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AN ASSAY FOR PLASMA MEMBRANE PHOSPHATASE ACTIVITY IN POPULATIONS OF INDIVIDUAL CELLS

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Abstract—Flow cytofluorimetric techniques have been used to assay plasma membrane phosphatase activity in populations of individual mouse mammary tumour cells. A new mathematical treatment, involving 'steady-state' kinetic assumptions to analyse the asymptotic portions of the progress curves, gave results which were compatible with normal Michaelian kinetics for the hydrolysis of the fluorogenic substrate 3-*O*-methyl fluorescein phosphate. The potential application of the methodology in the field of latent anti-neoplastic drugs is discussed.

High levels of phosphatases have been reported for a variety of human tumours (see review by Douglas [2]) and because of this a number of anti-cancer agents have been conjugated with *O*-phosphate groups to produce latent forms which might be activated in these neoplasms [3–8]. Latent anti-neoplastic agents designed for activation by a variety of other enzymes have also been synthesised [9].

To properly evaluate enzyme-activated anti-neoplastic agents of this type it is essential to investigate the possible relationship between drug response and the enzyme activities of both tumour and normal host tissues. However, care must be taken in choosing the type of enzyme preparation to be used in comparative studies of this nature. Artefactual results may be generated by the disruption of cellular and sub-cellular membrane permeability barriers in the preparation of cell-free extracts [10]. Intact cell preparations may therefore be more appropriate. Ionised drugs, such as *O*-phosphate esters, may be activated only by enzymes situated on the external cell membrane. Membrane preparations could be used to study the activation of these agents, but the preparation of clean fractions of homogenous character is technically difficult. The heterogeneity of neoplastic tissues also poses problems in the interpretation of enzyme activity data obtained by conventional assay techniques. This problem is compounded by 'contamination' of the sample with normal tissue components which may give rise to further artefacts.

We have recently developed a flow cytofluorimetric technique which allows the estimation of esterase activities in populations of intact cells [11, 12]. In the present communication we report the extension of these methods for the assay of membrane phosphatases in populations of intact cells and describe the reaction kinetics observed in EMT6 mouse tumour cells cultured *in vitro*.

MATERIALS AND METHODS

EMT6/M/CC cells are a tissue culture-adapted derivative of the EMT6 mouse mammary tumour line [14], and cells were prepared by two different methods. Firstly, cells were assayed in the early plateau phase of growth when the majority of the population is arrested in a 'G1' state [15, 21]. After removal of the monolayer by trypsinization the cells were resuspended in full growth medium containing 20 per cent calf serum to neutralize excess trypsin. Secondly, to test if artefacts in the assay system are produced by trypsinization, cells were harvested by mitotic selection and details of our methods have been published previously [22]. The selected cells were pooled, concentrated, placed in a universal plastic container and agitated periodically to stop any adherence to the plastic surface. After one hour the majority of the population had divided to produce a G1 population. These cells were never in contact with trypsin.

Following these various procedures the single cell suspension was then centrifuged at 200 *g* and the cell pellet was resuspended in 'Dulbecco A' phosphate-buffered saline (PBS), pH 7.3, at a concentration of 1.2×10^6 cells per ml.

Enzyme reaction. Phosphatase activity associated with intact single cells was assayed by measuring the fluorescence from 3-*O*-methyl fluorescein produced by the hydrolysis of the non-fluorescent substrate 3-*O*-methyl fluorescein phosphate (MFP).

Substrate preparation. MFP (monocyclohexyl-ammonium salt, A grade) was obtained from Calbiochem Ltd. (Herts., U.K.). As was noted previously [16] we found that unless extreme care is taken this compound can undergo very rapid 'spontaneous' hydrolysis. Chromic acid-washed glassware was therefore used for all experiments. A stock solution of substrate was prepared by dissolving 10 mg MFP in 25 ml PBS and subsequent dilutions were made in PBS.

