Table 1. *Effect of isocil and bromacil on induction of nitrate reductase by molybdenum or nitrate in cauliflower and radish leaf tissue*

Activities were measured as increase in nitrite produced proportional to $E_{540m\mu}$ during a 15 min. assay period after grinding 1 g. sub-samples of leaf material in phosphate buffer as described in the text. Induction of enzyme is indicated by increase over activity at 0 hr. resulting from infiltration with molybdenum or nitrate with or without the inhibitor and incubating at 25° in the light for 5 hr.

Inducer	Inhibitor	Species	Endogenous enzyme activity at 0 hr.	Concn. of inhibitor ($\mu\text{g./ml.}$) ...	Enzyme activity after incubation for 5 hr. with inducer and different concentrations of inhibitor			
					0	1	5	10
Molybdenum	Isocil	Cauliflower	0.009		0.543	—	—	0.044
Molybdenum	Isocil	Radish	0.091		0.474	0.180	0.102	0.063
Molybdenum	Bromacil	Cauliflower	0.019		0.273	0.020	0.057	0.021
Molybdenum	Bromacil	Radish	0.053		0.165	0.055	0.033	0.016
Nitrate	Isocil	Cauliflower	0.038		0.151	0.098	0.038	0.046
Nitrate	Bromacil	Cauliflower	0.024		0.174	0.081	0.053	0.041

leaves at concentrations between 0.4 and 16 mm (Afridi & Hewitt, 1965), and the negligible effects of isocil or bromacil on DNA metabolism reported by McGahen & Hoffman (1963*a,b*, 1966), suggests that the effects of bromacil and isocil now reported may relate to some other and quite different mechanism of action. It is possible that nitrate reductase in leaves, like nitrite reductase (Betts & Hewitt, 1966), may be located in chloroplasts. If so, a common site or mechanism for inhibition of some aspect of photosynthesis and formation of nitrate reductase by these compounds may be involved.

We thank Dr R. B. Hudson, and the E. I. du Pont de Nemours and Co. Inc., Wilmington, Del., U.S.A., for kindly providing the isocil and bromacil used in this work.

Afridi, M. M. R. K. & Hewitt, E. J. (1962). *Life Sci.* **1**, 287.
Afridi, M. M. R. K. & Hewitt, E. J. (1964). *J. exp. Bot.* **15**, 251.
Afridi, M. M. R. K. & Hewitt, E. J. (1965). *J. exp. Bot.* **16**, 628.

Betts, G. F. & Hewitt, E. J. (1966). *Nature, Lond.*, **210**, 1327.
Bucha, H. C., Cupery, W. E., Harrod, J. E., Loux, H. M. & Ellis, L. M. (1962). *Science*, **137**, 537.
Hewitt, E. J. (1966). *Tech. Commun. Bur. Hort., E. Malling*, no. 22: *Sand and Water Culture Methods Used in the Study of Plant Nutrition*, 2nd ed.
Hewitt, E. J. & Afridi, M. M. R. K. (1959). *Nature, Lond.*, **183**, 57.
Hewitt, E. J. & Nicholas, D. J. D. (1964). In *Modern Methods of Plant Analysis*, vol. 7, p. 67. Ed. by Linskens, H. F. Sanwal, B. D. & Tracey, M. V. Berlin, Gottingen and Heidelberg: Springer-Verlag.
Hilton, J. L., Monaco, T. J., Moreland, D. E. & Gentner, W. A. (1964). *Weeds*, **12**, 129.
Hoffman, C. E., McGahen, J. W. & Sweetster, P. B. (1964). *Nature, Lond.*, **202**, 577.
McGahen, J. W. & Hoffman, C. E. (1963*a*). *Nature, Lond.*, **199**, 810.
McGahen, J. W. & Hoffman, C. E. (1963*b*). *Nature, Lond.*, **200**, 571.
McGahen, J. W. & Hoffman, C. E. (1966). *Nature, Lond.*, **209**, 1241.

Biochem. J. (1966) **101**, 40 c

A Novel Method for Evaluating K_i/K_m and its Application to the Competitive Inhibition of Staphylococcal Penicillinase by Cephalosporins

By J. M. T. HAMILTON-MILLER

Department of Bacteriology, Guy's Hospital Medical School, London, S.E. 1

(Received 31 August 1966)

During a study of the competitive inhibition of staphylococcal penicillinase (EC 3.5.2.6) by the semisynthetic cephalosporins cephaloram, cephalo-

thin and cephaloridine, a rapid method was needed for calculating the ratio K_i/K_m . This ratio is conventionally obtained by plotting data by the method

of Hunter & Downs (1945), which involves several kinetic runs with different substrate concentrations; a simple three-part calculation has then to be made on each run to yield one point on a graph, the graph is drawn and the slope (and, where applicable, the intercept) calculated, to give K_i/K_m . By using the method described below, an accurate estimate of K_i/K_m can be obtained from runs with a single substrate concentration (or, alternatively, a single inhibitor concentration), and fewer calculations than are involved in the Hunter & Downs (1945) plot.

