- Pratt, R. F., McConnell, T. S. & Murphy, S. J. (1988) *Biochem. J.* **254**, 916–922
- Wharton, C. W. & Eisenthal, R. (1981) *Molecular Enzymology*, pp. 204–214, Blackie & Son, Glasgow

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Kinetic parameters of the acyl-enzyme mechanism

The preceding letter (Brocklehurst & Topham, 1990) comments on three points made by Christensen *et al.* (1990): two concern kinetic parameters and quasi-equilibrium in the acyl-enzyme mechanism and the third concerns the expected relationships between the rate constants for a fully efficient enzyme.

Their comments on this last point are discussed first. Our conclusions did indeed differ from those of Brocklehurst (1977); this was because we were interpreting our results according to Burbaum *et al.* (1989). For the acyl-enzyme mechanism [eqn. (1) of the preceding letter] Brocklehurst (1977) states that, unless substrate binding and product binding are equally strong, $k_{+2} \gg k_{-1}$; k_{+2}/k_{+3} is not specified. On the other hand, Burbaum *et al.* (1989) infer that $k_{+2} \cong k_{+3}$, and that at (or near to) the diffusion limit the barrier for the second transition state is likely to be as low as it needs to be to avoid slowing the reaction, and so $k_{-1} \cong k_{+2}$. Brocklehurst (1977) did suggest that the condition $k_{+2} \gg k_{-1}$ might be relaxed to $k_{+2} \cong k_{-1}$ with little loss in catalytic efficiency, and so this leaves the main difference between Brocklehurst (1977) and Burbaum *et al.* (1989) as the additional requirement that $k_{+2} \cong k_{+3}$ in the latter treatment. Our experimental results for β -lactamases (Christensen *et al.*, 1990) were that $k_{-1} \cong k_{+2} \cong k_{+3}$, and that the reactions were partly (or largely) diffusion-controlled; thus our results were entirely consistent with the theories of Burbaum *et al.* (1989).

We come now to the significance of the parameters obtained by steady-state and single-turnover kinetics. Single-turnover kinetics, applicable when the concentration of enzyme is much greater than that of the substrate, give an equation for P_1 in the acyl-enzyme mechanism [eqn. (1) of the preceding letter] that has two exponential terms (see, e.g., Kasserra & Laidler, 1970). The useful (and usual) approximate treatment of single-turnover kinetics for the acyl-enzyme mechanism (which neglects the first transient) does indeed give $(k_{-1} + k_{+2})/k_{+1}$, the parameter referred to as $K_{m(\text{acyl})}$ by Brocklehurst & Topham (1990), K' by Christensen *et al.* (1990) and K^* by Kasserra & Laidler. The approximation can be tested when all the rate constants are known. For example, if k_{+1} , k_{-1} and k_{+2} are $22 \mu\text{M}^{-1} \cdot \text{s}^{-1}$, 196 s^{-1} and 173 s^{-1} respectively [the values from Christensen *et al.* (1990) for β -lactamase PC1], and the concentration of enzyme is $15 \mu\text{M}$, then the approximate treatment would give a value for k_{+2} (given K') that is about 10% low; the first transient would be not normally be observed in practice. The concentration of substrate is assumed to be negligible compared with that of the enzyme in such treatments; in practice this is often inconvenient, and may be circumvented by the extrapolation procedure of Crompton & Waley (1989) [in that paper, the equation for $y+p$ should read $s[1 - \exp(-\lambda_2 \cdot t)]$ not $s[1 - \exp(-\lambda_2 \cdot t)]$ and the equation for λ_2 should read $k'_{+1} \cdot k_2 / (k_{-1} + k'_{+1} + k_{+2})$ not $k'_{+1} \cdot k_{+2} / (k_{-1} + k'_{+1})$].

It is clear from eqn. (2) of the preceding letter that $K_m/k_{\text{cat}} = (k_{-1} + k_{+2})/(k_{+1}k_{+2}) = K'/k_{+2}$; both single-turnover and steady-state treatments do indeed give K' as correctly pointed out by Brocklehurst & Topham (1990), and the method of determining the association rate (k_{+1}) in the Appendix of Christensen *et al.* (1990) is mistaken. However, to come to

practicalities, a useful way to determine k_{+1} is from the dependence of K_m/k_{cat} on viscosity (Hardy & Kirsch, 1984; Blacklow *et al.*, 1988; Christensen *et al.*, 1990); moreover this procedure also gives k_{-1}/k_{+2} and so shows directly whether the quasi-equilibrium assumption is valid. Thus the comparison of k_{cat}/K_m and k_{+1} which Brocklehurst & Topham (1990) commend as a test for quasi-equilibrium is unneeded if the viscosity method is used to find k_{+1} .

