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Full Length Article

Kinetics of the base catalysed hydrolysis of methyl paraben revisited: Implications for determination of the effective volume of flow-microcalorimeters used to study cell cultures

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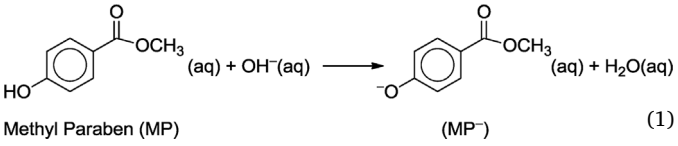
ABSTRACT

The kinetics of hydrolysis of methyl paraben (methyl 4-hydroxybenzoate, MP) by NaOH has been proposed as a reference system to calibrate the effective volume (V_{eff}) of flow-calorimetry vessels that are often used in studies of living cells. This parameter is essential to relate the observed calorimetric signal with a specific cell number or biomass and its determination relies on a rate constant, k, obtained under the assumption of pseudo-first-order kinetics. The significant discrepancies noted between published k values led us to test the reliability of this approximation for different NaOH/MP concentration ratios and at temperatures in a range typically used for living cell studies (292–310 K). Kinetic experiments carried out by spectrophotometry, isothermal microcalorimetry, and flow microcalorimetry indicated that the 9/1 NaOH/MP molar ratio typically used for V_{eff} determinations does not ensure the pseudo-first-order kinetic conditions assumed in the V_{eff} calculation model. It is recommended that a NaOH excess of at least 20/1 be used to warrant a $< 5\%$ error in k, which translates into an identical error in V_{eff}.

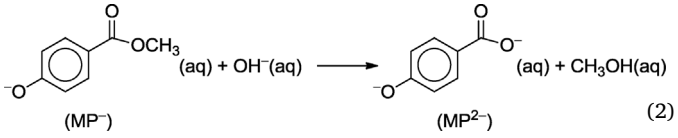
1. Introduction

Flow microcalorimetry has long been established as a very reliable technique to obtain information on cell metabolism [1,2]. One parameter that needs to be determined *a priori* in order to relate the observed calorimetric signal with a specific cell number or biomass is the so-called thermal or effective volume (V_{eff}) of the calorimetric cell. The effective volume differs from the actual physical volume of the calorimetric cell because part of the heat produced by the cell culture is carried away from the detection zone by the flowing process. Furthermore, since the amount of undetected heat depends on the flow rate, V_{eff} is also flow rate dependent [3,4].

The determination of V_{eff} for a given microcalorimetric system and flow rate normally involves the kinetic study of a chemical reaction that has been well-characterized in terms of rate constant and enthalpy change, based on the variation of the calorimetric signal over time. The most widely used reference reaction is the base catalysed hydrolysis of methyl paraben (methyl 4-hydroxybenzoate, MP) [3–6]. The recommended procedure [3] consists in adding solid MP to a 0.5 mol dm^{-3} NaOH aqueous solution outside the calorimetric cell and monitoring the calorimetric signal over a suitable period of time. The reaction comprises two sequential steps:



Methyl Paraben (MP) (MP⁻) (1)



(MP⁻) (MP²⁻) (2)

The first one corresponds to the deprotonation of the −OH group in MP, which immediately occurs upon solid dissolution because the pK_a for −OH ionization (pK_a = 8.42–8.87 in the 278–313 K temperature range [7,8]) is considerably smaller than the pH of the initial 0.5 mol dm^{-3} NaOH solution (pH = 12.7). The second one is the hydrolysis of the deprotonated species (MP^-) produced in the first step. The calorimetric system is normally blind to reaction (1), since acid-

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base reactions are very fast, and it takes the reaction mixture a few minutes to reach the calorimetric cell, for experimental set-ups and flow rates typically used in studies of cell cultures. The determination of V_{eff} relies, therefore, on the kinetic study of reaction (2). The amount of NaOH consumed in reaction (1) must nevertheless be taken into account to ensure that reaction (2) effectively occurs under pseudo-first order conditions.

For systems such as the LKB 10700-1 apparatus used in this work, the power, P, generated within the calorimetric cell as the MP^- hydrolysis proceeds can be related to the rate of MP^- consumption $(-dC/dt)$ through the equation:

$$P = \Delta_r H_m^o V_{eff}\left(-\frac{dC}{dt}\right) \quad (3)$$

where C is the concentration of MP^- and $\Delta_r H_m^o$ is the enthalpy of reaction (2). Because for a pseudo-first-order reaction $(-dC/dt) = kC$ and $C = C_o\, e^{-kt}$ Eq. (3) can be written as:

$$P = \Delta_r H_m^o V_{eff} k\, C_o e^{-kt} \quad (4)$$

from which it can be concluded that:

$$\ln P = a - kt \quad (5)$$

with $a = \ln(kC_o\Delta_r H_m^o V_{eff})$. A least squares fit of Eq. (5) to a plot of ln$P$ vs. time, should, therefore, lead to a linear relationship whose slope affords k and the ordinate gives V_{eff} as:

$$V_{eff} = \frac{\exp(a)}{kC_o\Delta_r H_m^o} \quad (6)$$

The determination of V_{eff} through Eq. (6) requires knowledge of C_o, $\Delta_r H_m^o$ and k, where the C_o value is fixed by the selected MP^- initial concentration.

In the case of $\Delta_r H_m^o$, a critical literature analysis previously showed that inconsistencies between the published values at 298.15 K could lead to discrepancies of up to ~20% in V_{eff} [6]. This led us to redetermine $\Delta_r H_m^o$ at 298.15 K [6]. Albeit the obtained result agreed with a previously proposed benchmark [3] a ~7 fold improvement in precision was achieved [6]. The $\Delta_r H_m^o$ determinations were also expanded up to 310 K so that the temperature range used in cell metabolism studies could be covered [6]. The fact that a good linear variation of $\Delta_r H_m^o$ with temperature was found enables reference values to be calculated at any temperature within the 298 K to 310 K interval.

