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NOTES AND BRIEF ARTICLES

(With Plates 15–19 and 8 Text-figures)

ESTIMATION OF INHIBITION CONSTANTS FROM COLONY GROWTH RATES OF FILAMENTOUS FUNGI

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Pirt (1973) has recently demonstrated that measurements of fungal colony growth rates can be used to estimate substrate affinities (called 'K_s' values). The method depends on the formulation (Monod, 1942) which describes the relationship between specific growth rate (μ) and the growth limiting substrate concentration (s) in bacterial cultures:

$$\mu = \mu_{\max} s/(s+K_s), \tag{1}$$

where $\mu_{\max}$ is the theoretical maximum specific growth rate and K_s, the saturation constant, is a measure of the affinity of the organism for the substrate. For a fungal colony the radial colony growth rate (K_r) can be substituted for μ since K_r is a direct measure of specific growth rate providing the width of the growing zone at the periphery of the colony remains constant (Trinci, 1971). Thus for a fungal colony relation (1) becomes

$$K_r = K_{r\max} s/(s+K_s). \tag{2}$$

Equations (1) and (2) are exact analogues of the Michaelis–Menten equation that describes the kinetics of an enzyme catalysed reaction:

$$v = V_{\max} S/(S+K), \tag{3}$$

where v is the reaction rate, $V_{\max}$ the maximum rate, S the substrate concentration, and K is the 'Michaelis' constant which, directly or indirectly, is a measure of the affinity of the enzyme for its substrate. Conformation of fungal colony growth rate data to equation (2) can therefore be demonstrated using any of the methods of graphical analysis which are commonly used in studies of enzyme kinetics, and Pirt (1973) shows that Lineweaver–Burk plots ($1/K_r \times 1/s$) generate straight lines except at the highest substrate concentrations which a degree of substrate inhibition is apparent.

Potentially the most useful aspect of this type of analysis is the determination of inhibitor constants. For if growth rate data can be processed using variants of the Michaelis–Menten equation which are known to apply to enzymic situations where both a substrate and an inhibitor are present, then it should be possible to establish the value of some constant equivalent

to the K_i (the dissociation constant of the enzyme-inhibitor complex). Such a constant would be of considerable use in comparisons of the effects of the same inhibitor on different fungi or of different inhibitors on the same fungus.

The various methods which can be used to analyse inhibition data derived from work with purified enzymes are extensively discussed by Webb (1963) and the derivations of the different graphical procedures will not be detailed here. Rather, the attempt is made in this note to apply what are essentially standard methods of analysis of enzyme kinetic data to fungal growth rate data. The main aim is to show that this approach is not as promising as it may appear and the intention is to emphasize the restrictions and illustrate some of the potential sources of error which are inherent in the method.

Although the methods of analysis are those of the enzymologist it cannot be too strongly stressed that any constants which might be evaluated are not necessarily characteristic of any identifiable enzyme reaction. Only correctly designed experiments using specific assay procedures and cellular extracts can be used to determine the constants which are termed K_i, K_m and K_s. In view of the very specific meanings that these constants have in terms of enzyme catalysed reactions it would be folly to use these designations for constants evaluated using far less direct measurements like colony growth rates. In order to emphasize the very different natures of these evaluations it is suggested that the terms I_k (inhibitor constant; being a measure of the affinity of the organism for an inhibitor) and S_k (substrate constant; a measure of the affinity of the organism for a substrate) be used in place of K_i and K_s when growth rate measurements are used.