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- Blacklow, S. C., Raines, R. T., Lim, W. A., Zamore, P. D. & Knowles, J. R. (1988) *Biochemistry* **27**, 1158–1167
- Brocklehurst, K. (1977) *Biochem. J.* **163**, 111–116
- Brocklehurst, K. & Topham, C. M. (1990) *Biochem. J.* **270**, 561–562
- Burbaum, J. J., Raines, R. T., Albery, W. J. & Knowles, J. R. (1989) *Biochemistry* **28**, 9293–9305
- Christensen, H., Martin, M. T. & Waley, S. G. (1990) *Biochem. J.* **266**, 853–861; see also erratum in *Biochem. J.* (1990) **268**, 808
- Crompton, I. E. & Waley, S. G. (1989) *Biochem. J.* **257**, 305–308
- Hardy, L. W. & Kirsch, J. F. (1984) *Biochemistry* **23**, 1275–1282
- Kasserra, H. P. & Laidler, K. J. (1970) *Can. J. Chem.* **48**, 1793–1802

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Questions about the use of [³H]thymidine incorporation as a reliable method to estimate cell proliferation rate

Control of cell proliferation is raising interest in an increasing number of research centres, appearing as it does to be one of the most promising fields of research. It involves studies on rapidly expanding subjects such as growth factors, hormones, transmembrane signalling, kinases, second messengers, ornithine decarboxylase, oncogenes, etc. It constitutes a basic approach to important questions such as embryogenesis, wound healing, cancer, etc. From a survey of the related recent literature, it appears that a large part, if not most, of the research teams use incorporation of radiolabelled thymidine (mostly ³H-labelled) into DNA as an index of cell proliferation. In many cases, number of c.p.m. after exposure to [³H]thymidine is reported as the sole index of cell growth.

Theoretically, the method is very attractive because it relies on a very simple principle: labelled thymidine added to the culture medium will be incorporated into DNA during the S phase of the cell cycle. As the rate of DNA synthesis expresses the mitotic activity, the radioactivity of trichloroacetic acid-precipitable material should reflect cell growth. Such a method is both easier to carry out and less time-consuming than direct cell counting under the microscope, an alternative to estimate cell growth. Besides, its wide use gives the feeling that there are few questions about its reliability.

In practice, authors use either the pulse labelling method or the continuous labelling method. Surprisingly, detailed protocols described in published papers rarely refer to first or previous authors having described one method or the other. It underscores the fact that there are multiple adaptations and variations of the protocols. It seems that the pulse method was described as early as 1953 [1]. Detailed descriptions of both methods and pertinent criticism of them can be found in a book published in 1980 [2].

Starting a study on the ability of some external signals to control proliferation of HT29 human colon cancer cells in

culture [3], we have tried for several months to use the [^3H]thymidine method, following classical pulse or continuous labelling protocols. Briefly, cells, routinely cultured in Dulbecco's medium supplemented with 10% fetal calf serum (FCS) [4], were tested during either exponential or stationary phases of growth in 24-well boxes or 35 mm Petri dishes. Standardization of the experimental protocol was achieved by previous FCS deprivation for 24 h in order to have most of the cells in G_0/G_1 . High percentage of synchronization (90%) was confirmed by flow cytometry analysis and growth arrest was demonstrated by measurement of total proteins and estimation of cell number. In test experiments, 10% FCS and/or 1700 μM -insulin were used to reinitiate cell growth and were found to be effective as shown by measurement of total proteins and estimation of cell number at 24, 48 and 72 h. Under the same experimental conditions, [^3H]thymidine (0.5 μCi /well) was added into 1 ml of medium and incorporation was allowed to proceed for 24 h (continuous labelling) or for 5 h after a 18 h delay (pulse labelling). Cell layers were then washed twice with ice-cold saline (PBS), twice with ice-cold 5% trichloroacetic acid and twice with absolute ethanol, then solubilized in 0.1 M-NaOH. Aliquots were counted into Picofluor scintillation liquid and radioactivity was expressed in d.p.m. after quenching and luminescence correction (Packard Tricarb 1600 beta counter).

