SUMMARY

1. An inhibitor of chymotrypsin has been found in extracts of body wall of *Ascaris lumbricoides*. It is inactivated by hot trichloroacetic acid and is therefore different from the trypsin inhibitor from the same source.

2. Both substances inhibit esterase and proteinase activity stoicheiometrically.

3. The ester substrates temporarily block combination between enzyme and inhibitor and this observation has been used to determine the rate constants for the combination.

4. Both these inhibitors and several naturally occurring trypsin inhibitors were found to be without effect on the activity of horse-liver esterase.

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The Activity and Specificity of Inducers of Penicillinase Production in *Bacillus cereus*, strain NRRL 569

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It has been recognized for many years that substances other than the normal enzyme substrate may act as specific inducers of enzyme formation in micro-organisms. Indeed, the fact that in numerous instances the product of enzyme action was found to be as efficient an inducer as the substrate itself was used by Yudkin (1938) as support for his 'mass action' theory of enzymic adaptation. Studies on β-galactosidase synthesis in *Escherichia coli* (Monod & Cohn, 1952), formation of protocatechuic acid oxidase in *Neurospora crassa* (Gross & Tatum, 1955) and maltase production in *Saccharomyces cerevisiae* (Spiegelman & Halvorson, 1954) have shown that inducers are not necessarily either substrates or specific inhibitors of the enzyme; neither are substrates nor enzyme inhibitors always active as inducers. It appears fairly well established that the specificity of enzyme induction is related to, but distinct from, the specificity of enzyme function.

A satisfactory interpretation of the pattern of inducer activity and specificity has not yet been provided. In the penicillinase induction system of *Bacillus cereus* transient contact with the inducer results in a constant rate of enzyme formation at a level dependent on the concentration of inducer employed (Pollock, 1950). This makes it particularly suitable for a quantitative analysis of inducer function. A number of compounds related to penicillin, in particular the cephalosporins N and C (see Abraham, 1956), have been so studied during the past few years. The results, reported here, have shown that the range of inducer specificity for penicillinase production is similar to that found with other enzyme induction systems, and support an earlier hypothesis (Pollock & Perret, 1951) that the primary site of inducer action is a specific cell receptor analogous to, if not identical with, the 'penicillin-binding component' (PBC) studied by several groups of workers in *Staphylococcus aureus* and other penicillin-sensitive bacterial species (see Cooper, 1956).

MATERIALS AND METHODS

Organism. Most of the work was done with *Bacillus cereus*, strain NRRL 569 (*B. cereus* 569), but in a few experiments *B. subtilis*, strain 749 (*B. subtilis* 749) was used.

Medium. 'S' peptone broth (Pollock & Perret, 1951) was used for growing *B. cereus* 569; the 'CCY' medium of Gladstone & Fildes (1940) was used for *B. subtilis* 749.

Penicillinase assay. The manometric method of Henry & Housewright (1947) was used under conditions employed previously (Pollock, 1952).

Induction tests. In the standard technique (used for all experiments unless otherwise stated) cells were allowed to grow at 35° on a shaker up to an opacity corresponding to between 0·15 and 0·20 mg. dry wt. of bacteria/ml., 10%

gelatin (Difco: 'purified') solution to a final concentration of 1% (w/v) was added and the culture distributed in 10 ml. lots in 100 ml. conical flasks. At the same time suitable quantities of the substances to be tested were added to the flasks, and shaking (5 cm. thrust; 120 times a min.) in a water bath at 35° was continued for a further 60 min., 2·5 ml. samples being removed into chilled 8-hydroxyquinoline solution (see Pollock, 1952) at 30 and 60 min. At the same time, a control culture with no added inducer was similarly incubated and sampled. The rate of penicillinase formation was calculated by subtracting the 30 min. assay value from the 60 min. value, and is expressed as a percentage of the rate found with benzylpenicillin as inducer at a concentration of 6·0 μg. (= 10 units/ml., or $1{\cdot}7 \times 10^{-5}$ M). This latter rate was usually about 300 times the 'basal' value (i.e. that obtained with no added inducer) and, on an average, amounted to 100 units of enzyme (as defined by Pollock & Torriani, 1953)/ml./hr. Although some variation in this optimum induction by benzylpenicillin did occur from day to day, it was possible to compare results in different experiments by always including a standard culture induced with benzylpenicillin at 6 μg./ml. None of the substances tested had any significant effect on growth (with the exception of benzylpenicillin at concentrations above 0·6 μg./ml. and cephalosporin C above 10 μg./ml., both only with *B. subtilis* 749).

Some induction tests were done by the 'cold pretreatment technique' (Pollock, 1950), in which washed cells from a logarithmically growing broth culture were suspended, at a concentration of 2 mg. dry wt./ml. in 0·01 M-potassium phosphate buffer, pH 7·0, containing the inducer, at 0° for 60 min. The cells were then centrifuged down and washed 3 times with 3 ml. of dilute buffer or water, resuspended at a concentration of 2 mg./ml. and inoculated into broth containing 1% (w/v) gelatin at an initial cell concentration of 0·2 mg./ml., and shaken for 60 min. at 35°, samples being taken for assay at 30 and 60 min. as in the standard technique.

