Lactamase[b]			β-Galactosidase		
		mU/10⁹ bacteria	Release[c] (%)	Recovery[d] (%)	mU/10⁹ bacteria	Release[c] (%)	Recovery[d] (%)
Escherichia coli 214T	C	18.0			2.2		
	OSC	18.8	0	104.4	2.1	0	95.5
	OSS	0			0		
E. coli 419	C	36.3			6.3		
	OSC	31.0	0	85.4	5.2	0	82.5
	OSS	0			0		
E. coli G11a1	C	28.7			1.8		
	OSC	28.2	0	98.3	1.8	0	100.0
	OSS	0			0		
E. coli D3	C	30.0			1.4		
	OSC	29.5	0	98.3	1.4	0	100.0
	OSS	0			0		
Klebsiella aerogenes 418	C	10.4			66.3		
	OSC	3.0	74.4	112.5	62.2	0	93.8
	OSS	8.7			0		
Escherichia coli K-12 (TEM)	C	604.2			2.1		
	OSC	34.3	95.9	138.9	2.6		123.8
	OSS	804.8			0	0	
E. coli K-12 (7268)	C	143.5			1.8		
	OSC	8.8	94.8	117.6	1.6	0	88.9
	OSS	160.0			0		
E. coli K-12 (1818)	C	34.6			1.9		
	OSC	26.9	11.5	87.9	1.7	0	89.5
	OSS	3.5			0		
E. coli J6-2 (TEM/P-*lac*)	C	457.8			5.0		
	OSC	13.3	97.0	98.2	5.4	0	108.0
	OSS	436.2			0		
E. coli J6-2 (7268/P-*lac*)	C	129.9			5.7		
	OSC	6.9	94.6	98.8	5.6	0	98.2
	OSS	121.4			0		
E. coli J6-2 (1818/P-*lac*)	C	128.8			4.9		
	OSC	122.3	5.2	100.2	4.5	0	91.8
	OSS	6.7			0		

[a] Key to extracts: C, control; OSC, osmotic shock cells; OSS, osmotic shock supernatant.
[b] Benzylpenicillin was used as the substrate for the β-lactamase assays except for *E. coli* J6-2 (1818/P-*lac*) when oxacillin was the substrate.
[c] From extracts OSC and OSS.
[d] From all three extracts-control, OSC, and OSS.

mirabilis F67 (P-*lac*). Osmotic shock of the R^+ J6-2 (P-*lac*) strains was done not only to test the behavior of R factor-mediated β-lactamases in a different host but also to test whether episomally mediated β-galactosidases behave differently to a β-galactosidase of chromosomal origin during osmotic shock. The results (Table 3) show that, whereas almost all the β-lactamase mediated by R factors TEM and 7268 could be released by osmotic shock, that mediated by R 1818 was essentially retained during osmotic shock treatment. The results were the same in *E. coli* strains K-12 or J6-2, and although the β-galactosidase gene of the former is on the chromosome whereas that of the latter is episomal, no release of this enzyme was observed during osmotic shock treatment. The retention of β-lactamase mediated by R 1818 therefore contradicts Neu's other hypothesis that β-lactamases mediated by episomes are released by osmotic shock treatment.

Osmotic shock of bacterial strains possessing both chromosomal and R factor-mediated β-lactamases. Since both the previous experimental sections have contained results which disagree with Neu's findings, the behaviors of R factor- and chromosomal-mediated β-lactamases were investigated in more detail in cells which simultaneously produced both types of enzymes. To do this, strains of *E. coli* 214T and

K. aerogenes 418 harboring R 7268 and R 1818 were subjected to the osmotic shock technique. (Strains of these bacteria harboring R TEM were not used since the previous results indicated that the β-lactamase mediated by R 7268 acted on osmotic shock similarly to the β-lactamase conferred by R TEM; indeed, Dale and Smith (7) have demonstrated that these enzymes are biochemically indistinguishable.) Since the bacteria being tested produced two different β-lactamases, it was necessary to differentiate between them to follow their separate retention or release during osmotic shock. This was done by using the different substrate profiles of the various enzymes as follows.