Fluorescence determinations. These were performed on populations of single cells with a Bio-Physics flow system, the Cytofluorograf model 4800A. These instruments constrain a monodispersed suspension to flow in an optical chamber so that one cell at a time passes through a focused laser beam and up to 5000 cells can be analysed per second. Each cell passing through the exciting beam gives rise to a

pulse of fluorescent light which is collected by a photomultiplier at 90° to the intersection of the laser beam and the cell stream. The resulting pulses are amplified and analysed by an analogue-to-digital convertor (ADC) which gives an electrical output measured in arbitary units, channels, which is directly proportional to the amount of light-emitted from each cell. Light scattered in the forward direction from cells, or subcellular particles, is simultaneously collected and similarly analysed. The quantity of forward scattered light is directly proportional to cross-sectional area of the particle, and it is possible to set the electronics of the instrument so that only fluorescent light from a predetermined size distribution is analysed. The cells used in these experiments had a mean diameter of 14μ and standard deviation of about 2μ. Thus by setting the instrument to record fluorescence from a size 'gate' of 10μ to 20μ we were able to exclude any 'background' fluorescence from fragments, clumps or in solution.

The reaction was started by mixing 1 ml of the single cell suspension and 3 ml substrate solution. The final concentration of MFP varied over the range 48–381 μM. Immediately after mixing, the sample was introduced into the instrument and the output signal from individual cells was fed to a PDP 11/40 computer which was instructed to record for 5 sec, then to wait for 10 sec sequentially over the first 60 to 90 sec. Thereafter, the population was sampled to give readings at 30 sec intervals. The flow rates were adjusted so that about 1000 cells were analysed per sec, and the median of the fluorescence distribution obtained during each 5 sec recording interval was printed out expressed in arbitrary units, channels. Details of this method for the assay of esterase activity have been published previously [11, 12]. No activity was observed in the absence of either cells or substrate and the determinations were duplicated.

Fluorescence microscopy. To determine the location of the fluorescence in individual cells a number of parallel samples were inspected by fluorescence microscopy using Zeiss epi-fluorescence equipment.

RESULTS

Flow cytofluorimetry. Figure 1 shows progress curves of the amount of cellular fluorescence plotted against time at five different substrate concentrations obtained with the trypsinized cells. Each point represents the average value obtained from two runs. In each set of data there was a very rapid initial increase in fluorescence which reached a well defined asymptotic value at 2.5 to 5 min depending on substrate concentration. Even though the first record was made at 15 sec after mixing the cells with substrate, it was impossible to obtain initial reaction velocities from these data due to the very rapid initial rate. Thus, we could not use conventional methods [20] to determine the reaction kinetic parameters and a new mathematical approach was therefore developed to analyse the type of curve shown in Fig. 1. Briefly, this involves analysis of the 'steady-state' conditions associated with the asymptotic portions of the curves in which allowance for loss of fluorescent product from the reaction site is made. The mathematics and assumptions required for this analysis are given in the appendix. The results produced by this analysis for both the trypsinized and non-trypsinized cell preparations, which were compatible with normal kinetic behaviour, are given in the table. At present the maximum velocity of the reaction must be expressed in arbitrary units, and the interrupted line in Fig. 1 depicts the maximum velocity predicted by this method if loss of product from the reaction site had not taken place.

Fluorescence microscopy. The flow system assay offers no information as to the site of enzyme activity.

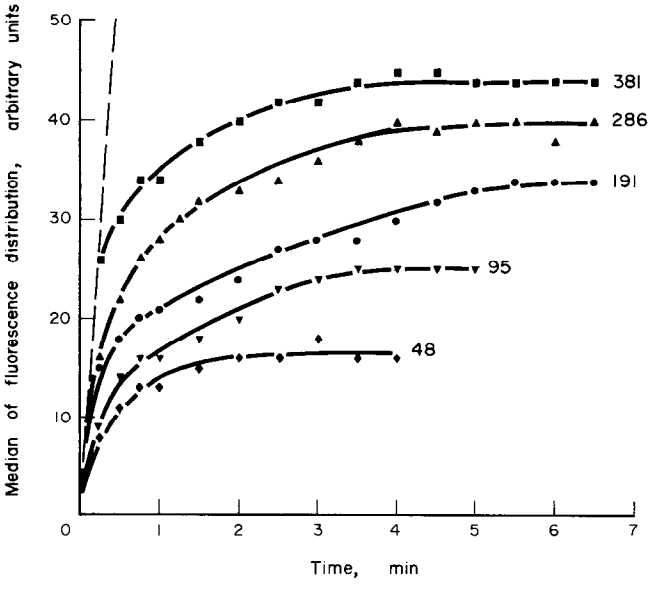

Fig. 1. Progress curves of the increase in fluorescence intensity vs time for the hydrolysis of the MFP substrate at the concentrations shown in μM. These data were obtained with the trypsinized cells, and the maximum reaction velocity assuming no loss of product from the reaction site is depicted by the dashed line.