The application of this method will first be demonstrated by analysing the effect of the glucose analogue 2-deoxy-D-glucose on the growth of *Coprinus*. A detailed report, using more traditional methods of analysis, has already been presented (Moore & Stewart, 1972), but in Fig. 1 the data obtained are shown analysed using four graphical procedures that are usually applied in studies of inhibition at the enzyme level. All of these plots use data derived from measurements of growth rates on media containing glucose (as substrate, S) and 2-deoxy-D-glucose (as inhibitor, I). These four methods of plotting are unanimous in suggesting a competitive inhibition and give an apparent S_k for glucose of 0·7 mM and I_k for 2-deoxy-D-glucose of 0·6 mM. Moreover, the data appear to comply with the necessary kinetic equations since not only do they generate straight lines but the constants derived from one plot can be processed through the appropriate equations and thereby used to successfully 'predict' the intercepts and slopes of other plots. This example, then, seems to indicate that the approach is well based in theory and is a reliable and relatively simple means of determining inhibitor constants. This apparent reliability and simplicity is misleading. A major difficulty with this approach is that it attempts to apply kinetic interpretations of a single enzyme reaction to an experimental system which measures the effect of substrate or inhibitor

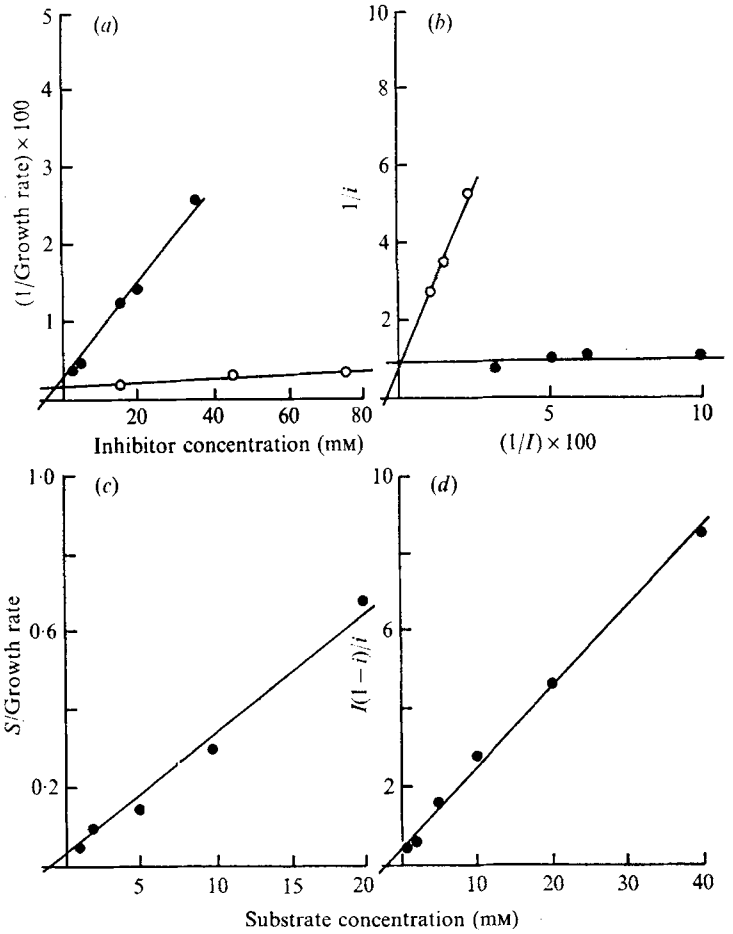

Fig. 1. Measurements of colony growth rates of *Coprinus lagopus* growing on medium containing glucose (as substrate, *S*) and 2-deoxy-D-glucose (as inhibitor, *I*) analysed using four standard methods of plotting enzyme-inhibition data. In (*a*) the reciprocal of the growth rate is plotted against inhibitor concentration for two different substrate concentrations (closed symbols 5 mM glucose, open symbols 150 mM glucose); the points fall on two straight lines which intersect just left of the ordinate. In (*b*) the reciprocal of the fractional degree of inhibition (i) is plotted against the reciprocal of inhibitor concentration; the points fall on two straight lines which intersect at an ordinate value of 1·0. In (*c*) and (*d*) the data derive from experiments in which the substrate concentration was varied while the inhibitor concentration remained the same.

