INDUCIBLE PENICILLINASE IN PROTEUS MORGANI

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In the course of a recent clinical trial of ampicillin carried out in this hospital (Trafford et al., 1962), and in other studies, a total of 29 representatives of the Proteus genus have been tested for penicillinase production, by the method of Knox and Smith (1961a); in no case could penicillinase activity be detected. In view of previous reports of penicillinase from the Proteus group (Abraham, 1951; Garrod, 1960), it was thought of interest to examine these strains more closely, especially to ascertain whether this enzyme could be induced in any of these organisms; this latter aspect of penicillinase production has recently become of great interest, as in Gram-negative bacteria studied so far it has proved to be non-inducible (Smith and Hamilton-Miller, 1963b).

Ten Proteus strains were grown overnight in infusion broth at 37°C; the cultures were adjusted to pH 7.4 and the penicillinase activity of each was measured by the hydroxylamine method (Knox and Smith, 1962), using benzylpenicillin as substrate. No breakdown was observed with eight of the strains, but with the remaining two, G and 185 (both Pr. morgani), although no breakdown was observed at first, continued incubation resulted in destruction of substrate, the rate of which increased progressively with time. However, when the experiment was repeated with cultures in the presence of 10^{-3}M p-chlormercuribenzoate (pCMB), no breakdown at all was observed. As pCMB has been shown, in Staph. aureus, to inhibit penicillinase induction (Steinman, 1961), it seemed possible that these two strains were

producing an inducible penicillinase during the course of the assay; this would account for the increasing rate of hydrolysis observed in the first experiment. To test if any of these strains were inducible, all ten were cultured overnight in concentrations of benzylpenicillin in infusion broth ranging from 100-500 μg./ml., depending on the sensitivity of each individual strain. On repeating the penicillinase assay on whole cell preparations after pCMB treatment, it was found that strains G and 185 hydrolysed benzylpenicillin at a linear rate, while no destruction could be detected with the other eight (five Pr.mirabilis, two Pr.rettgeri and one Pr.morgani). As pCMB has been shown to inhibit penicillinase activity in one bacterial species (Smith, 1963b), the seven inactive strains were again assayed in the absence of this reagent, but no activity could be demonstrated. Suspensions of induced cells from strains G and 185 were tested both in the presence of pCMB and of chloramphenicol (which was also found to prevent induction), at final concentrations of 10^{-3}M, and the rate of hydrolysis in each case was found to be the same as the initial rate using an untreated culture: thus neither of these reagents affected the penicillinase activity of these strains.

These findings are of interest for two reasons: firstly, this is apparently the first report of penicillinase induction in Gram-negative bacteria, and secondly it seems to be the first time that induction has been noted in bacteria whose basal penicillinase levels are too low to be detected

Cell-free enzyme preparations were made from concentrated, induced cultures of Pr.morgani G by disruption in the liquid extrusion press and centrifugation as described below. Some of the properties of this penicillinase preparation were then studied in greater detail: the nature of the hydrolytic cleavage of benzylpenicillin was elucidated by paper chromatography (Hamilton-Miller, 1963), which showed the reaction product to be penicilloic acid. This finding, taken in conjunction with the colour loss in the hydroxylamine assay, thus established that a penicillinase (β-lactamase (Pollock, 1961)) was produced by this organism. The enzyme was

found to be labile to heat and trypsin, had optimal activity against benzylpenicillin at pH 7.5, and showed an apparent activation energy of 4,700 cal./mole. The low value of the apparent activation energy, taken in conjunction with the values reported previously for penicillinases from a variety of bacterial sources (Smith and Hamilton-Miller, 1963a), suggests that this is a general characteristic of penicillinases from Gram-negative bacteria, not merely of non-inducible penicillinases.

The penicillinase activity was not affected by pCMB, sodium ethylenediamine tetraacetate, or iodoacetamide at concentrations of .001M, or by thioglycollate at .01M; of the compounds shown in Table I, only phenoxymethyl- and benzyl-penicillin were detectably hydrolysed, the former being destroyed at 38% of the rate of the latter. Thus the substrate specificity of this penicillinase somewhat resembles that of E.coli penicillinase (Smith, 1963a; Smith and Hamilton-Miller, 1963b).