However, parallel observations with the fluorescence microscope revealed that the fluorescence associated with the cells was localized in a 'ring' at the external surface of the cells. This was not seen when cells were incubated with fluorescein even when saturated solutions were used.

DISCUSSION

A flow cytofluorimetric method for the assay of phosphatase activity in populations of intact single cells has been described in the present paper. Progress curves for the hydrolysis of the MFP substrate showed a rapid initial increase in fluorescence which approached a substrate concentration dependent asymptotic value. Because of the rapid initial increase in fluorescence it was not possible to analyse the data by conventional methods. However, a new mathematical treatment, involving 'steady-state' kinetic assumptions and allowance for loss of product from the reaction site was developed. This gave values of the maximum velocity and Michaelis constant for the reaction which appears to follow a normal substrate dependent velocity pattern. The model on which the analysis was based assumes that the enzyme(s) and substrate combine with rate constant k_1 to form an enzyme–substrate complex which may then either dissociate into products and free enzyme with rate constant k_2, or revert to substrate and enzyme with rate constant k_{-1}. The shape of the progress curves in Fig. 1 suggested either inhibition of the hydrolysis reaction by one or both products, or loss of the product 3-*O*-methyl fluorescein from the 'site of analysis'. Reaction inhibition by the products was discounted as the asymptotic fluorescence value remained constant for over 45 min even at the highest substrate concentration. Furthermore, in one of the extended time course experiments it was discovered that the fluorescence response from the cells could be reduced considerably after subjecting the cells to shearing stress by rapid syringing of the suspension through a fine gauge nylon tube. However, once the agitation ceased the progress curves followed exactly the same time course as previously with the same asymptotic value being reached. This phenomenon was elicited a number of times during the same run and confirms that no product inhibition was occurring. The flow system can be set to analyse a predetermined size distribution and any loss of product from the cells into the supporting medium will not be recorded. Thus, in view of the shape of the progress curves, the temporary reduction in fluorescence by shearing stress and the direct observation that the fluorescence was located as a ring at the external membrane, we formulated the hypothesis that the fluorescent product is lost fairly rapidly from the immediate vicinity of the cell and hence from the volume which is 'seen' by the instrument. It was necessary, therefore, to add a further rate constant, k_3, to the model to account for product loss from the site of reaction and analysis. However, two possible back reactions governed by k_{-2} and k_{-3} were omitted as this simplifies the analysis of the results and is justified if both k_{-2} and k_{-3} are very much smaller in magnitude than the respective rate constants for the forward reactions. This simplification is also justified if an excess of substrate is present and its concentration does not alter significantly during the observed time course of the reaction. As we could not directly observe the two back reactions in question two additional extended time course experiments were conducted at the lowest substrate concentration. On both occasions the asymptotic fluorescence value remained constant for over 45 min which suggests that no significant substrate depletion occurred. As the results presented here were obtained over only 4 to 7 min we conclude that the substrate concentration was effectively constant and that our simplified model is valid.

Unlike the results presented in this paper, our previous studies using this technique to assay the hydrolysis of fluorescein diacetate, FDA, in populations of individual cells, demonstrated considerable deviation of the kinetics from Michaelis–Menten type [11, 12], and two major differences between the two fluorogenic substrates should be considered. Firstly, 3-*O*-methyl fluorescein, the reaction product of MFP, is considerably less polar than fluorescein, the reaction product of FDA [17]. Thus, if 3-*O*-methyl fluorescein enters the cell we would expect the rate constant for leakage from the cell to be greater than that for fluorescein. Our previous studies indicate that fluorescein leaks out of EMT6 cells with a 'half-time' of 7 to 8 min [11]. This compares with about 20 sec for 3-*O*-methyl fluorescein reported here which gives a leakage rate constant about 20 times greater than that for fluorescein. Secondly, FDA is relatively lipophilic and, therefore, penetrates the cell membrane rapidly. In contrast, MFP is ionized at physiological pH and would not be expected to penetrate the cell without prior dephosphorylation by membrane phosphatases. Thus, whereas membrane phosphatases would be responsible for the hydrolysis of MFP, intracellular as well as membrane esterases would catalyse the hydrolysis of FDA. This hypothesis is supported by the observations made with the fluorescence microscope. Previous studies [17], which have been confirmed by us, have shown that the fluorescence from FDA hydrolysis is distributed throughout the cell. In contrast, the fluorescence from MFP forms a ring which is localized in the region of the external membrane. We conclude from this direct observation that with intact whole cells MFP is hydrolysed by phosphatases located in the external membrane releasing 3-*O*-methyl fluorescein, some of which remains associated with the membrane. These observations are also very interesting, as they suggest that dephosphorylation at the membrane does not necessarily lead to appreciable uptake in the cytoplasm, and it may be that 3-*O*-methyl fluorescein is actively transported out of the cell. The possible inability of the reaction product to enter the cell, or the rapid active transport out of the cell are both compatible with the magnitude of the rate constant k_3 (see Table 1).