on the whole of metabolism. Since every stage in the metabolic sequence can contribute to the kinetic equation which relates substrate and inhibitor concentration to colony growth rate the situation can obviously be far more complex than any enzymic system. Any change in metabolism will affect the relationship and may completely alter the interpretation of the system. Unless the exact enzymic nature of the inhibition is known (in which circumstances the system would in any case be better analysed at

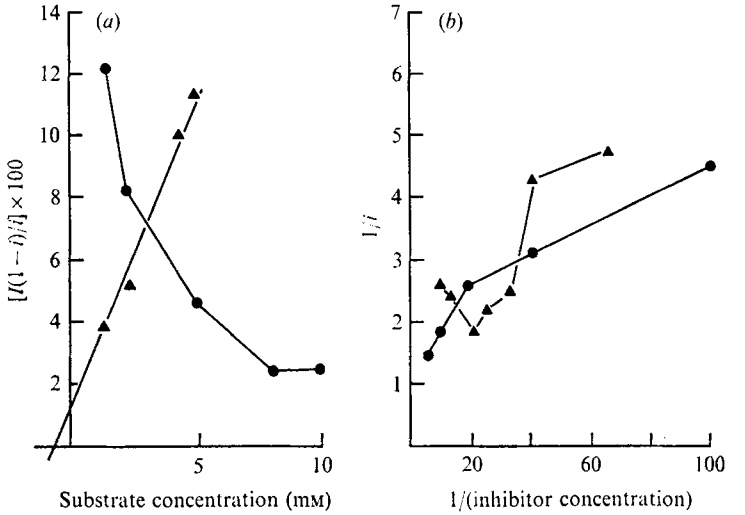

Fig. 2. Measurements of colony growth rates of *Coprinus lagopus* growing on media containing 2-deoxy-D-glucose (as inhibitor, *I*) and either fructose (●) or acetate (▲) as substrate (*S*) analysed using two standard methods of plotting enzyme-inhibition data.

the enzyme level) the possible effects of even apparently minor metabolic changes cannot be reliably predicted. Examples are provided by experiments similar to those considered above in which the inhibitory effects of 2-deoxy-D-glucose were examined but in the presence of the substrates fructose or acetate rather than glucose. The plots in Fig. 2 show that even these comparatively simple changes to the nutritional environment alter the whole pattern of the inhibition. An uncompetitive inhibition between 2-deoxy-D-glucose and fructose, and a competitive inhibition in the acetate system are suggested by the plots in Fig. 2(*a*) but neither of these conclusions are supported by the plots of Fig. 2(*b*). No inhibition or substrate constants can be derived and the only reliable conclusion is that the inhibition kinetics are, in both cases, different from those of the glucose/2-deoxy-D-glucose system. In these instances the method is not applicable but its unreliability can only be demonstrated by comparing the different types of plot.

Another example of the separation between the point of action of an inhibitor and its expression in terms of growth rate is provided by the effect of D-glutamate. The D-isomer of glutamic acid is known to inhibit extension growth in *Neurospora crassa* and seems to have a very specific influence on glutamic dehydrogenase (GDH) (Arkin & Grossowicz, 1970). The same is also true for *Coprinus lagopus* and detailed work has been done at the enzyme level so that it is known that inhibitions are competitive and true K_i values have been determined (Al-Gharawi, 1973). D-Glutamate inhibits the growth of *Coprinus* most severely on a medium (containing acetate as sole carbon source and urea as sole nitrogen source) which