As mentioned above, k is always accessible from the slope of the ln$P$ vs. t plot. Benchmark values at different temperatures in the range typically covered by biological assays (e.g. 288–313 K) are, nevertheless, needed if proper assessment of the calorimeter performance is to be achieved. A value $k = (3.15 \pm 0.11) \times 10^{-4}\,s^{-1}$ has been recommended for 298 K [3]. A literature survey (see Supporting Information) showed, however, that while the differences between most reported k values at 298 K [3,5,9–11] and that mentioned above are in the range of 1–4%, a discrepancy of up to ~60% ($k = 7.27 \times 10^{-4}\,s^{-1}$ [3], $k = 4.60 \times 10^{-4}\,s^{-1}$ [3], and $k = 4.79 \times 10^{-4}\,s^{-1}$ [11]) is, for example, found at 303 K, which is the temperature typically used in yeast studies [1,2]. These inconsistencies are not associated with the analytical methodology, thus are likely to be related with temperature calibration errors or flouting pseudo-first-order conditions. Particularly critical in the present context are the implications of those disparities for a reliable V_{eff} determination. For example, using $k = 4.60 \times 10^{-4}\,s^{-1}$ or $k = 7.27 \times 10^{-4}\,s^{-1}$ at 303 K, leads to a ~40% change in V_{eff}. The importance of V_{eff} for quantitative thermodynamic analysis of cell cultures based on flow calorimetry measurements carried out at temperatures different from 298 K, led us to revisit the kinetics of the base catalysed hydrolysis of methyl paraben in the range 293–313 K, using batch and flow calorimetric techniques as well as spectrophotometry.

2. Materials and methods

2.1. Materials

The methyl paraben sample used in the calorimetric and spectrophotometric experiments was the same previously used in the $\Delta_r H_m^o$ determinations [6]. Originally from Aldrich (Mass fraction > 0.99; CAS number 99-76-3) it was purified by sublimation at 338 ± 1 K. The final purity given by GC–MS corresponded to a mass fraction > 0.999. X-ray powder diffraction and differential scanning calorimetry analysis indicated that the sample corresponded to methyl paraben polymorph I (monoclinic, space group *Cc*, Refcode CEBGOF) [12,13]

The 0.500 ± 0.002 mol dm^{-3} sodium hydroxide solutions used in all calorimetric experiments were prepared by weight, diluting a 0.998 ± 0.001 mol kg^{-1} certified concentrate (Fisher Chemical), with deionized water (Milli-Q® Ultrapure Water Type 1) just before use. Those used in the spectrophotometric runs were prepared, every other day, by dissolving solid NaOH pellets (Panreac Mass fraction > 0.98; CAS number 1310-73-2) in Milli-Q® Ultrapure Water Type 1. The final concentration was adjusted to 0.050 ± 0.0005 mol dm^{-3} through the appropriate dilution factor calculated from standardization of 1:10 dilutions against potassium hydrogen phthalate (Merck Mass fraction > 0.995; CAS number 877-24-7).

2.2. Spectrophotometry

Spectrophotometric measurements were carried out on a Shimadzu UV-1800 apparatus equipped with a TCC-240A cell holder connected to a TCC temperature controller. Quartz cells with 1 cm optical path, closed by Teflon lids, were used for the reference and sample solutions. The temperature of the cell holder was set to a desired value and measured, before and after the kinetic run, with a precision thermistor (YSI 44001A, nominal resistance 100 Ω at 298 K) placed in the reference cell. The thermistor was connected in a four-wire configuration to a HP34972A multimeter. The whole temperature measurement set-up was calibrated against a reference platinum resistance thermometer which had been calibrated at an accredited facility in accordance to the International Temperature Scale ITS-90. The cell holder temperature was found to be stable to ± 0.1 K over more than 4 h.

The reference and sample cell were filled with 2.8 cm^3 of 0.050 ± 0.0005 mol dm^{-3} aqueous NaOH and left to equilibrate inside the spectrophotometer, at a previously selected temperature, during 15 min. Aqueous solutions of methyl paraben (0.002 ± 0.001) mol dm^{-3} were prepared by dissolving the solid material in 50 cm^3 of distilled and deionized water from a Milli-Q® Ultrapure Water system. The kinetic runs were initiated by adding 0.150 μL of MP solution to the NaOH solution inside the spectrophotometric cell and the absorbance of the reaction mixture was followed at 272 and 293 nm, until the observed change over 30 s was less than 0.001. The selected wavelengths correspond to the formation of the final MP^{2-} hydrolysis product and the reactant MP^- respectively, and were identified in preliminary scans of the reaction mixture over time (Fig. 1). Pseudo-first-order conditions were ensured with over a 4000 fold NaOH concentration. Absorbance values spanned the 0.3–1.4 range, most being between 0.3 and 0.8 in order to minimize absorbance measurement uncertainties.

2.3. Isothermal microcalorimetry

Isothermal microcalorimetry experiments were carried out at 298.02 K, 303.04 K, and 310.01 K using a LKB 2277 Thermal Activity Monitor (TAM). A stirred stainless steel cell of 15.0 cm^3 volume, including in-house designed systems for electrical calibration and solid sample dissolution, was used. The time constant of the apparatus was $\tau = 212 \pm 6$ s and no temperature dependence was noted. The general procedure was essentially that previously described [6]. In brief, ~30 mg of MP contained in a silica crucible were dropped into ~12 g