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From guinea pigs to cutting fluids — a microcalorimetric journey

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Abstract

This paper is a partial and selective review of the development of biological microcalorimetry with particular emphasis on kinetic aspects. The review describes, in outline, the concerns expressed for the determination of kinetic parameters in previous publications (mostly drawn from the author's own interests) and concludes with a new kinetic analysis of the output from flow microcalorimeters.

Keywords: Microcalorimetry; Micro-organisms; Kinetic parameters; Thermodynamics

1. Introduction

In the late 18th century Lavoisier and Laplace [1] developed calorimetry in the most imaginative fashion. They placed a guinea pig in an ice calorimeter and monitored the energy of metabolism through weighing the ice melted over the observation period and knowledge of the latent enthalpy of fusion of water. Of course they had to choose time intervals over which to measure this metabolic energy and thus, by definition, they were involved in the study of the kinetics of metabolism. Other investigators followed but normally [1] the study of thermochemistry was restricted to relatively rapid reactions since thermostatic controls and thermal stabilisation techniques were not well developed. However, some many years later, following rather serious calorimetric development, in particular the introduction [1] of the Calvet calorimeter, Prat described the study of rate of energy evolution in germinating seeds (Fig. 1), of plant parts and of small mammals [2]. These studies were semi-quantitative in the sense that use was made, in the interpretation of the data, of the rates of change in power but no quantitative data is calculated and cited in the publications. Forrest, in the same publication [3] showed data describing the rate of microbial fermentation as monitored microcalorimetrically. Indeed most publications of that period (the 1950s and 1960s during which there was an enormous increase in the number of publications that reported studies on biological subjects) drew attention to the fact that exponential growth of a microbe was accompanied by exponential power. There was, then, a keen perception that kinetic analysis was, in principle, possible. It was, therefore, a period characterised by studies of ingenuity and imagination but with, in general, only qualitative outputs.

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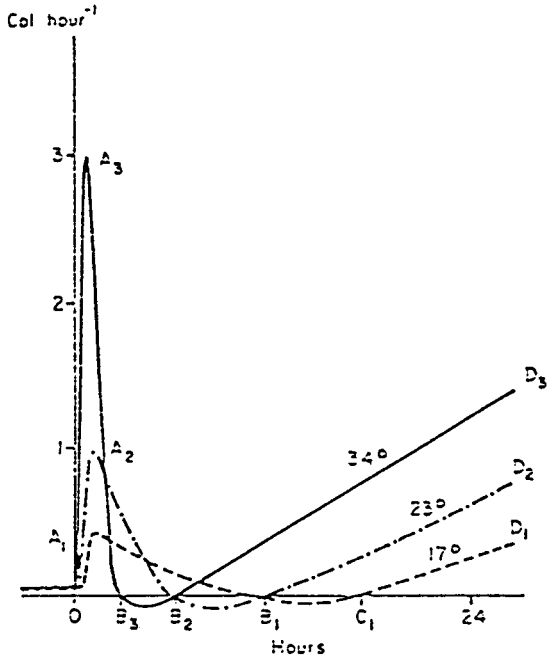

Fig. 1. Power–time curves for the germination of wheat at different temperatures [2].

By the mid-60s there appeared further calorimetric developments with the introduction of the LKB calorimetric system and further manufacturers came along soon after. All these instruments adopted the isothermal heat conduction principle — and such calorimeters were capable of stable, high sensitivity performance for extended (relative to the earlier instruments) periods of time. In 1972 Beezer and Tyrrell published [4] an analysis of the kinetic equations that described the output of a flow microcalorimeter operating in a zero-order or first-order kinetic regime. Eq. (1) shows the power expected for a first-order reaction

$$\frac{\mathrm{d}q}{\mathrm{d}t} = -R_2 C_2^0 \Delta_R H[1 - \exp(-k_1\tau)]\exp(-k_1 t) \quad (1)$$