At this point it is pertinent to speculate upon which enzyme(s) may be responsible for the hydrolysis of MFP in our intact cell preparations at neutral extracellular pH. Mammalian cells contain a wide variety of phosphatases with different substrate specificities and locations [1, 13, 24]. Since the enzyme must be present on the cell membrane the intracellular phosphatases, such as acid phosphatase (EC 3.1.3.2),

Table 1. Reaction kinetic parameters calculated by the method given in the appendix for trypsinized and non-trypsinized cells

Cells	$K_m(\mu M)$	P_∞ (channels)	k_3 (min^{-1})	V_{max} (channels min^{-1})
Trypsinized	110.0 ± 23	54.0 ± 2.9	1.98	107.0
Non-trypsinized	122.3 ± 31	54.5 ± 5.1	2.04	111.0

The limits were calculated at the 95 per cent confidence level.

can be ruled out. Those known to be present in the cell membrane include ATPase (EC 3.6.1.3), 5'-nucleotidase (EC 3.1.3.5) and alkaline phosphatase (EC 3.1.3.1) [1, 13, 24]. ATPase and 5'-nucleotidase have pH optima close to neutrality. Both enzymes have rather narrow substrate specificities but 5'-nucleotidase is able to catalyse the hydrolysis of aryl phosphate monoesters such as *p*-nitrophenyl phosphate [23]. This enzyme may therefore catalyse MFP hydrolysis in our intact cell preparations. Alkaline phosphatase (EC 3.1.3.1) catalyses the hydrolysis of a wide range of substrates [25] including MFP [16]. Although purified alkaline phosphatases exhibit alkaline pH optima with most substrates they are also active at neutral pH. Moreover, there is no guarantee that this enzyme exhibits an alkaline pH optimum *in situ*. Thus, alkaline phosphatase may also be involved in the hydrolysis of MFP in our intact cells.

The data given in Table 1 suggest that trypsinization has no effect on the enzyme reaction kinetic parameters as determined by this method. The K_m values obtained in the two preparations were not significantly different ($P > 0.05$). However, although the values for P_∞, k_3 and V_{max} were also almost identical in the two preparations the similarities must be viewed with a degree of caution for two reasons. Firstly, the experiments were carried out on different days and although the laser light output and photomultiplier gain settings were identical on the two occasions we did not have an *absolute* standard for calibration. Thus, we cannot be completely certain that the instrument was behaving identically on the different occasions. Absolute calibration can only be achieved with a physical as opposed to a biological standard and fluorescent microspheres should be used for this purpose. Unfortunately, the latter were not available when these experiments were conducted but our experiences over the past three years suggest that the instrument is very stable. Secondly, although we were performing the assay on predominantly 'G1' cells in both preparations, the trypsinized cells were obtained from a population which was partially arrested in G1, whereas the non-trypsinized cells were harvested from a monolayer during active division. Thus, we cannot exclude possible metabolic differences, including differences in membrane phosphatases, between the cells in the two preparations which may have been modified by the trypsin in the early plateau phase cells. However, the data at least demonstrate that no gross qualitative artefact in the assay system has been produced by the trypsin, though we cannot comment on any possible quantitative changes.

