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A KINETIC METHOD FOR THE DETECTION OF INHIBITORY CONTAMINANTS IN RADIOACTIVE SUBSTRATES

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Abstract—A simple kinetic test is proposed that will detect the presence of contaminating inhibitors in the radioactive substrate solutions used in the radiochemical assays of enzymes. Double-reciprocal plots are constructed from initial velocity data obtained from assays conducted under conditions of constant specific radioactivity and of constant radioactivity. A significant difference between the K_m values determined in this way will show the presence of an inhibitory impurity necessitating the purification of the radioactive substrate. It is possible to determine whether the contaminating inhibitor is competitive, uncompetitive or mixed by this method and to estimate the true K_m value in the first two cases.

The presence of inhibitory contaminants in substrate solutions is known to cause erroneous estimates in enzyme kinetic parameters [1–4]. Whilst it is generally a relatively simple matter to purify the substrates used in enzyme studies, the small amounts of radioactive materials used for radiochemical assays sometimes makes it inconvenient to carry out such purifications and in many cases kinetic studies have been reported in which the purity of radioactive component of the assay medium has not been checked. In this paper we propose a simple kinetic test to detect the presence of inhibitory impurities in the radioactive substrate.

THEORY

Most radiochemical enzyme assays that involve varying the substrate concentration are carried out either at constant radioactivity or constant specific radioactivity. In the former approach the amount of radioactive substrate present is held constant and the substrate concentration is varied by having different amounts of unlabelled substrate present. The constant specific radioactivity method involves maintaining the ratio of radioactive to unlabelled substrate at all substrate concentrations. The presence of an inhibitory contaminant in the radioactive substrate solution will have different effects upon the kinetic parameters for the two methods. In the constant radioactivity method the amount of inhibitor will be constant in each assay and thus the normal reversible inhibitor equations (see eg. [5]) will apply whereas in the constant specific radioactivity method the inhibitor concentration will vary with the substrate concentration and the kinetic equations derived for systems in which the substrate is contaminated by an inhibitor [1–4] will apply. The types of effects that will be seen will depend upon the type of inhibitor present and the effects of the common simple types are considered in terms of an enzyme obeying the simple Michaelis–Menten equation.

(a) *Competitive inhibitors.* Contamination of the radioactive substrate with a competitive inhibitor will give rise to the kinetic equation

$$v = \frac{V}{1 + \frac{K_m}{s}\left(1 + \frac{i}{K_i}\right)} \tag{1}$$

if the constant radioactivity method is employed, and where s and i represent the substrate and contaminating inhibitor concentrations. If the constant specific radioactivity method is used the inhibitor concentration will be a constant proportion of the substrate concentration and thus we can write $i = x.s$, where x is a constant. Under these conditions equation (1) will become

$$v = \frac{V}{1 + \frac{K_m}{s} + \frac{xK_m}{K_i}}. \tag{2}$$

The double-reciprocal plots that will be given by systems obeying equations (1) and (2) are shown in Fig. 1a. In both cases linear plots are obtained and the lines given by the two methods will intersect on the right hand side of the vertical axis. It can be seen that line given by equation (1) will intersect the vertical axis at a value of $1/V$ and the line given by equation (2) will have a slope of K_m/V allowing the true K_m value to be determined from the results of experiments carried out using both assay approaches.

(b) *Uncompetitive inhibitors.* Contamination with an inhibitor of this type will give the equation