where R is the flow rate, C^0 the initial concentration of material present as component 2 in a flow-mix experiment, $\Delta_R H$ is the enthalpy of reaction, k_1 the first-order rate constant, τ the residence time in the calorimetric chamber and t the time elapsed since initiation of the reaction external to the calorimeter. These equations were used to analyse data from a study of the urea–urease reaction [5]. Outputs were values for the enthalpy of the reaction, rate constants and of the Michaelis constant for urease. The treatment given [4] related only to integral orders of reaction as did the analysis presented by Becker [6] in the same publication. This latter treatment is more limited in that it attempted a kinetic analysis for batch calorimetric outputs for only simple solution phase-type reactions of first and second order.

In the field of biological microcalorimetry it is often the case that proteins, enzymes etc. are expensive and/or only available in small quantities. Following a design suggested by Biltonen [7], Poore and Beezer designed and tested a simple, rapid method for the generation of ligand binding isotherms [8].The operation of the device was examined and calibrated via study of the protonation of Tris by HCl — a reaction recommended (and questioned) for this purpose [9]. This device has, as yet, to find significant application but perhaps as the development of kinetic calorimetric equations grows then perhaps it, or its successor, will be found useful. It certainly allows for data to be accumulated over several orders of magnitude in concentration — and this may prove useful in binding studies, dilution studies etc.

There was, then, increasing concern for the development of kinetic forms of calorimetric equations but this concern for quantitative data did not keep pace with the rapid increase in publications relating to microbial metabolism. In 1977 the following [10] appeared in a volume reporting the proceedings of a conference on Applications of Calorimetry in the Life Sciences [11].

> Inevitably a new methodology appears to find its initial application in the more qualitative and applied aspects of research. The potential of the microcalorimetric approach to the study of microbiological subjects in quantitative aspects is yet largely untouched. The possibility of deriving both thermodynamic and kinetic parameters from one experiment on such complex subjects is appealing. The principal shortcoming at the present moment appears to be lack of precise analytical data to allow definition of the processes studied. Until precise thermodynamic equations can be written then the goals will prove elusive. Furthermore the interpretations of the kinetic detail will require a more complete understand-

ing of adsorption, transport and reaction (as a minimum) processes.

I would not revise the spirit of this quotation but I would now suggest that it really is the absence of satisfactory analytical equations that is the block to progress rather than the absence of clear chemical reaction sequences. Of course attempts have been and currently are made to analyse the outputs from microcalorimetric study of microbial systems in both batch and flow based experiments [12] however, the model used is concerned with deducing biochemical model based outcomes, e.g. the determination of Michaelis constants and Haldane-type inhibition kinetics. The equations presented are not general and do not, strictly, allow for development into other areas of study. They are, however, important in that they recognise that microbial numbers can be used as 'reagent' concentrations.

An important early conclusion derived from experiments on microbial systems was that the system could be forced to be zero order and hence that the previously developed equations [4] could be applied. Thus, inoculating an organism into a high concentration of buffered glucose solution resulted in a zero-order kinetic output — the power depending only on the number of organisms present in the inoculum [13]. Addition of drug to this flowing system allowed quantitative determination of drug concentration. Such bioactivity determination has the virtues of simplicity and rapidity — each experiment requiring only around 30 min. The bioassay procedure relied (and still does) on a phenomenological analysis of the output data. A simple determination of the response is taken as the power value in a drug treated system at a fixed time after drug addition. Typical outputs (Fig. 2) show the principle and from such data conventional log[dose]/response curves can be constructed (Fig. 3) from which a purely formal assignment of $\log[\text{dose}]_{\max}$ can be made ($\log[\text{dose}]_{\max}$ is the notional maximum concentration of drug that may be added without eliciting a microcalorimetric response). These analyses, therefore, have as their basis the kinetic response of the organism to the applied drug but there is no theoretical basis for the choice of the measured parameter — only convenience and simplicity. The calorimetric output is, however, faithfully recording the kinetics of the drug/microbe interaction. The common first step in the analysis of reaction mechanism (here to be construed as drug–cell interaction) is the creation of a model based upon kinetic analysis. Such detail is absent in the literature — and its absence has been noted for some 25 years [10].