Using the continuous labelling protocol, we found 54507 ± 5193 d.p.m. in FCS-deprived controls versus 9665 ± 2361 d.p.m. in FCS-treated cells. Using pulse labelling, values were 106141 ± 3016 d.p.m. versus 14426 ± 1180 d.p.m. respectively (mean values $\pm$ S.D., $n = 6$). By estimation of cell number, it was verified that FCS deprivation resulted in arrest of cell growth whereas number of cells was doubling each 24 h in the presence of FCS. In another set of experiments carried out on post-confluent cells, using the pulse labelling protocol, we found 3876 ± 514 d.p.m. in FCS-deprived cells versus 4169 ± 499 d.p.m. in FCS-treated cells and 1233 ± 153 d.p.m. in cells given 1700 nm-insulin (a dose which was found to stimulate growth).

It is clear that these results based upon d.p.m. values may lead to paradoxical interpretations such as that FCS and/or insulin inhibit cell growth. Discrepancies also appear when comparing results of 'continuous labelling' and 'pulse labelling'.

Our first inclination was to give up and keep an embarrassed silence about our results. However, discussing with many colleagues who use [^3H]thymidine 'incorporation' as an index of cell proliferation, we learnt that confusing results were not uncommon. From such informal discussions, it appeared to be widely admitted that the [^3H]thymidine method does not always work. We have verified that using the ^{14}C -labelled instead of the ^3H -labelled molecule does not solve the problem either. We also heard the story of hepatocytes in primary culture which incorporate [^3H]thymidine but do not divide [5,6].

Some reasons for such difficulties could be found in the analysis of the method as done by Adams [2] and by McGowan

[6]. Pulse labelling, when [^3H]thymidine is added during a short period of time, could 'miss' the S phase of DNA synthesis occurring earlier or later. In this respect the 'continuous labelling' protocol should overcome the difficulty. However, in such a case, there is more time for the cell to degrade labelled thymidine and to spread the label in its general metabolism. It is also possible that, in many cell models, growth-promoting factors strongly activate the synthesis *de novo* of dTTP which will dilute the exogenous precursor and reduce the labelling of synthesized DNA. Besides, we could suggest that specific characters of differentiation present in some cells (e.g., brush border, activated catabolic pathway) could render the method unreliable by strongly interfering at the level of thymidine transport through the cell membrane or thymidine breakdown in degradative metabolism.

Working with the HT29 cells, we also found that a pH change of 0.5 unit could modify by 2-fold or more the rate of incorporation of radioactivity. Such a change in pH is not uncommon when standard culture medium is used (i.e., without further addition of buffering agent) since many cell models also react to FCS or 'growth factors' by increasing glycolytic rate and lactic acid production, or just because the culture dishes have been taken out of the CO_2 incubator for some minutes.

Actually, we found that the [^3H]thymidine method (pulse-labelling protocol) applied to the HT29 cells could give satisfactory results only when used under strictly defined experimental conditions, i.e., cells must be in the early part of the exponential phase of growth and culture medium must be strongly buffered by adding 40 mM-Hepes.

The aim of this letter is (1) to request an answer to this puzzling problem or to open a debate if a sufficient number of scientists feel concerned, and (2) to underscore that the [^3H]thymidine method, in spite of its wide use, does not necessarily reflect the cell proliferation rate in all biological models. Therefore, we suggest that any result should be confirmed by at least another control of cell growth such as counting the number of cells or measuring the protein content.

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- Howard, A. & Pelc, S. R. (1953) *Heredity* (Suppl.), **6**, 261–266
- Adams, R. L. P. (1980) *Cell Culture for Biochemists*, Elsevier/North-Holland Biomedical Press, Amsterdam
- Fogh, J., Fogh, J. M. & Orfeo, T. (1977) *J. Natl. Cancer Inst.* **59**, 221–226
- Denis, C., Paris, H. & Murat, J. C. (1986) *Biochem. J.* **239**, 531–536
- Friedman, D., Claus, T., Pilgis, S. & Pine, G. (1981) *Exp. Cell Res.* **135**, 283–290
- McGowan, J. A. (1966) in: *Hépatocytes Isolés et En Culture* (Guillouzo, A. & Guen-Guillouzo, C., eds.), pp. 13–40, INSERM/John Libbey Eurotext

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