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THE EFFECT OF OSMOLALITY ON THE RESPONSE OF *ESCHERICHIA COLI* AND *PROTEUS MIRABILIS* TO PENICILLINS

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Summary.—The response of broth cultures of *Escherichia coli* and *Proteus mirabilis* to ampicillin was continuously monitored turbidometrically. The rapid lytic response which follows a few minutes after addition of the antibiotic as well as the later slow lysis of spheroplasts could be manipulated by altering the osmolality of the medium. A higher osmolality was found to be necessary to protect *Esch. coli* than *Pr. mirabilis* and this explains the differential response of the 2 organisms that we have previously described.

IN a number of recent papers (Greenwood and O'Grady, 1969; O'Grady *et al.*, 1970) we have drawn attention to the marked variability of response to penicillins of cultures of *Esch. coli* and *Pr. mirabilis*. One of the most striking findings was that populations of these organisms showed a trimodal response to the presence of inhibitory concentrations of benzylpenicillin and ampicillin (Greenwood and O'Grady, 1970). With both types of organism there was rapid lysis of part of the population, conversion of most of the survivors to spheroplasts and bacteristasis of the remainder, but a systematic quantitative difference in this respect was found between *Esch. coli* on the one hand and *Pr. mirabilis* on the other. Non-swarming variants of *Pr. mirabilis* were found to occupy an intermediate position (Greenwood and O'Grady, 1969).

Further investigation of these phenomena has led to a number of new observations which shed further light on inter- and intra-population differences of response.

MATERIALS AND METHODS

A strain of *Esch. coli* and one of a typical swarming strain of *Pr. mirabilis*, both originally derived from urinary tract infections, were used throughout. The minimum inhibitory concentration (MIC) of ampicillin for both organisms, as judged by a tube titration test using conventional inocula (*ca* 10^5 organisms/ml) was 4 μg/ml.

Growth measurements were made using the opacity monitoring system described by Watson and his colleagues (1969). The culture medium used was either Oxoid Nutrient Broth No. 2, enriched with 5 g/l yeast extract (medium A) or a " complete " medium containing (per litre) 10 g " Casitone " pancreatic digest (Difco), 5 g yeast extract (Difco), 5 g glucose, 3 g K_2HPO_4, 1 g KH_2PO_4 (medium B). Various additions were made to these basic media as indicated in the text.

Cultures were grown in 25 ml of broth and when the opacity trace showed the culture to be in the mid-to-late logarithmic phase of growth (viable count *ca* 5×10^7–10^8/ml), ampicillin (" Penbritin ", Beecham Research Laboratories) was added to give a concentration of 50 μg/ml.

Osmolality estimations were made by the cryostatic method using an " advanced " osmometer (Advanced Instruments Inc.).

RESULTS

The effect of ampicillin on log phase cultures of *Esch. coli* and *Pr. mirabilis* in medium A and medium B is shown in Fig. 1 and 2. The *Esch. coli* lysed rapidly in both media (Fig. 1) but the marked lysis of *Pr. mirabilis* seen in medium B was partially inhibited in medium A (Fig. 2). The osmolality of medium A (234 mOsm/kg) was found to be substantially higher than that of medium B (156 mOsm/kg), due probably to the salt content of Oxoid nutrient broth No. 2 (stated by the manufacturers to be 5 g NaCl/l). The effect on penicillin lysis of

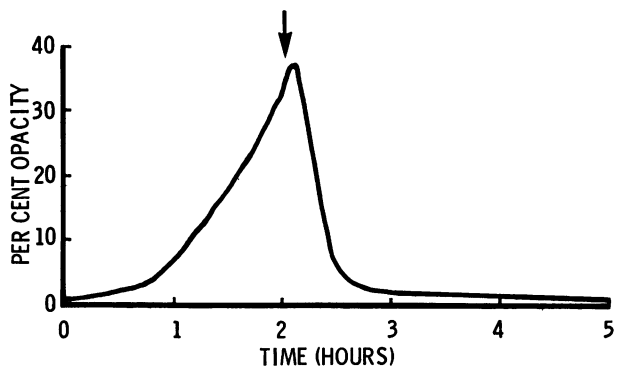

FIG. 1.—*Esch. coli* grown in medium A or medium B. 50 μg/ml ampicillin added at arrow.

