A FLOW CYTOFLUORIMETRIC METHOD FOR MEASURING ENZYME REACTION KINETICS IN INTACT CELLS

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1. Introduction

Flow cytofluorimetric techniques, introduced by Dittrich and Gohde [1] and by Van Dilla et al. [2], which enable the fluorescence from suitable stained constituents to be measured in individual cells at very rapid rates, are becoming increasingly used in various branches of biological science. The preliminary work presented in this letter was undertaken to test the feasability of studying enzyme reaction kinetics in populations of individual viable cells with these methods.

2. Materials and methods

2.1. *EMT6/M/CC cells*

EMT6/M/CC cells are a variant of a mouse mammary tumour line which can be grown either in vitro or in vivo [3]. The preparation of single cell suspensions and growth kinetic data have been described previously [4], and for this initial work in vitro cells were used during the early plateau phase of growth.

2.2. *Enzyme reaction*

Carboxylesterase (carboxylic-esterase hydrolase, EC 3.1.1), referred to subsequently as esterase(s), was assayed by monitoring the rate at which the fluorescence from free fluorescein accumulated in single cells after mixing with the nonfluorescent substrate fluorescein diacetate, FDA. This reaction has previously been studied by more conventional methods [5].

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2.3. *Substrate preparation*

FDA, 5 mg, (Koch-Light laboratories pure AR grade) was dissolved in 1.0 ml 'spectrograde' acetone (Fison's Ltd) and stored at −20°C in the dark. Stock solution, 20 μl, was added to 50 ml 'Dulbecco A' phosphate buffered saline (PBS) to give an FDA concentration of 4.8 μM. Aliquots, 1.5 ml, of substrate with concentrations varying between 4.8 μM and 0.24 μM were then prepared by dilution with FDA free PBS. These were then mixed with 1.5 ml of medium containing cells to give final FDA concentrations varying between 0.12 μM and 2.4 μM.

2.4. *Fluorescence determination*

Fluorescence determinations were performed with a Bio-Physics Cytofluorograf, model 4800A, with laser excitation at 488 nM. After mixing, the sample was introduced into the instrument, and the output signal from individual cells was fed to a PDP 11/40 computer via analogue to digital convertors.

2.5. *Computer sampling and analysis*

The cell concentration was adjusted so that about 2.10^5 cells were analysed per second. The output from the instrument was directed into the computer as soon as stable flow rates were attained in the optical flow cell, 25 s after mixing with FDA. The computer was instructed to record for 5 s, then to wait for 10 s sequentially over a 3-min period. The channel number (proportional to fluorescence intensity) of the median

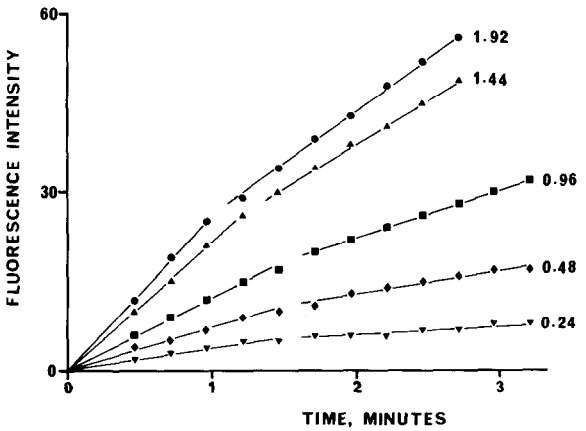

Fig.1. Progress curves of fluorescence intensity versus time for the substrate concentrations shown in μM.

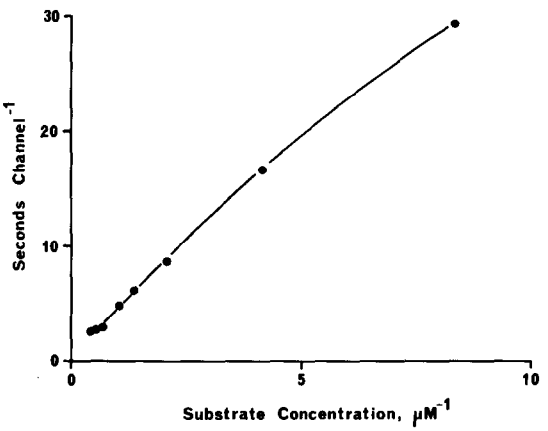

Fig.3. 'Lineweaver-Burk' double reciprocal plots of the data shown in fig.2.

of the distribution obtained during each 5 s record was then calculated and printed out.

3. Results

Figure 1 gives 5 selected progress curves of fluorescence intensity versus time for the various substrate concentrations shown. Each curve exhibits a biphasic pattern, and the reasons for this are discussed later. A 'Michaelis-Menten' plot of the initial velocity versus substrate concentration [6] is shown in fig.2. The initial velocities were calculated by regression analysis of the data in the first, apparently linear segment of the progress curves, and the limits indicate one standard error of estimate. The line has been drawn to pass within the error bars. Figure 3 gives the corresponding double reciprocal plots of $1/v$ versus $1/s$ [7], which shows a nonlinear pattern indicating a departure from 'Michaelis-Menten' kinetics.