Fixation of radiopenicillin. Fixation of radioactivity on cells after treatment with ^{35}S-labelled benzylpenicillin, alone and in the presence of other inducers, was measured by the technique previously described (Pollock & Perret, 1951) under the same conditions as those employed in the 'cold pretreatment technique' outlined above. Results are expressed as counts/min./mg. dry wt. of cells corrected for self-absorption at a standard thickness of 5 mg./sq.cm.

^{35}S-Labelled benzylpenicillin. This was prepared by E. J. Tridgell and H. V. Rickenberg by growing *Penicillium chrysogenum*, strain WIS 48–701, on a medium based on that described by Perret (1953). It was extracted by isolating the crystalline *N*-ethylpiperidine salt as described by Smith & Hockenhull (1952). Enough carrier-free [^{35}S]sulphate was added to give a final specific radioactivity of approximately 0·2 μc/unit.

Compounds tested. Benzylpenicillin (sodium salt) was obtained from Glaxo Laboratories Ltd. Phenoxymethylpenicillin and *n*-heptylpenicillin were obtained from Dr J. H. Humphrey. Cephalosporin N, cephalosporin C and benzylpenillic acid were gifts from Drs E. P. Abraham and G. G. F. Newton. Phthalamidopenicillinate (methyl ester) was a gift from Professor J. C. Sheehan. Dethiobenzylpenicillin, dimethoxypenaldyl-DL-penicillamine and the straight-chain tripeptide *N*-phenylacetyl-L-cysteinyl-D-valine were gifts from Merck and Co. Inc.; and the β-lactam of $\alpha\alpha$-dimethyl-(2-methylthiazolidin-2-yl)acetic acid was a gift from the Shell Development Co. Benzylimidazole was synthesized by Dr J. Cornforth. The cyclic tripeptide, *N*-phenylacetyl-L-cysteinyl-D-valine, was a gift from Dr H. R. V. Arnstein and Miss M. Clubb. The benzylpenicilloic acid was prepared by methanol extraction of an alkaline hydrolysate of benzylpenicillin (Pollock, 1950).

These substances probably contained only traces of impurities, with the exception of the cephalosporin N preparations, which varied in cephalosporin N content between 30 and 80% (as assayed biologically). Also some samples of cephalosporin N may have contained small quantities (up to 10%) of cephalosporin C (E. P. Abraham, personal communication). The concentrations quoted refer to the true cephalosporin N content. The mol.wt. of cephalosporin C (free acid) was taken as 415 (Newton & Abraham, 1956).

RESULTS

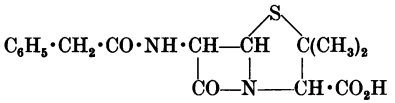
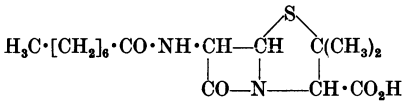
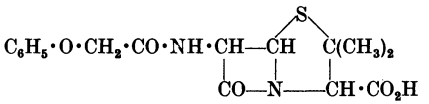
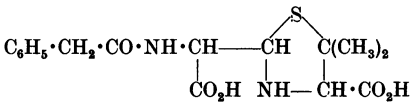
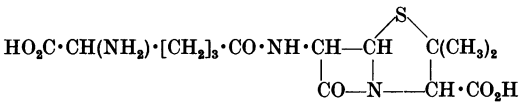
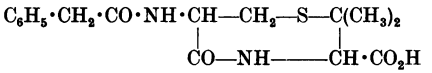
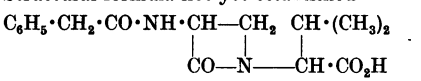
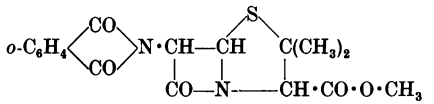
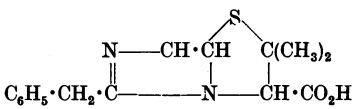
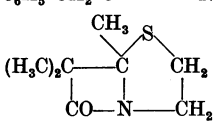
Qualitative tests

Table 1 summarizes the findings with a number of different penicillins and chemically related compounds. With the exception of cephalosporin C, all inducers were substrates for penicillinase and showed only slight differences in rates of hydrolysis (Abraham & Newton, 1956; Pollock, 1956). Cephalosporin C has recently been shown by Newton & Abraham (1956) not to be hydrolysed appreciably by penicillinase, although it will inhibit the hydrolysis of benzylpenicillin by penicillinase competitively, and must therefore be regarded as a specific enzyme complexant.

All compounds giving negative induction tests were also tested at the same concentration, together with benzylpenicillin at a concentration of 0·6 μg./ml. In no case was any significant inhibition of induction observed. Non-inducers having an intact β-lactam ring such that enzymic hydrolysis by a reaction similar to that which occurs with benzylpenicillin might be expected to liberate an acidic group and thus permit simple manometric assay in bicarbonate–CO_2 buffer were also tested as (*a*) substrates for, and (*b*) inhibitors of, penicillinase at up to twice the equivalent molar concentration of benzylpenicillin. None of the substances tested, however, showed any such activity.