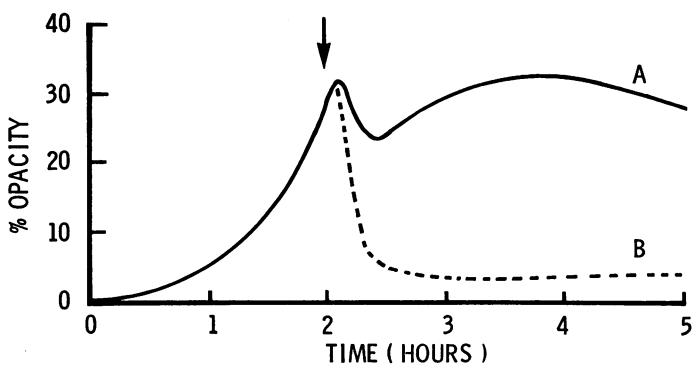

FIG. 2.—*Pr. mirabilis* grown in (A) medium A; (B) medium B: 50 μg/ml ampicillin added at arrow in each case.

altering the osmolality of the media is shown in Fig. 3 and 4. In order to minimize problems of cell permeability the osmolality of the media was raised by adding disaccharides which were non-fermentable substrates for the organisms—sucrose in the case of *Esch. coli*, lactose in the case of *Pr. mirabilis*. The addition of 10% (0·29 mol/l) sucrose to medium B totally abolished ampicillin lysis of *Esch. coli* (Fig. 3A) while 5% (0·15 mol/l) sucrose inhibited the rapid lysis although

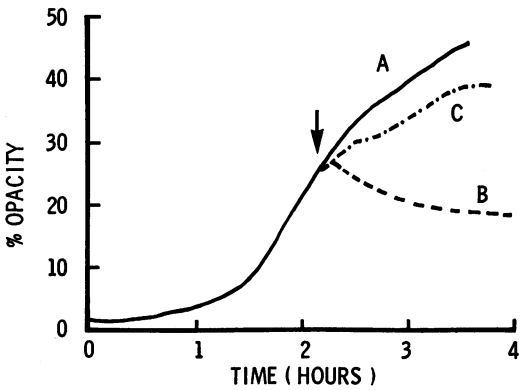

FIG. 3.—*Esch. coli* grown in (A) medium B containing 10% sucrose; (B) medium B containing 5% sucrose; (C) medium A containing 5% sucrose. 50 μg/ml ampicillin added at arrow in each case.

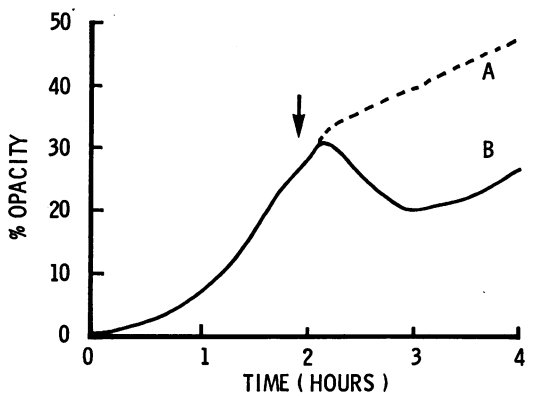

FIG. 4.—*Pr. mirabilis* grown in medium B containing (A) 5% lactose; (B) 3% lactose. 50 μg/ml ampicillin added at arrow in each case.

slow lysis ensued on continued incubation (Fig. 3B). Ampicillin lysis of *Esch. coli* in medium A was totally abolished by the addition of 5% sucrose (Fig. 3C).