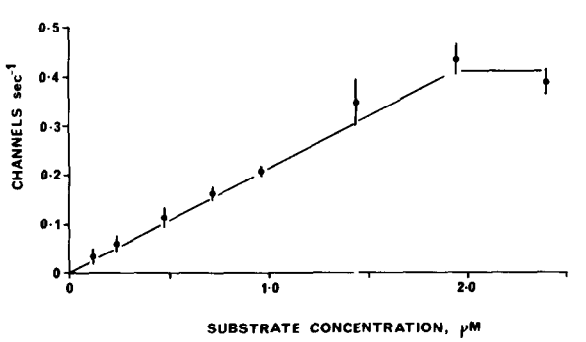

Fig.2. Plots of initial velocities versus substrate concentration. The limits represent one standard error of estimate.

4. Discussion

The method described here for studying enzyme reaction kinetics, which was illustrated with the conversion of FDA to free intracellular fluorescein catalysed by esterase(s), has a number of advantages and some disadvantages. Firstly, the characteristics of the instrument are such that only the fluorescence from the cell is recorded. Any leakage of product from the cell into the supporting medium (which may cause artefacts, see below) was so rapidly diluted by the 'streaming' sheath fluid in the optical flow cell that the background fluorescence was not recordable by the instrument with the photomultiplier gain settings used. This has the great advantage that the determinations can be performed on intact individual cells. Youdim and Woods [8] have pointed out that the properties of enzymes can be influenced by the type of tissue preparation used (eg., cell homogenates, tissue slices or purified enzyme extracts), and that results must be interpreted accordingly. Flow cytofluorimetric techniques should make it possible to overcome some of these interpretive problems by enabling enzyme reaction kinetics to be studied in

situ in viable cells with intact membranes. The perturbation of the cells is minimal prior to the determinations, as they are maintained in full growth medium until seconds before analysis.

Secondly, the distribution of fluorescence within the population can be studied with statistical precision. Histograms of fluorescence distribution taken at 45 s and 180 s after mixing with substrate at a final concentration of 0.48 μM, had coefficients of variation, CV, of 30% and 47%, respectively, and both were positively skewed. As the instrumental CV is constant with light intensity [9,10] the difference in the CVs of the two histograms is very highly suggestive of population inhomogeneity. During the early plateau phase of growth of this tumour cell line the [^{3}H] thymidine labelling index is between 20% and 25% with about 40% of the population in a cycling state [4,9]. Thus, from a cell kinetic standpoint the population is far from homogenous. As it has been shown that various enzymes exhibit cyclical changes through the cell cycle [11] it is possible that some of the increase in the CV at 180 s could be due to a mixture of cells with different enzyme content.

The reaction kinetics deviated considerably from the 'Michaelis-Menten' type [6]. A double reciprocal plot of the data [7] showed a non-linear pattern which is compatible with two enzymes acting on one substrate [12]. Corresponding plots (not shown) of velocity versus the ratio of velocity to substrate concentration ('Eadie-Hofstee' [13,14]), and the direct linear plot of Eisenthal and Cornish-Bowden [15] both suggest that two populations may be present. If this proposition is correct, the initial velocities must contain two or more components which are substrate-concentration dependent. Rate limitation, due to diffusion across intact membranes, or differing substrate-dependent reaction rate characteristics, could both be implicated in producing predominance of one enzyme reaction over another at varying substrate concentrations. Each of these factors is undergoing investigation, and the results will be communicated in due course.

The method has a number of disadvantages. Leakage of fluorescein has been reported to vary considerably from cell to cell, where measurements on individual cells gave rates of leakage with half-times between 8 min and several hours [5]. In our studies the half-time of fluorescence decrease from the population showed a biphasic pattern after cells were resuspended in substrate free medium. The first part of the curve declined exponentially with a half-time of 13 min, and the second section with a half-time of 7 min. This pattern is compatible with continuing production of fluorescein from intracellular substrate during the initial phase, followed by the faster rate of loss when all the intracellular FDA had been hydrolysed. Leakage of product is one factor which could contribute to the biphasic nature of the progress curves shown in fig.1. Although the results have been represented with straight lines through the points, the data probably describe curves; thus, the initial slopes will tend to be underestimated. Indeed, the regressions, which all included the origin as a point, had positive intercepts in all cases. Whilst in no case did this differ significantly from zero ($p > 0.1$) the fact that all deviations were in the same direction suggests that the initial velocities were all underestimated. However, the errors are likely to be small as the time course over which the regressions were performed was always less than 1.5 min, which is relatively short compared with the measured half-time of leakage. A more complete method of analysing the progress curves, to include leakage correction, will be undertaken.

A further disadvantage of our current computer sampling and analysis system is the inability to obtain a point between time zero and 27.5 s. However, this is only a technical problem which is also receiving attention. A further source of error can occur from the increase in fluorescence intensity during the sampling interval. For faster reaction rates this can be partly overcome by increasing the cell concentration and decreasing the sampling interval whilst maintaining the time between recordings. However, the maximum flow rate of cells through our instrument, without obtaining significant overlapping in the focal plane of the laser beam, is about 5000 per second. Thus, the ability to study fast reactions with this method may be limited unless coincidence correction is included in the analytic procedures.

Although many problems remain to be solved, we feel that this method could make a useful contribution to enzyme kinetic work by enabling reactions to be studied in situ in viable intact cells.

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