$$v = \frac{V}{1 + \frac{i}{K_i} + \frac{K_m}{s}} \tag{3}$$

if the constant radioactivity method is used, and

$$v = \frac{V}{1 + \frac{xs}{K_i} + \frac{K_m}{s}} \tag{4}$$

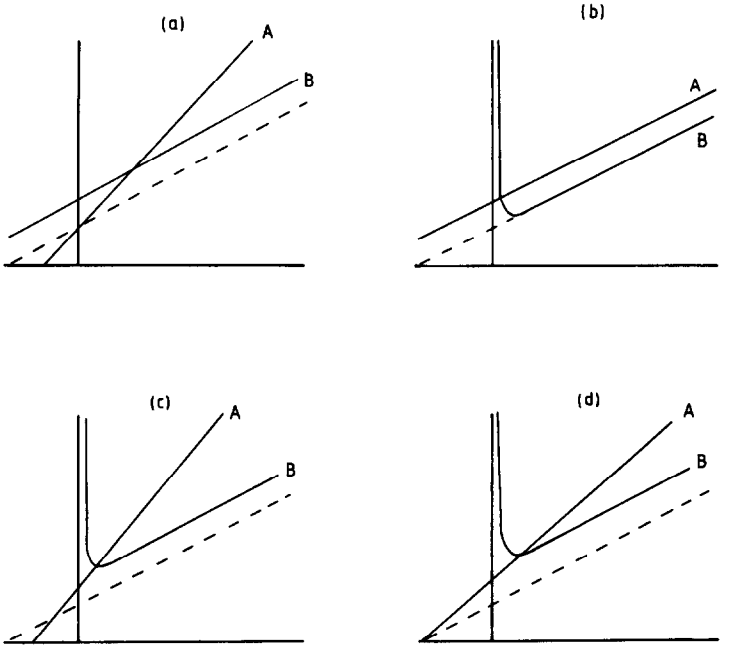

Fig. 1. Double-reciprocal plots [6] given by systems in which the radioactive substrate is contaminated by a reversible inhibitor. In all cases lines labelled (A) would be determined by the constant radioactivity method and those labelled (B) would be determined by the constant specific radioactivity method. The broken line represents that that would be expected in the absence of any inhibition. Plots for competitive (a), uncompetitive (b), mixed (c) and non-competitive (d) inhibition are shown. In the case of uncompetitive inhibition the linear portion of line B is superimposed upon that given in the absence of inhibition. For mixed inhibition line A can intersect with the broken line above or below the $-1/s$ axis.

if the constant specific radioactivity method is used. The types of double-reciprocal plots that will be obtained are shown in Fig. 1b. In this case the lines given by the two methods will be parallel but high substrate inhibition will be shown in the experiments carried out at constant specific radioactivity. Extrapolation of the linear portion of the line obtained at constant specific radioactivity (equation (4)) will allow V and K_m to be calculated from the intercepts on the vertical and horizontal axis in the normal way.

(c) *Mixed inhibitors.* Contamination with a mixed inhibitor will give

$$v = \frac{V}{1 + \frac{i}{K_i'} + \frac{K_m}{s}\left(1 + \frac{i}{K_i}\right)} \quad (5)$$

for the case where the constant radioactivity method is used and

$$v = \frac{V}{1 + \frac{xs}{K_i'} + \frac{K_m}{s} + \frac{xK_m}{K_i}} \quad (6)$$

when the constant specific radioactivity is used. The types of double-reciprocal plots that will be given are shown in Fig 1c. The lines given by the two methods will not be parallel but there is no way of predicting whether the intersection point will be to the right-hand or the left-hand side of the vertical axis. This type of inhibition can however be distinguished from a competitive contaminant since in this case apparent high-substrate inhibition would be given when the constant specific radioactivity method used but not with the constant radioactivity method. It is not possible to obtain the true K_m value in this case from these two lines.

In the special case of non-competitive inhibition where $K_i = K_i'$ in equations (5) and (6) the double-reciprocal plots (Fig. 1d) will not be distinguishable from those given by the mixed case.

Contamination of the radioactive substrates used in enzyme assays is not uncommon and frequently results from the instability of these compounds. In the assay of methyltransferases contamination of *S*-adenosylmethionine by *S*-adenosylhomocysteine [7] can lead to significant errors in the estimation of enzyme activities due to the powerful product inhibition given by the latter compound (see eg. [8]). The contamination of amine substrates used for the assay of monoamine oxidase by their corresponding aldehydes has also been shown to lead to incorrect kinetic conclusions [4]. Both in these and other cases the kinetic method outlined in this paper can provide a convenient means of checking for significant amounts of impurities in the radioactive substrates used.

DISCUSSION

The approach outlined in this paper provides a simple way of detecting the presence of a reversible inhibitor in the radioactive substrate solution used for radiochemical enzyme assays. A significant difference between the K_m values determined by the two

methods would indicate that the radioactive substrate solution should be purified. A contaminating irreversible inhibitor may readily be detected by determining the variation of initial velocity with enzyme concentration [5]. The method can be extended to cover contaminating inhibitors affecting enzymes that catalyse two substrate reactions (see [2,4]) and the effects that will be seen will be similar to those shown in Fig. 1. Despite the fact that the true K_m value may be extracted from results obtained with competitive and uncompetitive contaminants by using this method, it is not suggested that this represents an ideal way of doing so. It is, of course, not possible to determine a value of K_i (or K_i') by using this approach without knowing the concentration of the inhibitory contaminant. A limitation to this approach may occur if true high-substrate inhibition occurs, but in this case deviations from linearity will be seen with double-reciprocal plots determined using both the constant radioactivity and the constant specific radioactivity methods. In cases where high-substrate inhibition occurs without any inhibitory contamination of the substrate, the K_i values calculated for inhibition by substrate (see eg. [5]) will be the same whichever of the radiochemical assay approaches is used whereas an additional contamination of the radioactive substrate with either an uncompetitive or a mixed (or a non-competitive) inhibitor will result in unequal values.

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