The addition of 5% (0·15 mol/l) lactose to medium B offered *Pr. mirabilis* total protection from ampicillin lysis (Fig. 4A) while 3% (0·09 mol/l) lactose reduced the early ampicillin effect, giving a picture of partial lysis similar to that seen in medium A without added carbohydrate.

Similar protection was also obtained by adding various electrolytes, but with important differences. Fig. 5 shows the effect on the lytic response of *Esch. coli*

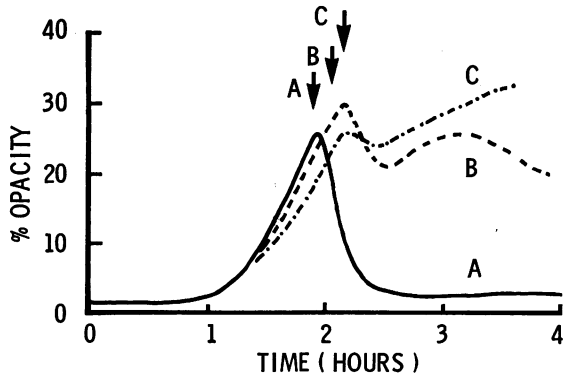

FIG. 5.—*Esch. coli* grown in medium B containing (A) 0·5% NaCl; (B) 1·0% NaCl; (C) 2·5% NaCl. 50 μg/ml ampicillin added at arrow in each case.

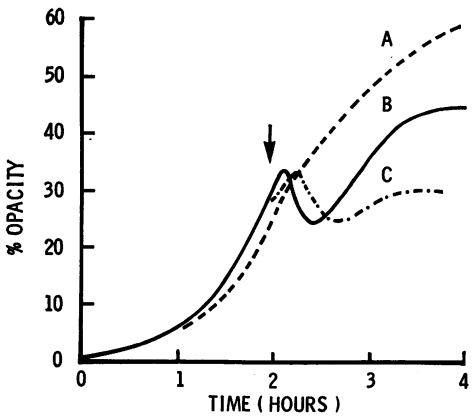

FIG. 6.—*Pr. mirabilis* grown in medium B containing (A) 1% NaCl; (B) 0·5% NaCl; (C) 0·7% KCl. 50 μg/ml ampicillin added at arrow in each case.

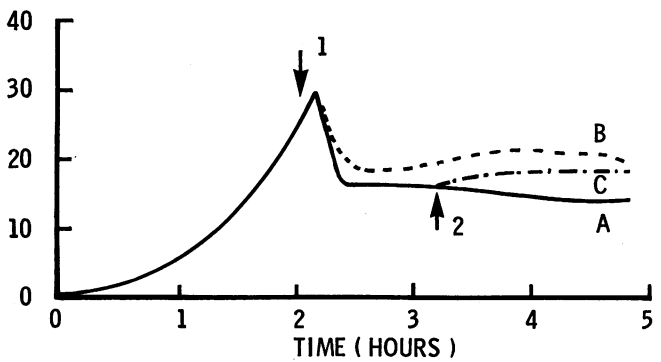

FIG. 7.—*Pr. mirabilis* grown in medium B containing (A) 0·5% $MgSO_4.7H_2O$; (B and C) 0·5% NaCl and 0·1% $MgSO_4.7H_2O$. 50 μg/ml ampicillin added at first arrow in each case. 1% dipotassium EDTA added at second arrow in case C only.