(i) E. coli 214T(1818). The β-lactamase conferred by R 1818 hydrolyzes oxacillin readily (38), whereas the β-lactamase mediated by the chromosomal gene of strain 214T (the 214T enzyme) has no significant activity on this substrate. The hydrolysis rates for oxacillin obtained with strain 214T(1818) were therefore ascribed to its R factor. Since both enzymes hydrolyze benzylpenicillin, their separate activities were calculated by assuming that strain 214T(1818) contained the same 214T enzymatic activity against benzylpenicillin as strain 214T which lacked an R factor, and by using the oxacillin-benzylpenicillin ratio obtained for the enzyme mediated by R 1818 alone in *E. coli* K-12(1818). These last two strains were treated in the same way as the control sample of strain 214T(1818).

(ii) E. coli 214T(7268). The 214T enzyme does not hydrolyze ampicillin (37), whereas the enzyme mediated by R7268 does (8). The hydrolysis rates for ampicillin obtained with strain 214T(7268) were therefore ascribed to its R factor. As both enzymes hydrolyze benzylpenicillin, their separate activities were calculated by assuming that strain 214T(7268) contains the same 214T enzymatic activity against benzylpenicillin as strain 214T, which lacked an R factor, and by using the ampicillin-benzylpenicillin ratio obtained for the enzyme mediated by R 7268 alone in *E. coli* K-12(7268). These last two strains were treated in the same way as the control sample of strain 214T(7268).

(iii) K. aerogenes 418(1818). The chromosomal and R factor-mediated enzymes were differentiated using the hydrolysis rates of strains 418(1818) and K-12(1818) against oxacillin and benzylpenicillin, and by using the benzylpenicillin hydrolysis rate of strain 418 lacking an R factor as detailed above for strain 214T(1818).

(iv) K. aerogenes 418(7268). There is no substrate which can clearly differentiate between the two β-lactamases of this organism, so the extracts were only assayed against benzylpenicillin. In this case it was assumed that the release of the enzyme mediated by the chromosome of strain 418(7268) during osmotic shock was 74.4% (i.e., the percentage release from strain 418 lacking an R factor [see Table 3]). The remainder of the activity on benzylpenicillin was then ascribed to R 7268.

The results (Table 4) show that the β-lactamase mediated by R 7268 and the β-lactamase of chromosomal origin in *K. aerogenes* 418 (the 418 enzyme) were substantially released by osmotic shock from any strain producing either or both of these enzymes. However, the 214T enzyme and that mediated by R 1818 were

TABLE 4. *Percentage of release and recovery of β-lactamase and β-galactosidase during osmotic shock treatment of bacteria producing two β-lactamases*

Organism	Type of β-lactamase	β-Lactamase		β-Galactosidase	
		Release (%)	Recovery (%)	Release (%)	Recovery (%)
Escherichia coli 214T (7268)	214T	0.3	101.0[a]	0	102.8
	R 7268	93.7	101.2[a]		
E. coli 214T (1818)	214T	0.4	88.9[b]	0	90.5
	R 1818	6.5	89.0[b]		
Klebsiella aerogenes 418(7268)	418	74.4[c]	89.2 (total enzyme)	0	77.3
	R 7268	87.8			
K. aerogenes 418(1818)	418	64.4	87.7[b]	0	91.7
	R 1818	2.3	85.6[b]		

[a] Results obtained by calculation from activities on benzylpenicillin and ampicillin.
[b] Benzylpenicillin and oxacillin.
[c] Assumed release; see text for details. Absolute enzyme activities were similar to those in Table 3.

almost completely retained during this treatment, again irrespective of the strain being tested. It should be noted that the release of the 418 enzyme from strain 418(1818) was 64.4%, and if this proportion was also released by strain 418(7268), then the release of the β-lactamase mediated by R7268 from the latter strain would be 88.5%. Thus, however calculated, the R factor-mediated enzymes acted similarly in two different bacterial host species regardless of whether the host bacterium produced a β-lactamase which was released or retained during osmotic shock. Again no release of β-galactosidase was observed in any experiment.

Osmotic shock of bacterial strains producing two R factor-mediated β-lactamases. The previous results have demonstrated that β-lactamases can be retained or released during osmotic shock independent of the presence of a second β-lactamase in the same cell. Since only organisms carrying both a chromosomal and an R factor-mediated β-lactamase had been tested, it was decided to investigate whether two R factor-mediated β-lactamases present in the same cell could react to osmotic shock treatment as they did when present alone. The *E. coli* K-12 strains harboring two different R factors were constructed by using an appropriate *E. coli* J6-2 donor: for example, strain K-12(TEM/1818) was obtained from a mating between strains J6-2(1818) as donor and K-12(TEM) as recipient. Strains K-12(1818/TEM), K-12(7268/1818), and K-12(TEM/7268) were similarly constructed.