Materials. *Staphylococcus aureus* 13137 (Jevons, 1961) was cultured statically in infusion broth (Southern Group Laboratories, Hither Green, London, S.E. 13) containing 0.5 μ g of methicillin/ml. at 37° overnight. Cells were collected by centrifugation (3000g for 20 min.) and resuspended in 10 ml. of 1 mM-sodium *p*-mercuribenzoate in 25 mM-sodium phosphate buffer, pH 7.4 (Dawson & Elliott, 1959). Penicillinase activity was assayed by the hydroxylamine method (Knox & Smith, 1962). Sodium benzylpenicillin (Crystapen), purchased from Glaxo Ltd., Greenford, Middlesex, was used as substrate throughout. The following cephalosporins, generously given by Glaxo Ltd., were used as inhibitors: cephaloram (7-phenylacetamidocephalosporanic acid), cephalothin [sodium salt of 7-(2-thienylacetamido)cephalosporanic acid] and cephaloridine [7-(2-thienylacetamido)-3-(1-pyridylmethyl)-3-cephem-4-carboxylic acid betaine].

Experimental. (a) Measurement of I_{50} . Kinetic runs were carried out in the presence of a fixed initial concentration of substrate (usually about 5 mM) and various amounts of inhibitor (usually between 50 and 0.5 μ M for cephalothin and cephaloridine, and from 250 to 50 μ M for cephaloram). Rates of substrate hydrolysis were calculated from the progress curves, and a graph was constructed of percentage inhibition versus log(inhibitor concentration); the concentration of inhibitor that caused 50% inhibition (I_{50}) was read off. Such graphs were found to be linear up to at least 75% inhibition. The expression I_{50}/S , where S is the initial substrate concentration, gives K_i/K_m (see below).

(b) Measurement of S_{50} . Kinetic runs were carried out in the presence of various initial concentrations of substrate (between 2.5 and 11 mM) and a fixed concentration of inhibitor (usually about 20 μ M for cephalothin and cephaloridine, and about 80 μ M for cephaloram). A graph was constructed of percentage inhibition versus substrate concentration, from which the value of S_{50} (the concentration of substrate at which the amount of inhibition was 50%) was determined. The expression I/S_{50} , where I is the inhibitor concentration, gives K_i/K_m (see below).

(c) Hunter & Downs (1945) plots. Results from all kinetic runs (those performed in preliminary

studies, as well as those carried out in the process of I_{50} and S_{50} determinations) were processed to give one point on a Hunter & Downs (1945) plot for each run; thus, with cephaloridine as inhibitor, there were 26 points on the graph; for cephalothin there were 27, and for cephaloram, 16. The straight line of best fit was drawn for each graph, by the method of least squares. In all three graphs, this line passed through the origin, indicating that the value of K_m for the penicillinase-benzylpenicillin system was too small to be measured by this means. K_i/K_m was given by the slope of the line.

Theory. Starting from the standard Michaelis-Menten expressions for reaction velocities in the absence of inhibition (v_0), and in the presence of a competitive inhibitor (v_i):

$$v_0 = \frac{V}{1 + K_m/S} \text{ and } v_i = \frac{V}{1 + K_m/S(1 + i/K_i)}$$

we can obtain the following expression:

$$v_i/v_0 = \frac{K_i(S + K_m)}{K_iS + K_iK_m + iK_m} \quad (1)$$

When v_i is $\frac{1}{2}v_0$ (i.e. 50% inhibition occurs), i is I_{50} , or, alternatively, S is S_{50} , depending on whether it is the substrate concentration or the inhibitor concentration that is being varied. Eqn. (1) thus simplifies to either:

$$K_iS_{50} + K_mK_i = iK_m \quad (2a)$$

$$\text{or} \quad K_iS + K_mK_i = I_{50}K_m \quad (2b)$$

If we can ignore K_iK_m as being so small as not to affect the situation (discussed below), we can obtain from eqns. (2a) and (2b):

$$K_i/K_m = i/S_{50} \quad (3a)$$

$$\text{and} \quad K_i/K_m = I_{50}/S \quad (3b)$$

Is it justified to ignore K_iK_m ? The true values of K_i/K_m obtainable from eqns. (2a) and (2b), when K_iK_m is not ignored, are:

$$K_i/K_m = \frac{I}{S_{50} + K_m} \quad (4a)$$

$$\text{and} \quad K_i/K_m = \frac{I_{50}}{S + K_m} \quad (4b)$$

Previous work on staphylococcal penicillinase has shown that K_m for benzylpenicillin is usually lower than 10 μ M (Batchelor, Cameron-Wood, Chain & Rolinson, 1963; Richmond, 1965), whereas S under the conditions used in the present work was never set at lower than 4 mM, and thus $S + K_m$ differs from S by not more than 0.2%; similarly, values of S_{50} were invariably found, owing to the inhibitor concentration selected, to be above 4 mM, and so the value of $S_{50} + K_m$ will differ from S_{50} by no more than 0.2%. It can be seen that, on the basis of these