to ampicillin of adding increased concentrations of NaCl to medium B. Addition of 0·5% (0.09 mol/l) NaCl gave no detectable protection (Fig. 5A). Partial inhibition of the lytic episode was achieved with 1% (0·17 mol/l) NaCl (Fig. 5B), but even 2·5% (0·43 mol/l) NaCl did not abolish lysis entirely (Fig. 5C). In keeping with the difference between *Esch. coli* and *Pr. mirabilis* protected by disaccharides, only 1% NaCl was needed to achieve total protection of *Pr. mirabilis* from ampicillin lysis in medium B (Fig. 6A), while 0·5% (0·09 mol/l) NaCl—the same concentration as that present in medium A—gave partial inhibition of lysis (Fig. 6B). Isomolal KCl (0·7%) in medium B had a very similar, but not identical, effect (Fig. 6C) but 0·5% (0·02 mol/l) $MgSO_4.7H_2O$ not only partially inhibited the early lysis of *Pr. mirabilis* but also abolished the increase in opacity which normally follows the lytic phase as spheroplasts emerge (Fig. 7A). The increase in opacity due to emergent spheroplasts of *Pr. mirabilis* in medium B containing 0·5% NaCl was also virtually abolished by incorporating as little as 0·1% $MgSO_4.7H_2O$ in the medium (Fig. 7B). Once this stabilization effect had been initiated (Fig. 7C), it could not be reversed by the addition of dipotassium EDTA even at a concentration as high as 1%. The protective effects of various additions to the 2 media used are summarized in Table I.

TABLE I.—*Comparison of Degrees of Protection Obtained by Various Additions to Medium B with* Esch. coli *and* Pr. mirabilis

Amount of addition to medium B (w/v)	Molality (mol/l)	Degree of protection obtained with	
		Esch. coli	*Pr. mirabilis*
5% sucrose	0·15	Partial	ND
10% sucrose	0·29	Complete	ND
3% lactose	0·09	ND	Partial
5% lactose	0·15	ND	Complete
0·5% NaCl	0·09	None	Partial
1·0% NaCl	0·17	Partial	Complete
2·5% NaCl	0·43	Partial	ND
0·7% KCl	0·09	ND	Partial
0·5% $MgSO_4.7H_2O$	0·02	ND	Partial

TABLE II.—*Osmolalities of Medium B With and Without Added NaCl Before and 1 hour After the Addition of 50 μg/ml of Ampicillin*

Organism	Medium	Ampicillin (50 μg/ml)	Osmolality (in mOsm/kg)
Esch. coli	B	0	140
Esch. coli	B	1 hour	133
Esch. coli	B + 1% NaCl	0	440
Esch. coli	B + 1% NaCl	1 hour	418
Pr. mirabilis	B	0	163
Pr. mirabilis	B	1 hour	157
Pr. mirabilis	B + 0·5% NaCl	0	342
Pr. mirabilis	B + 0·5% NaCl	1 hour	311

Table II shows that early lysis of some cells, followed by emergence of spheroplasts from others, was not due to the protective effect of increase in osmolality resulting from the addition to the medium of the contents of lysed cells, since the osmolality of the medium before and 1 hour after the addition of ampicillin to a growing culture of *Esch. coli* and *Pr. mirabilis* was unchanged both in the presence and absence of an osmotic stabilizer.

DISCUSSION

It is clear that the rapid lysis which follows exposure of cultures of *Esch. coli* and *Pr. mirabilis* to penicillins can be manipulated by altering the osmolality of the environment and that the difference in response we have previously reported between *Esch. coli* and *Pr. mirabilis* (Greenwood and O'Grady, 1969, 1970) can be explained in terms of differential osmotic susceptibility. The question remains why in medium of intermediate osmolality, which partially inhibits early penicillin lysis, some cells lyse while others convert to spheroplasts.

Microscopic observation shows that as the early lytic episode reaches its end, the protected cells undergo relatively slow transformation to spheroplasts.