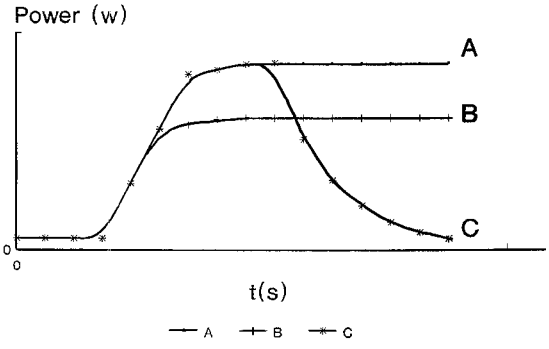

Fig. 2. Power–time curves for A: control (buffered glucose inoculated with a microbial suspension); B and C drug treated samples. Curves B and C reveal different modes of action of drugs on interaction with the organism.

A formal account of quantitative structure activity relationships (QSAR) is outside the scope of this review but general introductions to the subject are widely available (see, for example, [14]). One form of an equation which describes such an SAR is:

$$\log\left(\frac{1}{C}\right) = k_1\log(P^2) + k_2\log(P) + k_3\sigma + k_4 \quad (2)$$

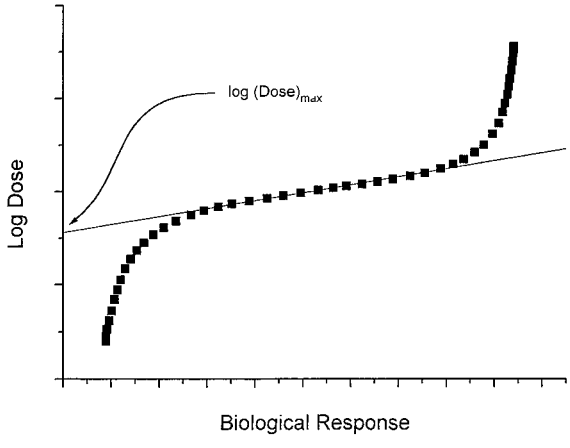

Fig. 3. Log[dose] vs. biological response plot derived from microcalorimetric data. $\text{Log[dose]}_{\max}$ is the notional maximum concentration that can be added to the microbial suspension without eliciting a response.

where C is the concentration required to elicit a specific effect in a specific time and hence $\log(1/C)$ is the Biological Response, k's are correlation constants, P is the partition coefficient and σ is the Hammett sigma function. Note that this definition of Biological Response incorporates a time base and hence is formulated kinetically — the other terms on the right hand side of Eq. (2) are equilibrium (time independent) terms. Despite the absence of a strict kinetic analysis microcalorimetric data has been shown to be a direct and unambiguous measure of Biological Response and to be useful (because of its simplicity, rapidity and high sensitivity and reproducibility) in QSARs [15].

All the regularly used QSARs are based on Gibbs Functions [14]. However, as is obvious the Gibbs Function is somewhat concealing and it is perhaps useful to enquire into the values of the enthalpies and entropies that contribute to the Gibbs Functions. This we did some years ago using a titration procedure [16,17] to add resorcinol monoethers to suspensions of *Escherichia coli*. The analysis of the results (Fig. 4) relied upon an assumption that the kinetics of the dilution process of the monoether into the suspending medium was fast but that the transfer process was slow. This assumption was untested, however, the calculated outcomes were regular and systematic through the homologous series. Comparison of these data with those for transfer of these some compounds from water into octan-1-ol, heptane and propylene

