A Method for Deriving Kinetic Constants for Two Enzymes Acting on the Same Substrate

By G. SPEARS, J. G. T. SNEYD and E. G. LOTEN

Department of Preventive and Social Medicine and Department of Clinical Biochemistry, University of Otago Medical School, Dunedin, New Zealand

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It has been demonstrated that 3':5'-cyclic nucleotide phosphodiesterase activity in several mammalian tissues displays two apparent K_m values for cyclic AMP (adenosine 3':5'-cyclic monophosphate) (Loten & Sneyd, 1970; Beavo, Hardman & Sutherland, 1970; Murad, Manganiello & Vaughan, 1970; Brooker, Thomas & Appleman, 1968; Loten, 1970). This phenomenon could be due to the presence of two separate enzymes acting on the same substrate, or to a single enzyme with unusual kinetics. Such negatively co-operative enzymes have been described (Levitzki & Koshland, 1969). To distinguish between these two possibilities is not always easy, and it may require physical separation of the two enzymes. In the case of phosphodiesterase there is some evidence that the phenomenon is caused by the presence of two separate enzymes (Thompson & Appleman, 1971; Senft, Munske, Schultz & Hoffman, 1968).

It is apparent that the derivation of kinetic parameters for two enzymes acting on a common substrate will be complicated (Thompson & Appleman, 1971; Dixon & Webb, 1964), but the present paper describes a procedure that will give a good approximation of such parameters.

Experimental. Male Wistar-strain albino rats (150–220 g) were obtained from the Otago Medical School Animal Department. The cyclic [^3H]AMP (3.0 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.) and was purified before use by passage through an anion-exchange resin column. Cyclic AMP, adenosine and *Crotalus atrox* snake venom were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). The ion-exchange resins were purchased from Bio-Rad Laboratories (Richmond, Calif., U.S.A.) and used without further purification. Bovine serum albumin (fraction V) was supplied by Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). All other chemicals used were of analytical grade.

Rat livers were homogenized in 0.25 M-mannitol containing 25 mM-tris-HCl buffer, pH 7.5, and 1% (w/v) of bovine serum albumin, and the homogenate was centrifuged at 48 000 g for 20 min. The clear red supernatant was used in the enzyme assays. It was found that omission of the albumin resulted in a loss of enzyme activity when homogenates were

diluted, and consequently it was included in all liver homogenates and dilutions.

Phosphodiesterase activity was measured by a method described previously (Loten & Sneyd, 1970). Briefly the procedure measures the amount of cyclic [^3H]AMP converted into adenosine by the combined actions of phosphodiesterase and an excess of snake-venom nucleotidase. Radioactive adenosine is separated from unchanged cyclic AMP by chromatography on a small column of Bio-Rad AG-1 anion-exchange resin (X2; 200–400 mesh; Cl^- form).

Results and discussion. When the enzyme velocities obtained experimentally for rat liver phosphodiesterase were used in a plot of s/v against s , there were two separate straight-line portions to the graph, as has been described previously for adipose tissue (Loten & Sneyd, 1970). Assuming that this phenomenon was caused by the presence of two enzymes acting on the same substrate, kinetic parameters for the low- K_m enzyme were derived from the straight line below a substrate concentration of $2\ \mu\text{M}$, and constants for the high- K_m enzyme from the straight line above a substrate concentration of $50\ \mu\text{M}$. The K_m values for the high- K_m and low- K_m enzymes were $61\ \mu\text{M}$ and $1.2\ \mu\text{M}$ respectively, and the V_{max} values were 5317 pmol/min per mg of protein and 312 pmol/min per mg of protein respectively.

Derivation of such constants directly from a graph assumes (a) that at substrate concentrations below $2\ \mu\text{M}$ the high- K_m enzyme does not contribute appreciably to the observed velocity and (b) that at substrate concentrations above $50\ \mu\text{M}$ the low- K_m enzyme does not contribute appreciably to the observed velocity. It can be calculated that in a mixture of two enzymes with the kinetic parameters set out above the high- K_m enzyme contributes 38% of the observed velocity at $1\ \mu\text{M}$ and that the low- K_m enzyme contributes 11.3% of the observed velocity at $50\ \mu\text{M}$. Thus the two assumptions are not justified and the kinetic parameters derived by such a method will be in error.

A procedure suitable for application to a computer was developed that enabled these parameters to be corrected and that gave a good approximation of assumed parameters in a model system.