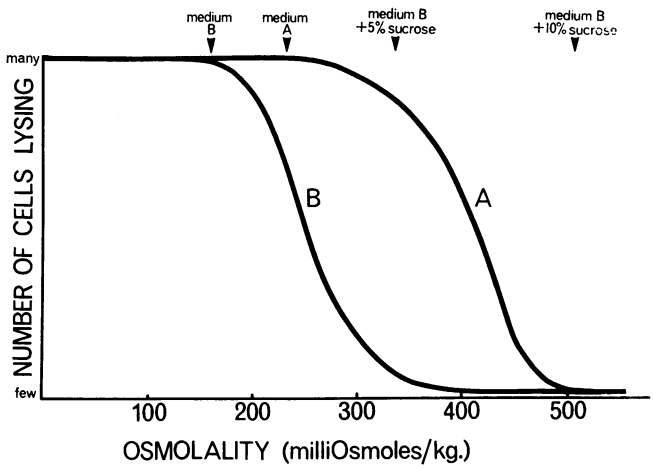

FIG. 8.—Postulated relationship between ampicillin lysis of (A) *Esch. coli* and (B) *Pr. mirabilis* and osmolality of medium.

This bimodal response could be due either to a spectrum of osmotic susceptibilities existing within the bacterial population or to the release of substances from early lysing cells which raise the osmolality of the medium and so protect the remainder of the population. This possibility was excluded by showing that *Esch. coli* and *Pr. mirabilis* cultures in complete medium with and without sodium chloride showed no increase in osmolality after 1 hour's exposure to ampicillin (Table II). We conclude that a spectrum of osmotic susceptibility must exist within each bacterial population and that *Esch. coli* and *Pr. mirabilis* differ in this respect. The situation is depicted diagrammatically in Fig. 8.

Table I shows that there was a good correlation between the molar concentrations of disaccharides and electrolytes required to achieve equivalent degrees of protection, with 2 notable exceptions: 2·5% (0·43 mol/l) NaCl, which did not completely protect *Esch. coli* in medium B whereas 10% (0·25 mol/l) sucrose did, and 0·5% (0·02 mol/l) $MgSO_4.7H_2O$ which offered partial protection to *Pr. mirabilis* despite its low molar concentration. It is possible that the anomalous lack of total protection by high concentrations of NaCl with *Esch. coli* is related to the complexities of ion flux in bacteria which are at present poorly understood.

The protective effect of relatively low concentrations of $MgSO_4.7H_2O$ may be due to a stabilizing effect on the cell membrane (McQuillen, 1960).

The finding that the response of Gram-negative bacilli to penicillins can be manipulated by altering the osmotic environment may be of two-fold importance. Awareness has been growing during the past few years of the possible role of spheroplasts in infections which recur following therapy with penicillins. In order to avoid such spheroplast-mediated recurrence in urinary tract infection, it has been recommended that urine osmolality should be kept low during penicillin therapy on the grounds that spheroplasts will not survive in dilute urine (Braude, Siemienski and Lee, 1968). The demonstration that the benefit of low osmolality is in enhancing the early non-spheroplast lysis of the organisms and prevention of spheroplasting on a massive scale means that the success of such a regimen would neither require a sustained lowering of the osmolality (because of the speed with which the lysis takes place) nor a sustained antibiotic concentration, as these lysed cells are incapable of the reversion to bacillary form which occurs with spheroplasts as the penicillin concentration falls below the minimum inhibitory concentration. However, it should be remembered that, particularly with the very high concentration of organisms likely to be found in infected urine, it is not necessary to invoke spheroplasts to explain persistence of infection as considerable numbers of organisms survive very high levels of penicillins without incurring any obvious morphological damage (Greenwood and O'Grady, 1969).

The other implication of these results is for the laboratory evaluation of the sensitivity of Gram-negative organisms to penicillins. In medium of suitably high osmolality, penicillins become purely bacteristatic agents in so far as their bactericidal activity resides in the lytic effects. It is already known that the sensitivity of *Proteus* to penicillin is enhanced on electrolyte deficient medium (Naylor, 1960; Barber and Waterworth, 1964) and our results suggest that the basis of this phenomenon may be osmotic. Recently, an international collaborative study has again recommended standardized procedures for antibiotic sensitivity testing (Report, 1971), and although the effects of different media were considered, osmolality differences were not taken into account. Our results suggest that such differences may influence sensitivity results, particularly in fluid media, and clearly osmolality is an important parameter to take into account in assessing the bactericidal efficacy of cell wall active antibiotics.

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