As with β-galactosidase induction in *E. coli* (Monod & Cohn, 1952) there appears to be no reason for believing that different inducers cause any changes in the nature of the penicillinase induced. For instance, the ratio of the rate of hydrolysis, by penicillinase, of heptylpenicillin to that of benzylpenicillin (0·65; see Manson, Pollock & Tridgell, 1954) was approximately the same whichever of these two substances had been used for induction of the enzyme. Moreover, cephalosporin C—the most 'unusual' of all the penicillinase inducers—was found to induce exactly the same proportion of the intracellular γ-penicillinase (see Pollock, 1956) as did benzylpenicillin.

Table 1. *Inducers of penicillinase formation in* B. cereus 569

Compound	Structural formula	Concn. used for test (μg./ml.)	Hydrolysis by penicillinase	Inhibition of penicillinase	Penicillinase induction
Benzylpenicillin		0·0006–6000	+	.	+
Heptylpenicillin		0·6–60	+	.	+
Phenoxymethylpenicillin		0·006–6	+	.	+
Benzylpenicilloic acid		0·006–60	–	–	–
Cephalosporin N		0·07–60	+	.	+
N-Phenylacetyl-L-cysteinyl-D-valine (cyclic)		100	–	–	–
Cephalosporin C	Structural formula not yet established	0·1–200	–	+	+
Dethiobenzylpenicillin		6	–	–	–
Phthalamidopenicillinate (methyl ester)		50	.	.	–
Benzylpenillic acid		100	.	.	–
β-Lactam of αα-dimethyl-(2-methylthiazolidin-2-yl)acetic acid		175	–	–	–

In addition, the following compounds were tested as inducers and gave negative results: dimethoxypenaldyl-DL-penicillamine (1000 μg./ml.), *N*-phenylacetyl-L-cysteinyl-D-valine (straight-chain: 100 μg./ml.), benzylimidazole (285 μg./ml.).

The following conclusions appear justified: (1) Penicillinase induction is very highly specific. Although modification of the penicillin side chain has relatively little effect, any disruption or other substitution of the β-lactam–thiazolidine 'nucleus' completely destroys inducer activity. The only possible exception is cephalosporin C, whose exact formula is so far unknown. (2) With all compounds so far tested there appears to be strict correlation between the ability to induce and the ability to combine specifically with the enzyme.

Benzylpenicilloic acid, the product of enzymic hydrolysis of benzylpenicillin, is totally devoid of inducing ability. This is in contrast to the behaviour of enzyme products in other induction systems. It may therefore be significant that although there may be reason for believing that penicilloic acid combines specifically with penicillinase, the facts indicate that its affinity for the enzyme is extremely low. No inhibition of enzymic hydrolysis of benzylpenicillin by benzylpenicilloic acid has ever been observed, even with molar concentration ratios of 10:1 in favour of the latter. The reaction is strictly linear until at least 90% of the substrate has been destroyed.

The most interesting of the penicillinase inducers are the two cephalosporins. Cephalosporin N has been shown to differ from benzylpenicillin only by having D-α-aminoadipic acid instead of phenylacetic acid as a side chain (Newton & Abraham, 1954; Abraham, Newton & Hale, 1954), but it is considerably more hydrophilic and very much less active antibiotically than benzylpenicillin against Gram-positive bacteria (Heatley & Florey, 1953). Cephalosporin C has the same side chain as cephalosporin N, but differs slightly, though significantly, in the so-called 'nucleus'; this markedly alters both its chemical and biological properties (Abraham & Newton, 1956; Newton & Abraham, 1956), so that it is much more stable in acid (pH 2·0) than benzylpenicillin and is antibiotically even less active than cephalosporin N. The inducing properties of these substances have therefore been compared quantitatively with benzylpenicillin and another 'typical' penicillin (phenoxymethylpenicillin) which is more acid-stable than the other penicillins but has approximately the same antibiotic activity (Goodey, Reed & Stephens, 1955).

Quantitative tests

The relative activities of different penicillinase inducers at varying concentrations have been compared graphically in Fig. 1 by expressing the rates of induced enzyme formation as percentages of the rate found with the optimum benzylpenicillin concentration of 6 μg./ml.

The curves differ from one another in two, apparently quite distinct, respects:

Maximal inducing action. The highest rates of penicillinase formation induced by benzylpenicillin, phenoxymethylpenicillin and cephalosporin N are approximately the same, whereas maximal induction with cephalosporin C is much higher.