The doubly R^+ strains were then subjected to osmotic shock treatment, and the extracts were assayed using benzylpenicillin as substrate. Oxacillin was additionally used as substrate for strains which harbored R 1818, this hydrolysis rate being ascribed solely to R 1818 in these strains. When R TEM was present in the same cell as R 1818, the benzylpenicillin rate was ascribed to R TEM, since the rate of hydrolysis of benzylpenicillin by the β-lactamase mediated by R 1818 is insignificant compared with that rate conferred by R TEM. For strain K-12(7268/1818), the R 7268-mediated β-lactamase activity against benzylpenicillin was calculated by using the oxacillin-benzylpenicillin ratio obtained in strain K-12(1818). There was no way of differentiating between the two β-lactamases produced by strain K-12(TEM/7268).

The results (Table 5) show that the β-lactamases conferred by R TEM or by R 7268 were almost completely released by osmotic shock and that this release was not affected by the presence of another R factor-mediated β-lactamase in the same cell. The additional presence of R 7268 did not increase the release of the enzyme conferred by R 1818. On the other hand, somewhat more R 1818-mediated β-lactamase seemed to be released on osmotic shock when R TEM was also harbored by the cell than when R 1818 was the sole R factor; 22.5 and 39.6% of the R 1818-mediated β-lactamase was released from strains K-12(1818/TEM) and K-12(TEM/1818), respectively.

DISCUSSION

It is generally considered that enzymes which can be released by osmotic shock treatment are surface or periplasmic enzymes; i.e., they are not found in the cytoplasm of the cell (28–30).

TABLE 5. *Percentage of release and recovery of β-lactamase and β-galactosidase during osmotic shock treatment of bacteria producing two R factor-mediated β-lactamases*

Organism	Type of β-lactamase	β-Lactamase		β-Galactosidase	
		Release (%)	Recovery (%)	Release (%)	Recovery (%)
Escherichia coli K-12 (TEM/1818)	R TEM R 1818	93.4 22.5	101.4[a] 103.7[a]	0	100.0
E. coli K-12 (1818/TEM)	R TEM R 1818	94.3 39.6	89.8[a] 95.6[a]	0	88.0
E. coli K-12 (7268/1818)	R 7268 R 1818	100.0 6.0	100.6[a] 91.9[a]	0	95.7
E. coli K-12 (TEM/7268)	R TEM R 7268	96.4	100.2[b]	0	100.0

[a] Results obtained by calculation from activities on benzylpenicillin and oxacillin.
[b] Benzylpenicillin alone; see text for details. Absolute enzyme activities were similar to those in Table 3.

However, on fundamental grounds it seems probable that such enzymes have merely become located outside the plasma membrane subsequent to their synthesis on ribosomes in the cytoplasm. With the β-lactamases studied here, a completely external location can be ruled out, since a differential permeability barrier between them and their substrates has been shown for the β-lactamases of chromosomal origin in *E. coli* (strains 214T and 419) and *K. aerogenes* 418, as well as for the β-lactamases mediated by R factors TEM, 7268, and 1818 when *E. coli* K-12 is host (8, 15, 37, 39, 40). Therefore, if the β-lactamases are located externally to the plasma membrane, the cell wall or outer cell membrane must act as a permeability barrier. Hamilton-Miller (14) reported that the crypticity to penicillins was reduced by EDTA treatment, and this could suggest that lipopolysaccharide or those other cell wall constituents removed by EDTA function as a permeability barrier. This was also proposed by Monner, Jonsson, and Boman (25) who found that ampicillin-resistant mutants of *E. coli* K-12 which were less permeable to ampicillin had alterations in the lipopolysaccharide layer. Normark, Boman and Bloom (32), working with their chain-forming mutant of *E. coli* K-12, also found that the outer layers of the cell can have a barrier function. Scherrer and Gerhardt (36), working with *Bacillus megaterium*, demonstrated that the cell wall, which in this organism consists mainly of a teichoic acid-mucopeptide complex (i.e., without lipids), acts as a molecular sieve. It is clear, therefore, that several layers of cell wall can function as permeability barriers and retain bacterial enzymes under normal physiological conditions.

