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HANDLING EQUILIBRIUM PROCESSES EMBEDDED IN METABOLIC SYSTEMS

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ABSTRACT

Thermodynamic constraints are essential for understanding the behaviour of living systems, but they are far from sufficient, because they allow a wide range of possibilities. Additional constraints imposed by kinetic considerations are often crucial in determining not merely whether a process can occur, but whether it does. Entropies of activation are in principle very useful for analysing experimental properties, but in practice they are rendered almost useless by the impossibility of estimating them accurately from observations spread over a narrow temperature range. Such estimation typically involves extrapolating more than 10-times the range of the data, and involves huge errors. Another common problem in the literature, results from confusion between actual and standard Gibbs energies of reaction: supposedly unfavourable equilibrium constants can suggest that processes necessary for life, such as reduction of sulphate by organisms that use sulphate as a terminal electron acceptor, are impossible; but as long as an organism has efficient mechanisms for maintaining reactant concentrations far from their standard states, a reaction can be driven in either direction regardless of the magnitude of its equilibrium constant. The increasing importance of computer modelling in studying metabolism has now focused attention on the question of how to handle reactions that are essentially irreversible. It has often been assumed that if the reverse reaction is negligible then all the effects of products can be neglected, but that is a potentially serious error: many enzymes are known where product inhibition has important effects on the rate of a reaction that is for practical purposes irreversible.

INTRODUCTION

Living organisms are chemical systems that operate far from thermodynamic equilibrium, and it is therefore difficult to predict their behaviour from simple thermodynamic considerations alone. This does not mean, of course, that metabolic processes do not obey thermodynamic rules: they do, but although thermodynamic constraints limit what is permitted they still allow a wide range of possibilities. More generally, the laws of physics place limits on what is possible in a living system, but they fall far short of defining what a living system is, or even of predicting that life can exist [1]. The difference between the standard Gibbs energy and the Gibbs energy of a particular physiological state, which may be very large, can never be neglected. Furthermore, kinetic constraints are also important, and are often crucial in determining whether a process occurs. In this article we discuss a number of different thermodynamic aspects of biochemistry that are sometimes forgotten or badly understood, though they need proper attention if valid metabolic models are to be created. Most of our points are not new, and some, indeed, date back to the early years of thermodynamics. Accordingly, we prefer to provide a general discussion, with references to more detailed arguments, rather than presenting all of the supporting details here.

We begin by examining why thermodynamics has always been regarded as a difficult subject, suggesting that the problems has its roots in the rather obscure way in which the essential concept of entropy was originally introduced and defined. This has led, on the one hand, to major examples of its misuse, for example in most evocations of the idea of entropy-enthalpy compensation, and, on the other hand, to reluctance to accept perfectly valid concepts such as enzymes that are more effective as catalysts in one direction of reaction than the other. Most serious, from the point of view of metabolic modelling, uncertainty about the proper way to deal with reactions with very large equilibrium constants has led both to valid models that are more complicated than they need to be, and to others that are invalid because they use thermodynamic arguments to reach invalid kinetic conclusions.

ORIGINS OF ENTROPY

The concept of entropy was introduced to thermodynamics by Clausius, who deliberately chose an obscure term for it, wanting a word based on Greek roots that would sound similar to "energy".

In this way he hoped to have a word that would mean the same to everyone regardless of their language, and, as Cooper [2] remarked, he succeeded in this way in finding a word that meant the same to everyone: nothing. From the beginning it proved a very difficult concept for other thermodynamicists, even including such accomplished mathematicians as Kelvin and Maxwell; Kelvin, indeed, despite his own major contributions to the subject, never appreciated the idea of entropy [3]. The difficulties that Clausius created have continued to the present day, with the result that a fundamental idea that is absolutely necessary for understanding the theory of chemical equilibria continues to give trouble, not only to students but also to scientists who need the concept for their work.