The maximal effect of cephalosporin C varied somewhat in different experiments, but was always 100–250% greater than that of benzylpenicillin. Since cephalosporin C is known not to be destroyed appreciably by penicillinase, it was thought possible that the higher maximal induction effect might be due to its continued presence in the medium during the standard induction test, where benzylpenicillin is known to be destroyed completely within the first few minutes (Pollock, 1952). However, a comparison of the two substances by using the 'cold

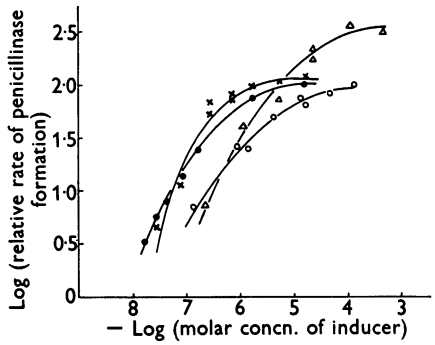


Fig. 1. Relationship between concentration of different inducers and rate of induced penicillinase formation by *B. cereus* 569, expressed as percentage of the rate obtained with benzylpenicillin as inducer, at the optimum concentration of 6 μg./ml. 'Standard' technique (see text). ●, Benzylpenicillin; ×, phenoxymethylpenicillin; ○, cephalosporin N; △, cephalosporin C.

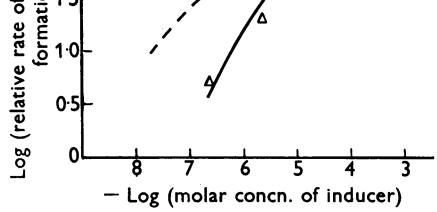


Fig. 2. Relationship between concentration of benzylpenicillin and of cephalosporin C as inducers and rate of induced penicillinase formation by *B. cereus* 569, expressed as percentage of the rate obtained with benzylpenicillin as inducer at the optimum concentration of 6 μg./ml. 'Cold pretreatment' technique (see text). △, Cephalosporin C; – – –, benzylpenicillin [copied from summary of results previously published (Pollock, 1950)].

pretreatment technique' (where all free inducer is removed by washing before induced enzyme production begins) showed the same difference (Fig. 2). A simple and satisfactory interpretation of the cephalosporin C effect has not been found, and the problem is discussed below.

Concentration of inducer necessary to evoke a given rate of enzyme formation. Inducers differ markedly in the concentration necessary for a given degree of induction, whether this is expressed in terms of absolute rate of induced enzyme formation or as a percentage of the maximal rate attainable. It seemed possible that such differences might be expressed loosely in terms of differing 'affinities' for some sort of induction 'centre'.

It has previously been pointed out (Pollock, 1950) that the relation between enzyme-formation rate and inducer concentration in the penicillinase system is reminiscent of that existing between enzymic activity and substrate concentration interpreted by Michaelis and Menten on the basis of a reversible combination between enzyme and substrate. This is illustrated by the fact that if the reciprocal of the rate of enzyme formation (expressed as a percentage of the maximal rate obtained with benzylpenicillin) is plotted against the reciprocal of the inducer concentration, the points lie approximately on a straight line (Fig. 3). Although the theoretical considerations of reversible enzyme–substrate complexes cannot strictly apply to analysis of the irreversible interaction between inducer and cell in the penicillinase system, the relationship can be analysed mathematically in the same way as that suggested by Lineweaver & Burk (1934) for enzyme Michaelis constants, and 'induction constants' thus calculated. These constants (summarized in Table 2) give convenient quantitative expression to differences in inducer action. They are equal to the concentration of any one inducer which gives half-maximal induction with that inducer. There is a large difference in

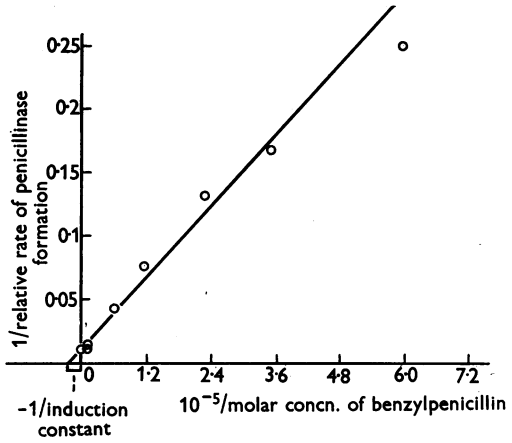


Fig. 3. Calculation of the penicillinase 'induction constant' for benzylpenicillin in *B. cereus* 569, by plotting the reciprocal of the benzylpenicillin concentration against the reciprocal of the relative rate of induced penicillinase formation (expressed as a percentage of the rate obtained at the optimum concentration of 6 μg./ml.).

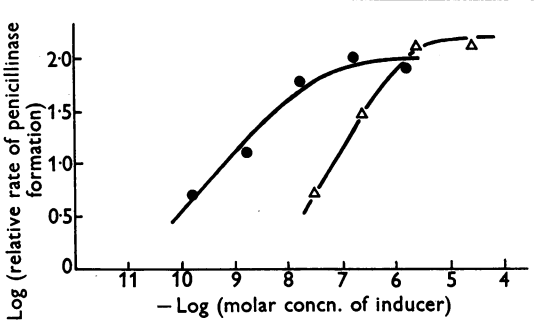


Fig. 4. Relationship between concentration of benzylpenicillin (●) and of cephalosporin C (△) as inducers and rate of induced penicillinase formation by *B. subtilis* 749, expressed as percentage of the rate obtained with benzylpenicillin as inducer at the optimum concentration of 6 μg./ml. Standard technique (see text).