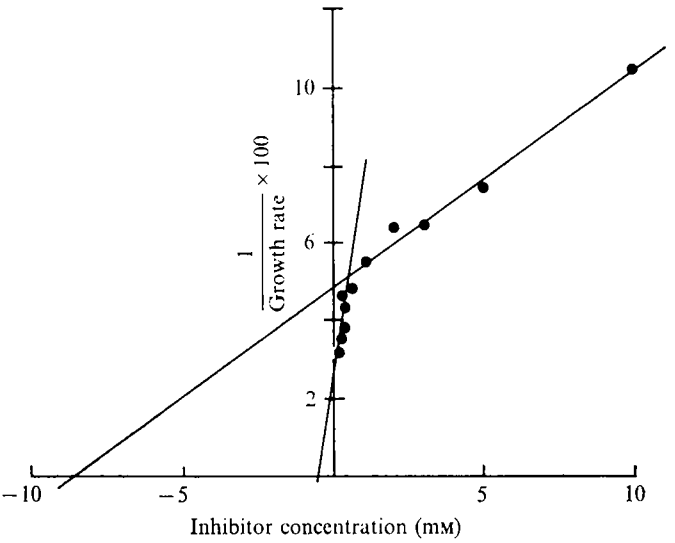

Fig. 3. Measurements of colony growth rates of *Coprinus lagopus* growing on a medium containing the inhibitor D-glutamate analysed with a standard plot for enzyme-inhibition data.

favours the production of the NAD-specific GDH. Growth-rate inhibition data obtained with such a medium can be plotted as in Fig. 3 (plots involving substrate concentration are not applicable in this case because the endogenous concentrations are unknown). Despite the specificity and relative simplicity of the enzyme inhibition involved here, the kinetics of the growth rate inhibition are quite complex. The plot in Fig. 3 is clearly at least biphasic, and perhaps more complex. Taking the simplest assumption two I_k concentrations can be calculated, 0·5 and 15·2 mM, from the abscissa intercepts. Neither of these is close to the true K_i values determined for the enzyme reaction itself which were: 7·2 and 8·6 mM (for the amination reaction) with 2-oxoglutarate and ammonium as substrate respectively. So although this is a very specific inhibition there is no simple relationship between the point of action of the inhibitor and the expression of the inhibition in the form of an effect on the rate of hyphal growth.

Another potential difficulty with this approach arises from the dependence of equation (2) on the constancy of the width of the peripheral growth zone. Certain inhibitors cause changes in the pattern of growth which seriously influence the peripheral growth zone; the hexose sugar L-sorbose is just such an inhibitor (Trinci & Collinge, 1973). Data from experiments in which growth rates were determined in the presence of sorbose (Moore & Stewart, 1972) produce an uninterpretable scatter of points in the various inhibition plots illustrated here, demonstrating categorically that the method is not applicable to inhibitors of this type.

The examples presented here suggest that it is possible in some situations to make use of the graphical methods of the enzymologist to analyse data

obtained by measuring colony growth rates. More importantly though, they emphasize the restrictions of this approach. It is clear that a number of different graphical analyses must be carried out and the plots critically compared in order to demonstrate that the methods are applicable. Plots of a single type may give rise to completely erroneous conclusions because they can quite fortuitously give curves which approximate to those of a successful plot. This situation can be recognized providing a number of different graphical procedures are utilized. Once the method is shown to be applicable the interpretation of the inhibition kinetics indicated by the form of the plots, and of any constants that are evaluated must be tempered by the recognition that the approach is very indirect and cannot be relied upon to reveal the characteristics of any specific enzyme reaction. The indirectness of the approach also gives rise to the sensitivity of the method to changes in the nutritional environment. It may be demonstrated that this method works satisfactorily for one inhibitor in a particular medium; it cannot then be assumed that it will work with the same inhibitor in a different medium or with a different inhibitor in the same medium. The approach must be validated for each individual set of conditions.

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STIMULATION OF GROWTH OF WHEAT STEM RUST IN AXENIC CULTURE BY DITHIOTHREITOL (DTT)

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Mycelium and spores of *Puccinia graminis* Pers. f. sp. *tritici* (race 126-ANZ-6, 7) were first cultured in vitro by Williams, Scott & Kuhl (1966). Since then there have been a number of reports of the growth in axenic