ENTROPY-ENTHALPY COMPENSATION

Partitioning temperature effects on reactions into entropy and enthalpy components is very useful for understanding why they behave as they do. A major practical problem arises however, from the fact that the actual numbers are almost impossible to estimate accurately from temperature-dependence measurements over the narrow ranges of temperature typically accessible for biological systems. Calorimetric measurements, as used for example by Freire and co-workers [4], are another matter, of course and are not open to the objections that we shall discuss, but unfortunately they are not the ones most frequently encountered; in the biochemical literature, and much other chemical literature, thermodynamic parameters are nearly always the result of analysing the temperature dependencies of equilibrium or kinetic constants. Even today, reputable journals provide examples of entropy estimates given with a precision of about 10%, even though they come from measurements at four or five temperatures in a narrow range. The entropy estimate is then obtained by extrapolating a small amount of information over more than ten times the range of the data (Fig. 1).

A study of the dependence on temperature of the kinetic parameters of an enzyme is typically restricted to a range from a little above 0ºC to no more than 40ºC. For various practical reasons the range is often substantially smaller than this. For example, a study of ATPases of various fishes [7], used as an example in a current textbook of biochemical kinetics [8], involved measurements in the range 0-18ºC.

In the ordinary units used for Arrhenius plots, this is a 1000/*T* range of 3.44-3.66 K^{-1}, and so the extrapolation needed to estimate the intercept on the ordinate axis, and hence the entropy of activation, extends more than 15 times the range of the data. Despite this, the apparent correlation between entropies and enthalpies of activation of the ATPases of seven different (and in most cases very distantly related) fishes is excellent, with no perceptible deviation from a perfect linear relationship. In our view, the only reasonable interpretation of such a perfect correlation is a statistical artefact caused by the extremely high correlation that inevitably appears between a slope and an intercept estimated from an excessively long extrapolation [9]. In fact, simulated data with mean catalytic activities varying randomly over a 10-fold range and Arrhenius activation energies varying randomly in the range 25-160 kJ/mol still show an excellent, but meaningless, correlation when transformed into a compensation plot [10].