Table 2. '*Induction constants*' *of different inducers of penicillinase production in* B. cereus 569 *and* B. subtilis 749

The constants have been calculated in a manner similar to that employed for determination of enzyme Michaelis constants (see text), and are equivalent to the concentration giving half-maximal rate of penicillinase formation with each inducer.

Organism	Inducer	Technique	Induction constant μg./ml.	Induction constant μM
B. cereus 569	Benzylpenicillin	Standard	0·21	0·6
	Benzylpenicillin	Pretreatment	0·13	0·37
	Phenoxymethylpenicillin	Standard	0·19	0·49
	Cephalosporin N	Standard	1·22	3·25
	Cephalosporin C	Standard	5·0	11·6
	Cephalosporin C	Pretreatment	5·5	12·7
B. subtilis 749	Benzylpenicillin	Standard	0·0042	0·012
	Cephalosporin C	Standard	0·50	1·16

'affinity' between benzylpenicillin and cephalosporin C, and the position of cephalosporin N is intermediate.

A further comparison of benzylpenicillin and cephalosporin C was made, by using the standard technique, with *B. subtilis* 749, and the results are summarized in Fig. 4. The findings are of interest because, although benzylpenicillin induces at much lower concentrations than with *B. cereus* 569, the relative inducing activities of cephalosporin C and benzylpenicillin are similar in the two species.

Evidence has already been provided to show that benzylpenicillin induces the formation of penicillinase by first reacting with some specific penicillin receptor within or on the cell (Pollock & Perret, 1951). If, as seems likely, other inducers react with the same receptor, differences in activity could be explained on the basis of differences in 'affinity' for the receptor molecules (as indicated by the varying induction constants). This hypothesis predicts that an inducer of high 'affinity' (low induction constant) such as benzylpenicillin would inhibit induction by an inducer of low 'affinity', such as cephalosporin C. This, in fact, proves to be true, as shown by two experiments summarized in Table 3. In Expt. I, induction (as measured by the standard technique) with a mixture of benzylpenicillin at optimum concentration (6 μg./ml.) and cephalosporin C at suboptimum concentration (10 μg./ml.)—added to the culture simultaneously—occurred at a rate intermediate between the rates with each inducer alone. In Expt. II (with the cold pretreatment technique) addition of benzylpenicillin at a concentration of 6 μg./ml., together with cephalosporin C at a concentration (100 μg./ml.) 17 times as great, resulted in induction at a rate about two-thirds that obtained with cephalosporin C alone, and barely significantly higher than with benzylpenicillin alone. These results suggest that these inducers act, in the first instance, by combination with the same receptor. However, further, and more satisfying, evidence is provided by experiments on fixation of ^{35}S-labelled benzylpenicillin by cells of *B. cereus*.

Inhibition of radiopenicillin fixation by the cephalosporins

It has already been shown (Pollock & Perret, 1951) that there is a striking similarity, in *B. cereus* 569, between the relative rates of induced penicillinase formation and the relative amounts of radioactivity irreversibly fixed to the cells, following treatment with different concentrations of ^{35}S-labelled benzylpenicillin. A benzylpenicillin 'fixation constant' for *B. cereus* 569 can, in fact, be calculated in the same way as an induction constant by measuring the slope of the straight line obtained by plotting the reciprocal of the amount of radioactivity fixed/mg. dry weight of cells against the reciprocal of the benzylpenicillin concentration (Fig. 5). It has been found to be approximately 0·17 μg./ml., and this corresponds closely to its induction constant. This fact has already been used to support the hypothesis that the primary interaction of penicillin as an inducer involves its fixation to some specific receptor in the cell. If, as suggested above, other inducers react with the same receptor, but to an extent corresponding to their greatly differing 'affinities' for this receptor, they would, at any particular concentration, be expected to

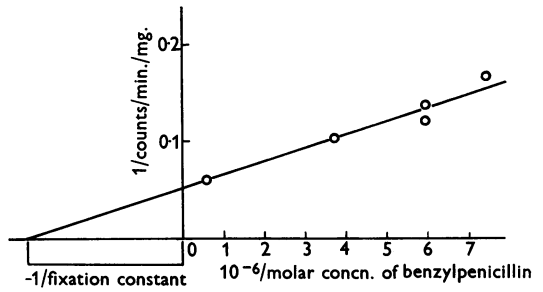


Fig. 5. Calculation of the 'fixation constant' for benzylpenicillin on cells of *B. cereus* 569 by plotting the reciprocal of the ^{35}S-labelled benzylpenicillin concentration used for treating the cells against the reciprocal of the quantity of radioactivity irreversibly fixed (expressed as counts/min./mg. dry weight of cells).

Table 3. *Inhibition, by benzylpenicillin, of penicillinase induction by cephalosporin C in* B. cereus 569

	Inducers added	Rate of induced penicillinase formation (units/ml./hr.)
Expt. I (standard technique)	Benzylpenicillin, 6 μg./ml.	127
	Cephalosporin C, 10 μg./ml.	211·5
	Benzylpenicillin (6 μg./ml.) + cephalosporin C (10 μg./ml.)	177·5
Expt. II (pretreatment technique)	Benzylpenicillin, 6 μg./ml.	109·5
	Cephalosporin C, 100 μg./ml.	167·5
	Benzylpenicillin (6 μg./ml.) + cephalosporin C (100 μg./ml.)	118·5

inhibit the fixation of radiopenicillin to a degree determined by these 'affinities'.