To investigate induction, organisms were cultivated overnight at 37° in the presence of varying concentrations of different penicillins, 10^{-3}M pCMB added to prevent further induction and the cultures centrifuged down, washed and resuspended in phosphate-buffered saline. The number of organisms in each suspension was determined from the value of the extinction at 700 mμ, measured on a Unicam SP 500 spectrophotometer, and comparison with a standard suspension. Each suspension was divided into two portions, one of which was treated for five minutes in the M.S.E. 60 w ultrasonic disintegrator, and penicillinase activities of both disrupted and intact suspensions were measured. The results, expressed in rates of hydrolysis per 10^{9}cells/ml., are shown in Table I; it can be seen that benzylpenicillin, phenethicillin and propicillin are the best inducing agents for this organism. As it has been shown (Crompton et al. 1962; Knox and Smith, 1962; Novick, 1962; Smith et al. 1962; Swallow and Sneath, 1962) that methicillin and the isoxazolyl penicillins are the most effective inducing agents for staphylococcal penicillinase there is a marked difference between the penicillinase of this Proteus strain and that of Staph. aureus, as regards the relative inducing abilities of various

penicillins. Furthermore, the reported correlation between inducing ability and biological activity (Crompton et al, 1962; Smith et al, 1962) for Staph. aureus does not apply to this Proteus strain. Table I also shows that this organism is highly resistant to all the penicillins tested; as reduction of the inoculum size by one million fold only slightly reduced the minimum inhibitory concentration of the two hydrolysable penicillins, it appears likely that this resistance is "inherent" (Knox and Smith, 1961b).

TABLE I. Inducing ability and antibacterial activity of various penicillins tested with Proteus morgani strain G.

Penicillin	Concentration for optimum induction (μg./ml.)	Amount of penicillinase produced *	Minimum inhibitory concentration (μg./ml.)
Benzylpenicillin	1100	22.2	3000
Phenoxymethylpenicillin	2200	6.9	4000
Phenethicillin	425	22.9	700
Propicillin	350	23.8	700
Phenbenicillin	200	12.3	500
Ampicillin	175	12.0	500
Methicillin	250	6.2	500
Oxacillin	500	6.4	1000
6-Aminopenicillanic acid	40	2.2	125

* = μg. benzylpenicillin hydrolysed per minute/10^9 organisms/ml., at 37°C and pH 7.4, using whole cells.

Minimum inhibitory concentrations were determined by the tube dilution method (as described by Smith, 1963a), using an inoculum of 5 x 10^6 organisms. Other results were obtained as described in text.

Disrupted preparations from cultures induced in phenoxymethylpenicillin, methicillin, oxacillin and 6-aminopenicillanic acid showed approximately twice

as much enzymic activity against benzylpenicillin as did intact suspensions induced by these compounds; however, disruption did not increase the activity of cultures induced with benzylpenicillin, propicillin, phenethecillin, or phenbenicillin; ampicillin-induced cultures occupied an intermediate position, as those grown in high levels of this inducing agent showed no further increase in activity after disruption, while those grown in lower concentrations showed a twofold increase. Further work is being carried out to clarify this issue. It seems, then, that the extent of the permeability barrier (the presence of which appears to be a characteristic feature of penicillinase-producing coliform organisms (Smith and Hamilton-Miller, 1963b)) is possibly dependent upon the nature of the agent used to induce the enzyme, and, in the case of ampicillin, on the concentration of inducing agent used. Smith (1963a) and Hamilton-Miller (1963), have also reported damage to permeability barriers by growth in the presence of sub-inhibitory levels of certain penicillins. Smith (1963a) has further reported penicillinase activity in disrupted preparations of a strain, E. coli 214 0, whose intact cells showed no such activity; consequently, uninduced cells of strain G were disrupted and tested for activity, but none was found.

Experiments have also been carried out on cell-free preparations of induced Pr. morgani 185, which were found to hydrolyse only benzyl- and phenoxymethyl-penicillin (the latter was destroyed at 44% of the rate of benzylpenicillin destruction). After disruption in the extrusion press the penicillinase activity, like that of Pr. morgani G, remained in the soluble fraction after centrifugation at 30,000 *g* for 50 minutes; such a preparation showed optimal activity against benzylpenicillin at pH 7.4. The apparent activation energy for the hydrolysis of benzylpenicillin was found to be 3,700 cals./mole. Thus this penicillinase shows striking resemblances to that obtained from Pr. morgani G.

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