Some of the advantages of using flow systems for enzyme activity determinations have been itemized previously [11]. Two particular advantages may be of importance in the study of enzyme-activated anti-neoplastic agents, or indeed any metabolized drug. The first is the ability to study enzyme activities and kinetics using whole intact cells. This is often difficult using conventional methods due to problems with the mixing and settling of cells and their absorbance properties. Nevertheless, intact cell preparations may be less artefactual than cell-free extracts for the study of drug metabolism *in vitro*. Our previous studies have shown that abnormal reaction kinetics are observed for FDA hydrolysis in whole cells [11, 12]. But in contrast, homogenized preparations exhibit normal kinetics for this reaction [18]. Furthermore, the quantity of substrate converted per mg protein per unit time was about 5 times greater in whole cells than homogenates [18]. Differences in reaction kinetics between whole cells and homogenates may also be found for ionized drugs which require dephosphorylation. These include anti-neoplastic phosphate derivatives which are activated by phosphatases [3–8]. If these agents are activated exclusively at the cell membrane then artefactual results must be obtained if the kinetics to determine potential efficacy are studied in homogenates. In at least one case it was not possible to explain the anti-tumour selectivity of an agent activated by phosphatases on the basis of enzyme activity measurements obtained with homogenates [7]. Plasma membrane preparations may provide a better model than homogenates, but uniform samples of these types of preparations are difficult to prepare.

The second advantage of flow systems in this particular field is the ability to determine the distribution of enzyme activities within the sampled population. The cellular heterogeneity of neoplastic tissues, compounded by contamination of biopsy specimens with normal tissue elements, including inflammatory cells and stroma, makes the interpretation of enzyme activity data obtained with tissue homogenates and organnelle preparations rather difficult. By using flow cytofluorimetric techniques it may be possible to resolve at least some of these problems. Wilder and Cram, for example, have shown that peripheral lymphocytes exhibit a bimodal fluorescence distribution after incubation with FDA [19]. This was attributed to different properties of T and B lymphocytes.

Although this method of estimating membrane phosphatases may overcome some of the disadvantages of more established techniques there is a particular disadvantage that should be considered. Our aim was to study viable cells which must, therefore, be maintained in a buffered solution at optimum physiological pH. In these studies we used PBS as we

knew that this would maintain viability for the duration of the experiments. However, phosphate is a competitive inhibitor of alkaline phosphatase (EC 3.1.3.1) with an affinity comparable to that for good substrates [25]. Although the concentration of phosphate was not high (10 mM) this is sufficient to cause some inhibition. However, if the inhibition is proportionally the same at each substrate concentration this will make no difference to the value of the Michaelis constant as this was calculated by comparing ratios. It will, however, tend to cause an underestimation of V_{max}. At present we have no data on the viability of this cell line after suspension in non-phosphate containing buffers, but work is being undertaken in this area to find such buffers which will maintain viability and their influence on the assay system will then be determined.

In view of the number of latent anti-neoplastic agents which have been synthesized for selective activation in tumour tissues, it is essential that comparative estimates of enzyme activity in normal and neoplastic cells should be as relevant to the *in vivo* situation as possible. We believe that flow cytofluorimetric analysis will find a very useful application in this field.

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APPENDIX

The mathematics developed here were evolved to produce the reaction kinetic parameters by analysis of the 'steady-state' portions of the progress curves.

Consider the system,

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k'_2} P \xrightarrow{k_3} p'$$
$$\qquad\qquad\qquad\qquad\quad +$$
$$\qquad\qquad\qquad\qquad\quad E$$

where S, E and ES are the concentrations of substrate, free enzyme and an enzyme–substrate complex respectively. In this system the product is lost from the compartment under analysis, thus p' represents product which is no longer within either the cell or cell membrane and which is not 'seen' by the instrument. P is the compartment which is estimated by the instrument. This system is a simplification as two possible back reactions, $ES \underset{k_{-2}}{\leftarrow} P$ and $P \underset{k_{-3}}{\leftarrow} p'$, have been omitted. The initial substrate concentration S_0 is equal to the sum $(S + ES + P + p')$. However, if the substrate concentration remains effectively constant during the time course of the experiment, ie $S_0 \simeq S$, the two back reactions can be ignored as P and $p' \ll S$. The evidence for our belief that the substrate concentration remains effectively constant is considered in the discussion, but the rate constants for $ES \rightarrow P$ and $P \rightarrow p'$ have been 'primed' and written as k'_2 and k'_3 in acknowledgment of the simplification.

The rate equations for this system are,

$$\frac{\delta ES}{\delta t} = k_1 E.S - k_{-1}ES - k'_2 ES \tag{1}$$

$$\frac{\delta P}{\delta t} = k'_2 ES - k'_3 P. \tag{2}$$

The concentration of free enzyme, E, at any time is given by,

$$E = E_0 - ES$$

where E_0 is the total enzyme concentration.