Table 4 summarizes the results of all the radiopenicillin fixation experiments (unfortunately limited by supplies of the two cephalosporins) that it was possible to do. At the low concentrations of radiopenicillin employed, the proportion of 'non-specific' fixation of radioactivity [due either to trace non-penicillin sulphur impurities in the radiopenicillin preparation or to fixation of penicillin on some other non-specific receptor site (see Pollock & Perret, 1951; Pollock, 1953*a*)] amounted to less than 10% of total radioactivity fixed, and has been ignored.

It is clear that both cephalosporin N and cephalosporin C inhibit the fixation of benzylpenicillin, but to very different extents. The most plausible explanation is that they combine—but, at equivalent concentration, to a different degree—with the same receptor substance within the cell as that which fixes benzylpenicillin. Relatively high concentrations of both substances are needed in order to produce significant inhibition of benzylpenicillin fixation, and even a molar ratio of cephalosporin C/benzylpenicillin of about 1000:1 inhibited fixation by only 50%. As controls, it can be seen that the non-inducer benzylpenillic acid at a concentration (100 μg./ml.) equivalent to the highest concentration of cephalosporin C employed caused no significant inhibition (in confirmation of findings by Daniel & Johnson, 1954), whereas phenoxymethylpenicillin (which has the same induction constant as benzylpenicillin) almost completely inhibited fixation at a concentration of 10 μg./ml.

Benzylpenicillin-fixation inhibitor constants of the cephalosporins can be calculated in a manner similar to that suggested by Dixon (1953) for the determination of K_i (enzyme-inhibitor constant) by plotting the reciprocal of the quantity of benzylpenicillin sulphur fixed on the cell against concentration of inhibitor, at varying concentrations of benzylpenicillin. It must, however, be admitted that the fixation figures are subject to considerable errors, particularly at low concentrations of radiopenicillin, and agreement was not always very good. Such a plot indicated that the inhibition of fixation by cephalosporin N is of the competitive type, and that by cephalosporin C probably so. The fixation

Table 4. *Inhibition of the fixation of radiopenicillin on cells of* B. cereus 569 *by cephalosporins N and C*

Cells were treated at 0° for 30 min. at a concentration of 2 mg. dry wt./ml. in 5·0 ml. of 0·01 M-potassium phosphate buffer, pH 7·0, containing the radiopenicillin and additions. They were then centrifuged, washed three times, dried, weighed and counted (see text for full details). Standard errors of counts are given in parentheses.

Concn. of ^{35}S-labelled benzylpenicillin (μg./ml.)	Additions		Amount of radioactivity fixed after treatment for 30 min. at 0° (counts/min./mg. dry wt. of cells)
	Compound	Concn. (μg./ml.)	
0·048	Nil	—	5·97 (±0·32)
	Phenoxymethylpenicillin	10	0·37 (±0·02)
	Cephalosporin N	6·25	2·88 (±0·17)
	Cephalosporin N	12·5	1·43 (±0·13)
	Cephalosporin N	18·75	2·21 (±0·14)
	Cephalosporin C	40	7·85 (±0·39)
	Cephalosporin C	70	3·94 (±0·22)
	Cephalosporin C	100	3·13 (±0·17)
0·06	Nil	—	7·19 (±0·24)
	Benzylpenillic acid	100	7·00 (±0·24)
	Cephalosporin N	25	2·22 (±0·12)
	Cephalosporin N	100	1·27 (±0·15)
	Cephalosporin C	1	6·95 (±0·46)
	Cephalosporin C	10	6·21 (±0·48)
	Cephalosporin C	100	5·27 (±0·42)
0·096	Nil	—	9·70 (±0·45)
	Phenoxymethylpenicillin	10	0·70 (±0·01)
	Cephalosporin N	6·25	5·22 (±0·26)
	Cephalosporin N	12·5	4·75 (±0·22)
	Cephalosporin N	18·75	1·44 (±0·12)
	Cephalosporin C	40	7·50 (±0·12)
	Cephalosporin C	70	6·04 (±0·18)
	Cephalosporin C	100	5·30 (±0·26)
0·60	Nil	—	14·7 (±0·29)
	Cephalosporin C	1	18·5 (±0·27)
	Cephalosporin C	10	16·8 (±0·31)
	Cephalosporin C	100	14·6 (±0·29)

inhibitor constants so determined can obviously be regarded only as crude approximations; but they do indicate the very low 'affinity' of cephalosporin C and the intermediate positon of cephalosporin N. The values, given in Table 5, are compared with those of the constants indicating the absolute and relative 'affinities' for the induction receptor and for the enzyme itself, together with the range of antibiotic activities of the three compounds against various species of Gram-positive bacteria.