Briefly the program followed the following steps.

(1) The experimental enzyme velocities are printed and s/v is calculated for each substrate concentration.

(2) From these values a regression line of s/v on s is calculated for substrate concentrations above $25\mu\text{M}$. From this line V_{max} and K_m for the high- K_m enzyme can be estimated.

(3) From these values of K_m and V_{max} , the velocity of such an enzyme can be calculated by using the Michaelis-Menten equation at each of the substrate concentrations used below $25\mu\text{M}$. These calculated velocities are then subtracted from the observed velocities at each substrate concentration, giving new values for s/v at low substrate concentrations. From these corrected values a regression line of s/v on s is calculated and values for the V_{max} and K_m of the low- K_m enzyme are derived. These parameters should represent those of the low- K_m enzyme in the absence of activity contributed by the high- K_m enzyme at low substrate concentrations, and should therefore more closely represent the true parameters than those derived directly from the original plot of s/v against s .

(4) The new parameters for the low- K_m enzyme can now be used to calculate the velocity of such an enzyme at each of the high substrate concentrations (i.e. above $25\mu\text{M}$). These velocities are subtracted from the observed velocities and hence new s/v values obtained. From a regression line of s/v against s new values for V_{max} and K_m of the high- K_m enzyme can be calculated. These values should refer to the high- K_m enzyme in the absence of activity contributed by the low- K_m enzyme at high substrate concentrations.

(5) The new parameters for the high- K_m enzyme can now be used to correct those for the low- K_m

enzyme again, as in (3) above, and the whole process can be repeatedly recycled until a pre-selected criterion of convergence is achieved.

This procedure was used on a model system that had the following kinetic constants:

$$\left. \begin{array}{l} K_1 = 1\mu\text{M} \\ V_1 = 1 \end{array} \right\} \text{low-}K_m \text{ enzyme}$$

$$\left. \begin{array}{l} K_2 = 50\mu\text{M} \\ V_2 = 50 \end{array} \right\} \text{high-}K_m \text{ enzyme}$$

(units are omitted from the velocities for simplicity).

Additive reaction velocities for two such enzymes were calculated at substrate concentrations from 0.05 to $1\mu\text{M}$ and from 50 to $333\mu\text{M}$.

These velocities were then used to derive constants for the two enzymes by the above procedure. The results of successive correction cycles can be followed on Table 1, which shows that a close approximation to the original assumed constants is obtained after only a few correction cycles.

A computer program written in PL/I incorporating the principles set out above has been deposited as Supplementary Publication SUP 50006 with the National Lending Library for Science and Technology, Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies may be obtained on the terms indicated in *Biochem. J.* (1971), **121**, 7. The program has additional facilities: it enables a dividing point other than $25\mu\text{M}$ to be selected between the data for the high- K_m and low- K_m enzymes; it is able to indicate which such dividing point gives the best fit to the data; it also gives an estimate of the goodness of fit of the experimental data to the two straight lines finally drawn.

This program was used to correct experimental results for liver phosphodiesterase and gave K_m

Table 1. Successive corrections of kinetic parameters derived for a model system of two enzymes acting on the same substrate

Additive reaction velocities for two enzymes with kinetic constants at the head of the table were calculated over a substrate range from $0.05\mu\text{M}$ to $1\mu\text{M}$ and from $50\mu\text{M}$ to $333\mu\text{M}$. These velocities were then used in the procedure described in the text to derive constants for the two enzymes. Units are omitted from the velocity values, for simplicity.

	Low- K_m enzyme		High- K_m enzyme	
	K_m (μM)	V_{max}	K_m (μM)	V_{max}
Assumed constants	1	1	50	50
Constants derived directly from a plot of s/v against s	2.963	5.814	48.185	50.891
1st correction	1.320	1.060		
2nd correction			50.115	49.948
3rd correction	0.993	1.001		
4th correction			50.027	50.002
5th correction	0.987	0.994		
9th correction	0.986	0.992		
10th correction			50.006	50.010

values for the high- K_m and low- K_m enzymes respectively of $65\mu\text{M}$ and $0.5\mu\text{M}$, and V_{max} values of $5266\text{pmol/min per mg of protein}$ and $92\text{pmol/min per mg of protein}$. These differ significantly from the values derived graphically from the experimental results, indicating once more the inaccuracies inherent in simple graphical derivation of such constants under these circumstances.

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