Substituting for E in equation 1 gives,

$$\frac{\delta ES}{\delta t} = k_1 S(E_0 - ES) - ES(k_{-1} + k'_2)$$
$$= k_1 SE_0 - ES(k_1 S + k_{-1} + k'_2). \tag{3}$$

When steady-state conditions are reached $\delta ES/\delta t = 0$. Thus, from equation 3 we get,

$$ES = \frac{k_1 SE_0}{k_1 S + k_{-1} + k'_2}$$
$$= \frac{SE_0}{S + ((k_{-1} + k'_2)/k_1)} = \frac{SE_0}{S + K_m} \tag{4}$$

where $K_m = ([k_{-1} + k'_2]/k_1)$.

(a) *Estimation of the Michaelis Constant.* Substituting the solution for ES from equation 4 into equation 2 gives

$$\frac{\delta P}{\delta t} = k'_2 \frac{SE_0}{S + K_m} - k'_3 P \tag{5}$$

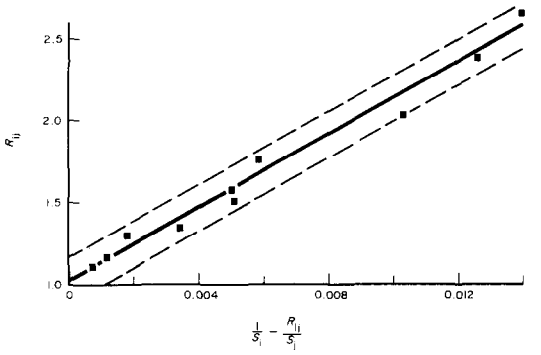

Fig. 2. Derivative plots of R_{IJ} vs $((1/S_I) - (R_{IJ}/S_J))$, see text. The slope of the regression line gives the value of K_m and the 95% confidence limits for the regression of y on x are shown by the interrupted lines. The ordinate intercept does not differ significantly from unity, P > 0.1.

Under steady-state conditions $\delta P/\delta t = 0$ and thus,

$$P = \frac{k'_2}{k'_3}\frac{SE_0}{S + K_m}. \tag{6}$$

Equation 6 gives the asymptotic fluorescence response from the population for a given substrate concentration, enzyme content, K_m and the rate constants k'_2 and k'_3 when loss of product is taking place from the system.

The family of curves depicted in Fig. 1 all reach well defined asymptotic values from which K_m for the reaction can be calculated as follows.

Let P_1 be the asymptotic fluorescence value at the lowest initial substrate concentration S_1. Thus P_2, P_3, etc. are the asymptotic values associated with substrate concentrations S_2, S_3 etc.

From equation 6 it can be seen that by taking the ratio of P_2 to P_1 we obtain an expression from which k'_2, k'_3 and E_0 vanish. Thus,

$$\frac{P_2}{P_1} = \frac{k'_2}{k'_3}\frac{S_2E_0}{S_2 + K_m} \times \frac{k'_3}{k'_2}\frac{S_1 + K_m}{S_1E_0}$$

$$= \frac{S_2}{S_1} \times \frac{S_1 + K_m}{S_2 + K_m} = R_{12}. \tag{7}$$

The ratio (equation 7) is designated R_{12} and by taking a similar ratio between P_3 and P_1 we get R_{13}. In all, there are 10 such ratios which can be obtained from the 5 sets of experimental data shown in Fig. 1.

Equation 7 can be rearranged as follows.

$$R_{12}(S_2 + K_m) = \frac{S_2}{S_1}(S_1 + K_m)$$

$$\therefore R_{12}S_2 + R_{12}K_m = S_2 + \frac{S_2K_m}{S_1}$$

$$\therefore R_{12}S_2 = K_m\left(\frac{S_2}{S_1} - R_{12}\right) + S_2$$

$$\therefore R_{12} = K_m\left(\frac{1}{S_1} - \frac{R_{12}}{S_2}\right) + 1$$

In general notation for 5 sets of data where I varies from 1 to 4, and where J varies from $I + 1$ to 5 we get a triangular matrix for the ratios where,

$$R_{IJ} = K_m\left\{\frac{1}{S_I} - \frac{R_{IJ}}{S_J}\right\} + 1$$

By plotting R_{IJ} against $((1/S_I) - (R_{IJ}/S_J))$ we obtain a line with slope K_m which should intersect the y-axis at 1.0.