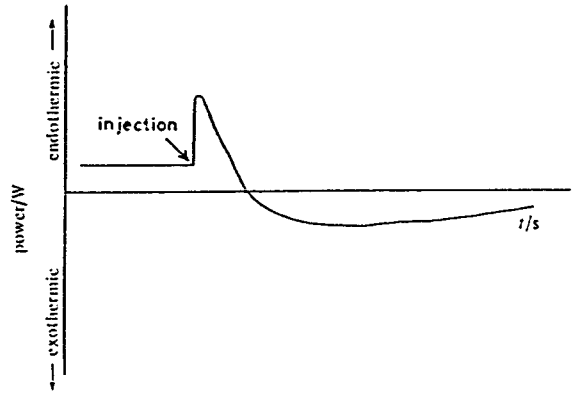

Fig. 4. Representative power–time curve for the addition of pure liquid solute to isotonic aqueous suspension of *E. coli*. It was assumed that the dilution was rapid and that the slower process was the transfer of solute from water to the organism [16,17].

Table 1
Values of $\Delta_{trans}H$ (kJ { mol^{-1}}) for solute transfer from water to the described solvents[a]

Solute	Cells	Octan1-ol[a]	Heptane[b]	Propylene carbonate[b]
m-methoxy	−0.22	−8.03	20.9	23.2
m-ethoxy	−1.1	−6.95	19.3	23.4
m-propoxy	−2.02	−6.96	16.0	23.9
m-butoxy	−4.06	–	13.9	23.4
m-pentoxy	−5.14	–	12.0	23.2

[a] Error limits are given in the original publications [16,17].

carbonate showed that none of these solvents adequately mimicked the biological cell (Table 1). This is a disturbing outcome — and perhaps indicates that basing QSARs on Gibbs Function related terms (such as P, pK_a) could be misleading and that entropy may be a better basis for such relationships. The data could not be better analysed for its kinetic dependence because of its form although there was concern for analysis of such titration data in separating fast and slow reactions. Fast and slow in this context relate to the time constant of the instruments used. In 1972 (a good year for microcalorimetrically based kinetic analysis) Hunt, Ross and Ginsburg described [18] a method for dissection of (effectively) titration results into two first-order processes. Poore subsequently [19] used this procedure to suggest that in the interaction of an antigen with an antibody there is a rapid binding process between the two proteins followed by a slower unfolding process (Fig. 5). It is notable that there is no indication in contemporary microcalorimetric titration studies that interaction is anything other than rapid. Publications in this area seem not to be concerned to demonstrate that the dynamically corrected data does indeed involve only one process.

It is also of note that the recent publications [20,21] in the area of plant calorimetry are all concerned with the rates of processes in the studied systems. Indeed the ingenuity and imagination of the early calorimetrists has surfaced again in these experiments designed to probe the metabolism of plant materials. What is heartening about these reports is the clear assertion that a successful outcome is dependent upon the development of detailed kinetic analysis in addition to the thermochemical result naturally available.

This same statement could not have been made about the study of the stabilities of raw drug materials