Table 1. Comparison of values of K_i/K_m obtained, by two methods, with three competitive inhibitors of cell-bound penicillinase of *Staph. aureus* 13137, with benzylpenicillin as substrate

Hunter & Downs method: lines of best fit were determined by method of least squares. I_{50}/S and I/S_{50} method: values given are means $\pm$ s.e.m., with the numbers of separate determinations in parentheses.

Inhibitor	K_i/K_m	
	Hunter & Downs method (graphical)	I_{50}/S and I/S_{50} method
Cephaloridine	3.42×10^{-3}	$3.35 \times 10^{-3} \pm 1.75 \times 10^{-4}$ (5)
Cephalothin	4.04×10^{-3}	$3.94 \times 10^{-3} \pm 2.39 \times 10^{-4}$ (4)
Cephaloram	1.88×10^{-2}	$1.86 \times 10^{-2} \pm 1.2 \times 10^{-3}$ (4)

figures, the premise that $K_m K_i$ may be ignored is justifiable.

It can be seen from eqn. (2b) that if I_{50} (y axis) is plotted against S (x axis), the resultant graph will be a straight line, of slope K_i/K_m , the intercept on the x axis being K_m .

Results. The values of K_i/K_m obtained for the three inhibitors both by the Hunter & Downs (1945) method and by the method suggested in the early part of this paper are shown in Table 1. There is good agreement between the values obtained by the two methods. Although the value with cephaloridine as inhibitor is smaller than that obtained with cephalothin, the difference is not significant at the 5% level (t test); however, the value of K_i/K_m with cephaloram was significantly larger ($P < 0.01$) than either of the other two values.

Discussion. The method described here for the evaluation of K_i/K_m has been found to be considerably more convenient than the method of Hunter & Downs (1945). A great advantage of the I_{50}/S method is that, with one enzyme, it enables comparisons to be made of the behaviour of a series of inhibitors with one substrate, and also of a series of substrates with one inhibitor. This characteristic should prove especially useful in the field of penicillinase, where there exist several different substrates and inhibitors for the various enzymes (Hamilton-Miller & Smith, 1964).

The affinity of cephaloridine and cephalothin for cell-bound penicillinase from *Staph. aureus* 13137 is

some five times as great as the affinity of cephaloram. The value of K_i/K_m for this latter substance, 1.86×10^{-2} , is very close to the value obtained by Crompton, Jago, Crawford, Newton & Abraham (1962), 1.7×10^{-2} , with a cell-free preparation of penicillinase from a different strain (R2), benzylpenicillin being the substrate in both cases. The possibility that cephaloridine and cephalothin may act synergistically with a hydrolysable penicillin against penicillinase-producing strains of *Staph. aureus* is at present being pursued, and preliminary results indicate that some synergism does in fact occur.

I am very grateful to Dr D. C. Watts for valuable discussions and advice on inhibitor kinetics. I acknowledge generous gifts of cephalosporins from Glaxo Research Ltd., and a grant from the Guy's Hospital Endowment Fund for Medical Research.

- Batchelor, F. R., Cameron-Wood, J., Chain, E. B. & Rolinson, G. N. (1963). *Proc. Roy. Soc. B*, **153**, 311.
 Crompton, B., Jago, M., Crawford, K., Newton, G. G. F. & Abraham, E. P. (1962). *Biochem. J.* **83**, 52.
 Dawson, R. M. C. & Elliott, W. H. (1959). In *Data for Biochemical Research*, p. 200. Ed. by Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. Oxford University Press.
 Hamilton-Miller, J. M. T. & Smith, J. T. (1964). *Nature, Lond.*, **201**, 999.
 Hunter, A. & Downs, C. E. (1945). *J. biol. Chem.* **157**, 427.
 Jevons, M. P. (1961). *Brit. med. J.* **i**, 124.
 Knox, R. & Smith, J. T. (1962). *J. gen. Microbiol.* **28**, 471.
 Richmond, M. H. (1965). *Biochem. J.* **94**, 584.