Neu (26) postulated that β-lactamases which were episomally mediated were released by osmotic shock treatment and were therefore surface or periplasmic enzymes. The present paper shows that the β-lactamases conferred by R factors TEM and 7268 were released by osmotic shock treatment, but the β-lactamase mediated by R 1818 was retained during osmotic shock. Moreover, these three β-lactamases exhibited the same responses in a variety of host bacterial species. Hence, our results with R factor β-lactamases contradict the hypothesis of Neu (26). Since the molecular weight of the β-lactamase mediated by R 1818 (44,600) is the largest known for an enzyme of this type (5, 6), it seems possible that this fact has a bearing on its response to osmotic shock. The molecular weights of the β-lactamases conferred by R TEM (21,110 [7]) and by R 7268 (20,600 [7], 22,000 [20]) are about 20,000, and hence it would seem that osmotic shock treatment can release the β-lactamases of R factors in this range but not those of molecular weight 44,600.

Neu (26) also suggested that β-lactamases specified by chromosomal genes are bound more firmly in bacteria, since he found that at least 90% of each enzyme of this type was retained during osmotic shock treatment. However, Curtis et al. (4) showed that the β-lactamase of *E. coli* K-12 (TEM) was periplasmic regardless of whether its structural gene was on the chromosome or carrier on an R factor. The present work shows that only some enzymes of chromosomal origin are retained by bacteria during osmotic shock; i.e., the β-lactamases of *E. coli* strains 214T, 419, G11a1, and D3 were retained. In contrast, 74.4% of the *K. aerogenes* 418 chromosomally mediated β-lactamase was found to be released by osmotic shock. The extent of this release of the β-lactamase from *K. aerogenes* 418 can possibly be equated with its complete release since Neu and Chou (28) showed that enzymes which were totally released from *E. coli* were at best only 50% released in *Klebsiella pneumoniae* or 70 to 80% released in *Enterobacter aerogenes*.

Thus it seems that the retention or release of β-lactamases specified by chromosomal genes during osmotic shock is also governed by their molecular weight. The *K. aerogenes* 418 β-lactamase has a molecular weight of 20,080 (7), whereas the *E. coli* enzyme of chromosomal origin has molecular weights of 29,000 in strains G11a1 and D3 (21), 31,150 in strain 214T (7), and 31,530 in strain 419 (7). Therefore, taking the results of the R factor-mediated and chromosomally specified β-lactamases as a whole, it would seem that the location of the gene for their synthesis is unimportant but that their behavior during osmotic shock is a function of the molecular weight of the enzyme molecule. Hence, only β-lactamases of molecular weights in the region of 20,000 are released, whereas any β-lactamase of molecular weight about 30,000 or higher is retained during osmotic shock treatment. Sawai, Takahashi, Yamagishi, and Mitsuhashi (35) obtained release by osmotic shock treatment of two β-lactamases mediated by R factors GN14 and GN823 of molecular weights 20,600 and 22,600, respectively, and these results support our hypothesis. Against our hypothesis is the work of Neu and Winshell (31) who seemed to obtain release by osmotic shock of four β-lactamases of molecular weights of about 30,000—those mediated by R factor B1H9, those mediated by an R factor in *Klebsiella*, and even from strains of *E. coli* and *Salmonella St. Paul* lacking R factors—but the

actual percentage of release was not given for each enzyme.

On the other hand, our results could be taken to suggest that the β-lactamases mediated by R TEM and R 7268 and the β-lactamase of *K. aerogenes* 418 were periplasmic, whereas the β-lactamase mediated by R-1818 and the β-lactamases of chromosomal origin in *E. coli* strains 214T, 419, G11a1, and D3 were not periplasmic. However, since all these β-lactamases can be released by spheroplast formation (J. M. Wyatt, Ph.D. thesis, Univ. of London, 1972), a process also considered to release surface enzymes (22, 29, 30), we conclude that osmotic shock treatment simply damages some layer of the cell wall so that β-lactamases of about 20,000 molecular weight escape while larger β-lactamases with a molecular weight of 30,000 or more are retained despite the damage to the cell wall. It is tempting to assume that such molecular sieving may be taking place at the mucopeptide layer which is not damaged by osmotic shock treatment.

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