DISCUSSION

These results support the contention that the primary interaction between inducer and cell involves combination with a specific receptor. Differences in activity of various inducers appear to be largely explicable on the basis of differences in 'affinity' for this receptor. The interpretation of the word 'affinity' in this connexion must be tempered by emphasizing that it is simply a convenient way of expressing the over-all relation between low concentrations of inducer and their quantitative effect, either on rate of induced enzyme production or on irreversible fixation on the cell. This effect must obviously be the end-result of a number of different factors, such as rates of penetration into the cell and rates of possible destruction within the cell, and cannot be strictly compared with true affinity relations between soluble enzymes and their substrates.

The fact that penicillinase inducers are all either enzyme substrates or competitive enzyme inhibitors, and vice versa, shows that the specificity range for combination with the receptor is broadly very similar to that for combination with the enzyme itself. This suggests that the specific combining groups of the penicillin receptor and of penicillinase may be structurally related. However, it seems rather unlikely that they are actually identical since the 'affinities' of benzylpenicillin and cephalosporin N for the induction or fixation 'centre' are much higher than their respective affinities for the enzyme. In order, therefore, to maintain the hypothesis that the specific combining groups of the penicillin receptor and of penicillinase are identical it would be necessary to introduce an assumption that there must be some special system responsible for concentrating benzylpenicillin and cephalosporin N within a relatively localized area in the cell before combination with the receptor. As is shown below, there is considerable evidence against this possibility, and so far none in its favour.

Further, the antibiotic activities of the cephalosporins and penicillins appear to bear some relation to their fixation and induction constants. There is already good evidence [see particularly the review of Cooper (1956) and the work of Eagle (1954)] in support of the idea that the sensitivity of micro-

Table 5. *Comparison of affinity relationships and antibiotic activities of benzylpenicillin and cephalosporins N and C*

Induction and specific fixation constants have been calculated by methods similar to those employed for determining enzyme Michaelis constants (see text for details), and are therefore approximately equivalent to the concentrations giving half-maximal induction and fixation respectively. The relative 'affinities' are the reciprocals of these constants, expressed as percentages of the figure obtained for benzylpenicillin.

	Penicillinase action				Penicillinase induction		Fixation on cells		Range of antibiotic activity *v.* different species of Gram-positive bacteria	
	In *B. cereus* 569									
	Affinity for enzymes		Rate of hydrolysis							
	K_m or K_i (μM)	Rel. affinity	Molecular activity (mol. of substrate hydrolysed/mol. of enzyme/min. at 30°)	Rel. activity	Induction constant (μM)	Rel. 'affinity' for induction receptor	Specific fixation constant (μM)	Rel. 'affinity' for fixation receptor	Lowest concn. preventing growth (μM)	Rel. activity
Benzylpenicillin	48*	100	$1{\cdot}60 \times 10^5$†	100	0·48	100	0·45‡	100	0·11–1·10§	100
Cephalosporin N	48‖	100	$9{\cdot}7 \times 10^4$**	61	3·2	14	4·8	9	13–53§	1–6
Cephalosporin C	110‖	43	$<2{\cdot}4 \times 10^4$¶	<0·015	12·1	3	2100	0·02	230–460**	0·1

* Pollock (1956).
‖ Abraham & Newton (1956).
† Kogut, Pollock & Tridgell (1956).
¶ M. R. Pollock (unpublished).
‡ Pollock & Perret (1951).
** Newton & Abraham (1956).
§ Heatley & Florey (1953).

organisms to penicillin is, in many cases, directly dependent on the amount of antibiotic irreversibly fixed to the cells by means of a specific receptor substance (penicillin-binding component, PBC). This, in turn, may depend on two factors: (*a*) the quantity of receptor present (which determines the sensitivity of different strains to the same penicillin); (*b*) the 'affinity' relationship of the receptor (which determines the sensitivity of the same strain to different penicillins). Now, Heatley & Florey (1953) and Abraham *et al.* (1954) have reported that cephalosporin N shows 17–100 times less activity than benzylpenicillin against Gram-positive bacteria, while Abraham & Newton (1956) state that cephalosporin C has about 10 times less activity than cephalosporin N against *Staphylococcus aureus*. Unfortunately, it is not possible to test the relative activities of these substances against *B. cereus* 569 independently of the effects of the penicillinase which the organisms produce. It can, however, be reasonably assumed that the 'intrinsic' sensitivity of this strain will be of the same order as that of *B. subtilis* and other Gram-positive organisms.

In antibiotic activity the intermediate position of cephalosporin N is apparent, as in induction or fixation. Indeed, very approximately, the antibiotic activity of all these three compounds is directly proportional to their 'affinity' for the specific penicillinase-receptor substance in the cell. Their chemical similarity makes it almost certain that their fundamental modes of antibacterial action are identical. Thus there is here further support for the hypothesis that PBC is the primary cell reactant by means of which penicillin exerts its antibiotic effect.