A plot of these derivatives is shown in Fig. 2. The asymptotic fluorescence values were obtained by averaging the last 4 data points on each curve. Regression analysis of these data gave,

$$K_m = 110.0 \pm 23.0\ \mu M$$

$$\text{Intercept} = 1.03 \pm 0.14.$$

The limits were calculated at the 95 per cent confidence level and the intercept does not differ significantly from unity, P > 0.1.

(b) *Estimation of the maximum attainable asymptotic fluorescence value.* Let P_∞ be the asymptotic fluorescence value at infinite substrate concentration, S_∞. It is apparent from equation 7 that,

$$\frac{P_\infty}{P_n} = \frac{S_\infty}{S_n}\frac{(S_n + K_m)}{(S_\infty + K_m)}$$

where P_n is the asymptotic fluorescence value associated with substrate concentration S_n. The term $S_\infty/(S_\infty + K_m)$ is unity and thus,

$$P_n(S_n + K_m) = P_\infty S_n$$

Therefore, by plotting $P_n(S_n + K_m)$ against S_n we get a line with slope P_∞. This is shown in Fig. 3 and regression analysis gave $P_\infty = 54.0 \pm 2.9$ channels.

(c) *Estimation of maximum reaction velocity.* Equation 6 relates P_n, S_n, E_0 and K_m. As S_n increases the ratio $(S/(S + K_m))$ tends to unity, thus at S_∞ equation 6 reduces to,

$$P_\infty = \frac{k'_2}{k'_3}E_0$$

$$\therefore E_0 = (P_\infty k'_3)/k'_2 \tag{8}$$

Substituting this solution for E_0 in the rate equation 5 we get,

$$\frac{\delta P}{\delta t} = k'_3\frac{S}{(S + K_m)}P_\infty - k'_3P$$

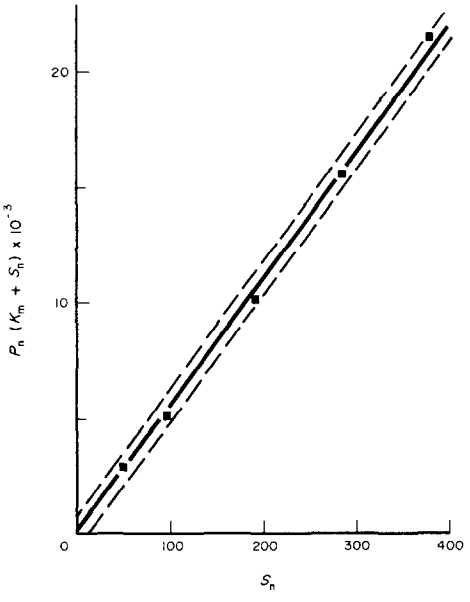

Fig. 3. Regression analysis of $P_n(K_m + S_n)$ on S_n, see text. The slope of the line gives P_∞, defined as the asymptotic fluorescence intensity at infinite substrate concentration. The 95 per cent confidence limits are shown by the interrupted lines, and the ordinate intercept does not differ significantly from zero, P > 0.1.

Integration of this equation gives,

$$P = P_\infty \left\{\frac{S}{(S + K_m)}\right\} \times (1.0 - \exp(-k'_3 t)) \qquad (9)$$

Thus, each of the curves depicted in Fig. 1 should follow a time course described by equation 9. An approximate estimate for k'_3 can be obtained from the time taken for each curve to reach 50 per cent of the asymptotic value. Rough estimates give '$\frac{1}{2}$-height' times varying between 15 and 26 sec with an average of about 21 sec. We appreciate that this is merely an approximation, but in order to obtain better conditioned values a more formal mathematical technique would be needed with a greater number of points in the initial reaction phase. We also appreciate that between about 1.0 and 2.5 min some of the curves tend to 'flatten out' and that there is a fair amount of scatter in the data. However, as this communication is intended as a description of the methodology in which technical improvements can be made (e.g. more data points in the initial phase) we feel content with this approximation for illustrative purposes.

Using this value of 21 sec for the "$\frac{1}{2}$-height" time we get,

$$k'_3 = (\ln 2 \times 60)/21 = 1.98 \text{ min}^{-1}$$

Referring back to equation 8 and using the value for P_∞ found in the previous section, we get,

$$\begin{aligned} E_0 k'_2 &= k'_3 P_\infty \\ &= 1.98 \times 54.0 \text{ channels min}^{-1} \\ &= 107 \text{ channels min}^{-1}. \end{aligned}$$

It will be recognised that $E_0 k'_2$ is the maximum reaction velocity; thus V_{max} is defined.