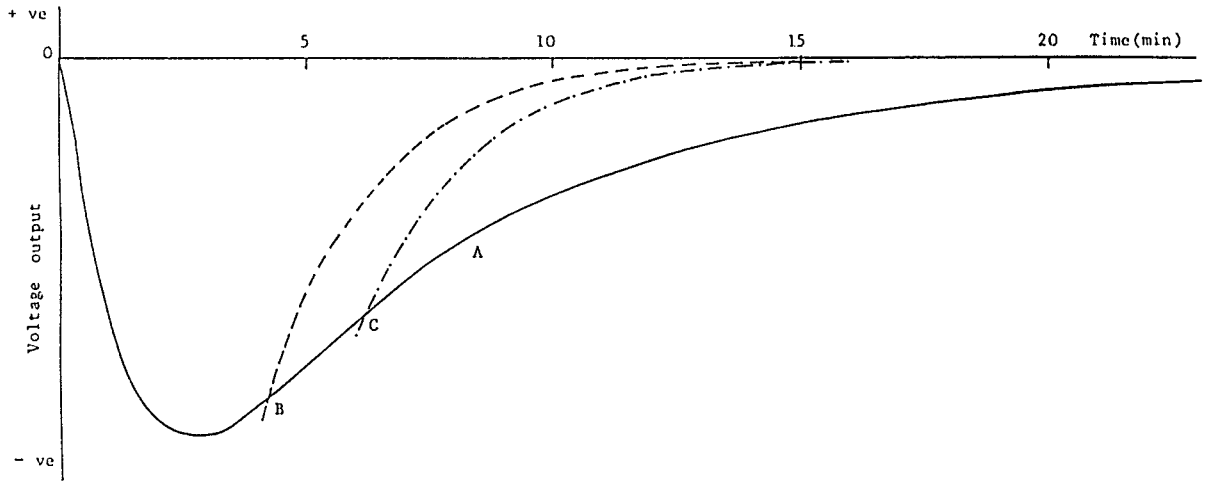

Fig. 5. Power–time curves for the interaction of rabbit ATPase antiserum with ADP.Mg (A). Curves B and C represent the method of analysis current in 1972 to distinguish fast and slow reactions. For details see [18,19].

and of compatibility studies of drug/excipient combinations. Most of the early data — a natural extension of the concern for drug/microbe interactions — was only qualitative. The principal conclusions being only that the evidence of an observed power from a solid sample, say, in a microcalorimeter was conclusive of a damaging degradation reaction. In 1990 Hansen and co-workers published [22] a paper that was concerned with quantitative prediction of shelf life for pharmaceuticals. However, the published methods were not entirely general and did not, therefore, describe solution phase and solid phase reactions. In 1995 Willson et al. [23] published a general procedure for the analysis of microcalorimetric data which also allowed the unambiguous assignment of order of reaction, rate constant and enthalpy of reaction. Equations are also presented that describe a range of reactions such as solution phase, solid phase, auto-catalytic, coagulation and enzyme based systems. The published equations are not meant to be an exhaustive list but only to represent the generality of the approach. In these publications it was shown that the calorimeter could detect 50 nW and this meant that, in principle, 50 h of measurement should allow reactions with half-lives up to 2500 year to be studied. Subsequent publications [24,25] described the application to both solid phase and to solution phase reacting systems. The derived equations were couched properly in terms of the quantities of reacting material and not in terms of the concentrations of the reagents. Thus whilst formally correct they were inconvenient for comparison with published kinetic data. The equations have now been re-written to incorporate concentration and an example (Eq. (3)) is shown that describes the output expected from the microcalorimeter from study of the imidazole catalysed hydrolysis of triacetin.

$$\frac{\mathrm{d}q}{\mathrm{d}t} = k\Delta HV\left\{A_0 - \frac{q}{\Delta HV}\right\}^n \tag{3}$$

In this equation k is the reaction rate constant, ΔH the reaction enthalpy, A_0 the concentration of the reagent which is added in volume V to the calorimeter and n is the order of reaction which for the imidazole reaction is set at 2. It is easy to convert from the quantity based rate constant to the concentration based rate constant via the relationship:$k_{\mathrm{conc}} = k_{\mathrm{quant}}V^{n-1}$.

This reaction is now proposed as a calibration reaction for isothermal heat conduction microcalorimeters [26,27]. It has been the subject of an International study involving inter- and intra-laboratory collaborations and the final publication will include, necessarily, a protocol for the performance of the test. The reaction will have not only the capacity to act as a chemical calibrant for the analysis of microcalorimetric kinetic data but it can serve as a training reaction for new personnel and for the examination of the stability and performance of a microcalorimeter. The reaction can yield excellent thermochemical