If it can be accepted, as seems reasonable, that the 'induction receptor' (as characterized by the value of induction constants with different inducers) is identical with the 'fixation receptor' (PBC), as characterized by values of fixation constants with different penicillins, it would appear that PBC has two distinct functions. Cooper (1956) has attempted to equate these two functions by suggesting that PBC catalyses the production of a substance, 'S', which combines with a specific penicillinase precursor to form an essential cell metabolite, diverting the path of penicillinase synthesis and thus inhibiting enzyme production. He postulates that addition of penicillin inactivates PBC so that less 'S' is formed and some of the precursor material is thereby released for transformation into penicillinase. Induction of penicillinase is thus necessarily coupled with a proportional decrease in the production of a substance essential for cell growth. This hypothesis (I) is made unlikely, though not excluded, by the fact that maximal induction of penicillinase in *B. cereus* cultures will occur after addition of penicillin at concentrations that are completely without effect on growth rate.

An alternative possibility (II) is that the penicillin–receptor complex is further involved in simultaneous but independent metabolic pathways, one leading to inhibition of growth and death of the cell and the other to penicillinase induction. The possibility of the penicillin–PBC complex functioning, directly or indirectly, as some sort of specific catalyst or template for penicillinase formation has been discussed previously in consideration of the 'organizer' hypothesis for enzyme induction postulated by Pollock (1953*b*) and Cohn & Monod (1953).

A third possibility (III) which, in a sense, is a special case of the second, would be to regard PBC as a penicillin-concentrating site from which the penicillin may be subsequently released, perhaps in a modified form, to function at specific points within the cell in more than one independent reaction.

On this last view, PBC might be considered as a 'point of entry' to the cell acting in a manner analogous to the 'Y' transporting and concentrating system of *E. coli*, described by Rickenberg, Cohen, Buttin & Monod (1956). This 'Y' system ('permease') is stated to be specifically responsible for the accumulation of inducers and substrates for β-galactosidase in a cell not freely permeable to these substances. In other ways, however, PBC functions quite differently from 'Y'. First, the cell structure of *B. cereus* does not appear to offer any barrier to the free entry of penicillin, at least so far as the action of intracellular penicillinase is concerned (Pollock, 1956); whereas Rickenberg *et al.* (1956) claim to have shown that the accessibility of *E. coli* β-galactosidase to its substrate, normally greatly restricted in intact organisms, is determined largely by the functioning of the 'Y' system. Secondly, the reaction between penicillin and PBC in *B. cereus* is essentially irreversible (Pollock & Perret, 1951; H. V. Rickenberg, personal communication), and methyl β-thiogalactoside, accumulated in cells of *E. coli* from the medium as a result of the action of 'Y', is rapidly lost when the methyl β-thiogalactoside is removed from the surrounding medium. Thirdly, penicillin fixation in *B. cereus* is unaffected either by addition of 20 mM-sodium azide (H. V. Rickenberg, unpublished observations) or by disruption of cell structure (Pollock, 1953*a*), in contrast to the 'Y' system of *E. coli*, which is almost completely inhibited by 20 mM-sodium azide and which does not function after damage to cell structure from treatment with toluene (Rickenberg *et al.* 1956). Thus in most respects penicillin fixation in *B. cereus*, as in *S. aureus*, behaves as a non-enzymic chemical reaction, whereas the 'Y' system of *E. coli*, which apparently requires energy and an intact cell structure, appears to function like a continuously acting metabolic pump.

No explanation has been offered for the higher maximal induction effect of cephalosporin C. It is

unlikely that maximal fixation of cephalosporin C on PBC is higher than that of other inducers. The quantity of inducer fixed, at optimum concentration, is determined by the number of combining sites on PBC, and there is good reason for believing, as argued above, that these combining sites are common to all penicillinase inducers. It is possible, though also unlikely, that if inducers are subsequently released within the cell before functioning in the specific penicillinase induction system (hypothesis III) a non-substrate inducer, such as cephalosporin C, might be relatively more stable than those other inducers that are liable to attack by intracellular penicillinase. It seems more probable, however, that the higher maximal inducing action of cephalosporin C is due to some other, unknown property of the molecule, effective in the induction mechanism at some point beyond the primary reaction with PBC.

It is clearly impossible to draw any conclusions at present on the fate and function of the penicillin–PBC complex. An attempt has been made to explain some of the properties and relative activities of penicillinase inducers on the basis of their primary reaction with PBC, but the subsequent role of the inducer–PBC complex is a matter for speculation.

SUMMARY

1. The ability to induce the formation of penicillinase in *Bacillus cereus* is restricted to compounds very closely related to benzylpenicillin; almost any modification to the fused β-lactam–thiazolidine 'nucleus' results in complete loss of activity.

2. Apart from the usual penicillins, the only substances found to be inducers were the cephalosporins N and C. At high concentrations cephalosporin C is the most powerful penicillinase inducer known.

3. Induction specificity is similar to, but distinct from, enzyme specificity. Inducers appear to be either enzyme substrates or competitive enzyme inhibitors, but their affinities for the enzyme differ both relatively and absolutely from their 'affinities' for the hypothetical induction 'centre'.

4. The relative inducing activities of different inducers appear to depend largely upon their 'affinities' for a specific penicillin-binding component present in the cell (as studied by their ability to inhibit the fixation of ^{35}S from ^{35}S-labelled benzylpenicillin).

5. The 'affinities' of these inducers for the penicillin-binding component bear some relationship to their activities as antibiotics.

6. The possible functions of the inducer–penicillin-binding component complex are discussed.

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