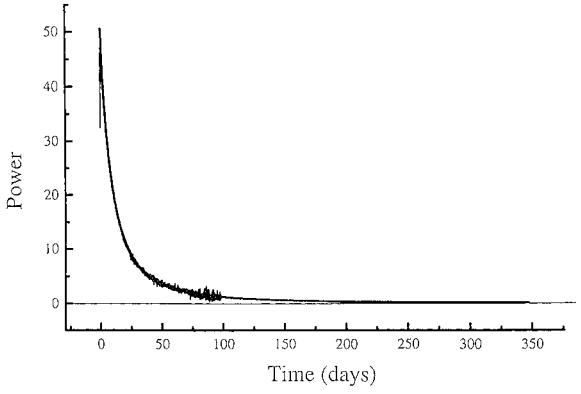

Fig. 6. Comparison of experimental data for the imidazole catalysed hydrolysis of triacetin run for 100 days with simulated data.

and kinetic results from studies from12 h to 100 days (Fig. 6).

Inevitably in the study of biological subjects we might expect much greater complexity in the kinetic forms of the microcalorimetric outputs for, for example, the process and progress of metabolism of a micro-organism To explore, initially, complex reaction systems before proceeding to biological systems it is more straightforward to investigate chemical non-biological systems and hence the acid catalysed hydrolysis of potassium hydroxylamine trisulfonate was selected for study. This reaction which is of the A→B→C→D type cannot be studied by classical kinetic techniques at 298 K and at constant acid conditions since the individual reaction steps differ widely in rate. However, when monitored in the microcalorimeter at 298 K it was necessary to include all reaction steps and enthalpies, order and rate constants were determinable for each of the individual steps [28].

Since 1972 there has been no effective extension of the equations noted above that describe microcalorimetric output for flow based experiments. It is often more convenient to conduct microbial growth and related studies — such as, for example, drug/cell bioassays — in a flow system. Recently Hills [29] has derived equations that describe flow microcalorimetric output in a general fashion. As with the introduction [23] of the general equations outlined above the equations satisfy all reaction orders — integral and non-integral — and permit, again, the introduction of well-characterised calibration reactions. As noted above the imidazole catalysed hydrolysis of triacetin fulfils all the requirements for such a calibrating reaction. The materials are easily obtained pure, the system is non-demanding in preparation and the reaction has a consistent behaviour over several hours to 100 days. Indeed Wadso noted this much earlier [30] when he proposed the reaction as a calibrant for flow microcalorimeters. However, this early report made only phenomenological deductions — there is no determined rate constant nor enthalpy for the reaction. In the light of the newly developed equations [23,29] the paper [30] does raise a subtle point in that calibration proposed by Wadso [30] is only satisfactory for the then standard design of microcalorimeter with a flow cell of a specified volume and for concentrations of reagents which are, likewise, closely specified. It is not possible to transfer the data for calibration purposes to any other design of microcalorimeter nor to use other than the specified concentrations. The reason is clear when one considers that a calorimetric equation requires that quantity of reagent be used (enthalpy is a per mole quantity and its value is also essentially independent of concentration) whereas a kinetic equation requires concentration to be used. The equations presented here and in detail in [30,31] allow the flexibility to select all the important variables — the only requirement being that the reaction is conducted under conditions in which the reaction mechanism is the same as that described the initial calibration reaction process.

Finally, therefore, I believe that there are now equations which adequately describe the output of microcalorimeters from experiments conducted in batch and flow based processes. There is a wealth of information available on the growth and metabolism of organisms, in both planktonic culture and on surfaces [32], on their interaction with drugs and other metabolic modifiers and perhaps really the time is now to exploit the unique property of microcalorimetry — its capacity to report all the detail of the process under study without being selective or partial in its recording. Biological subjects are often well characterised by end-point biochemical studies yet microcalorimetry may have even more detail available through its indiscriminate eye.

We hope, therefore, to be able, using the approach outlined above, to give a more detailed report on a

recent study [33] of a complex biological system. The system involved probing the microbial growth in, and treatment of with an appropriate biocide, a cutting fluid emulsion. Such a heterogeneous, complex reaction system is not amenable to classical investigation and is, therefore, a perfect test-case for microcalorimetry.Time will tell!

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