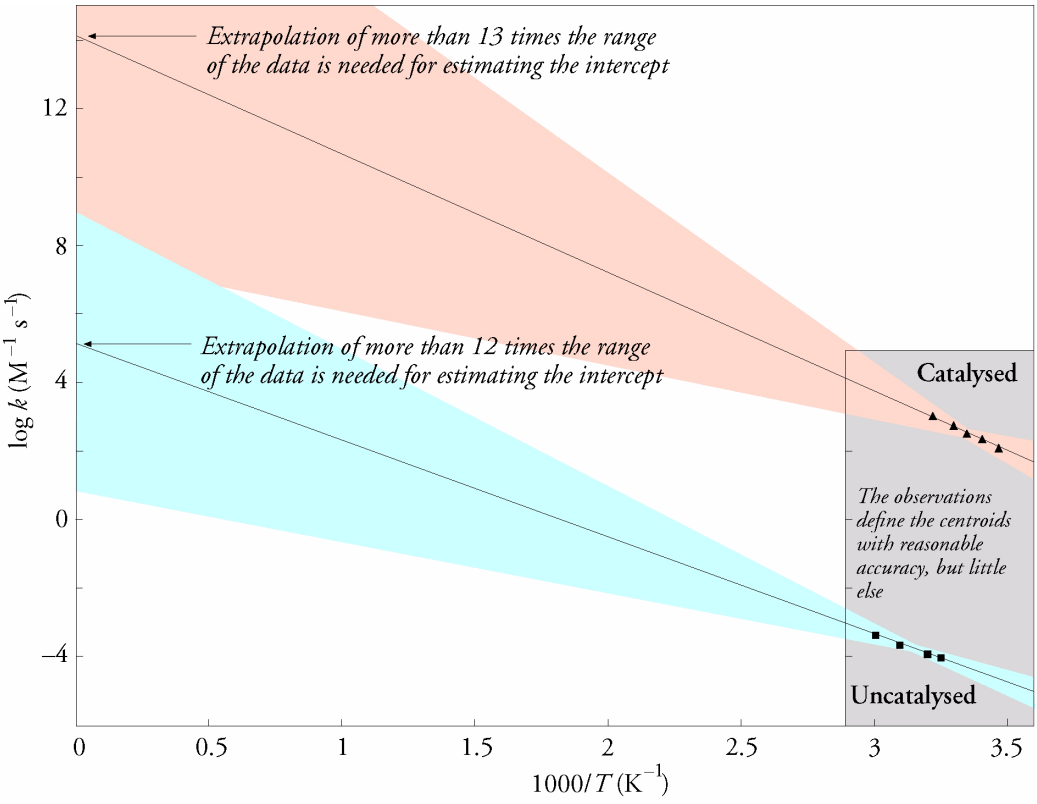

Figure 1. Extrapolation implicit in estimation of entropies from Arrhenius plots. The shaded area is redrawn from Fig. 4 of [5] and shows the temperature dependence of rate constants for uncatalysed and catalysed formation of peptide bonds. The data were said to provide estimates accurate to ±10% of the entropies of activation in the two cases. However, it is evident from the expanded versions of the plot shown here that estimating these entropies (whether graphically or by computer fitting) is equivalent to extrapolating the trends more than 12 times the ranges of the observations. In both cases the data define the centroids with fair accuracy, but, as shown by the shaded envelopes, a very wide range of straight lines could be drawn that would fit the points adequately. (More quantitative accounts of the effects of long extrapolations on parameter estimates may be found in textbooks of statistics, e.g. [6].)

ONE-WAY CATALYSIS

Some reactions, such as the one catalysed by methionine adenosyltransferase, have equilibrium constants that do not favour either the forward or the reverse direction very strongly, and yet have kinetic constants that make them far more effective catalysts for the forward than for the reverse reaction [11]. Such examples are sometimes thought to violate thermodynamic restrictions, but in fact they present no thermodynamic problem, because the equilibrium constant of a reaction only constrains the ratio between the kinetic parameters for the forward and reverse directions, not the individual values, and so large deviations from equality of catalytic constants can be compensated for by equally large deviations in the opposite direction of the corresponding binding constants. This type of consideration was thoroughly discussed by Jencks [12], and more recently by one of us [13].

The equilibrium constant certainly determines the direction in which a reaction will proceed in any particular conditions, and efficient removal of one or more reaction products can ensure that it proceeds in the opposite direction from what naive inspection of the equilibrium constant might suggest. Aspartokinase, for example, catalyses conversion of aspartate and ATP, an anhydride of phosphoric acid, into ADP and phosphoaspartate, a mixed anhydride of phosphoric acid and a carboxylic acid. This is chemically very unfavourable, with an equilibrium constant of 6.4×10^{-4} [14], but the phosphorylation of aspartate still occurs, because the phosphoaspartate is converted so rapidly into aspartic semialdehyde that its concentration is very small (normally too small to measure) in all physiological conditions. In his oral presentation to this symposium Tom Leyh discussed the even more extreme example of the physiological strategy that permits activation of sulphate for use by *Escherichia coli* in spite of an equilibrium constant of the order of 10^{-8}.

In all such cases one needs to take care to avoid interpreting thermodynamic data in an excessively naive way. Although the standard free energy of any reaction unambiguously defines the direction in which the reaction will proceed from the standard state, this will be very misleading if the possibility that the physiological state may be very far from the standard state is not taken into account, and suitable manipulation of one or more product/substrate ratios can ensure that it proceeds in what may appear at first sight to be the wrong direction.

NEARLY IRREVERSIBLE REACTIONS IN METABOLIC MODELS

Most metabolic reactions have equilibrium constants close enough to unity for it to be imperative to represent them with fully reversible rate equations in metabolic models. However, some proceed virtually irreversibly in the physiological direction of reaction, and it is then important to decide whether they can be safely represented by irreversible rate equations. The reaction catalysed by pyruvate kinase provides the classic example: it is needed for models of glycolysis and has an equilibrium constant of the order of 10^5, and it has divided investigators since the earliest simulations of metabolic systems in the 1960s. Some authors, such as Garfinkel and Hess [15], and, others more recently [16,17], have insisted on the need to use reversible equations throughout, regardless of the magnitudes of the equilibrium constants; others have treated such reactions as irreversible and product-insensitive [18-23].

The question is not trivial, as there are two opposing dangers to be avoided: ignoring reversibility when it must not be ignored leads to an invalid model that makes incorrect predictions; not ignoring it when it is safe to do so leads to a model that is more complicated than it needs to be. The second may appear the lesser of the two dangers, especially in the light of the enormous increases in computing power that have occurred in recent years, but it is a danger nonetheless. Reversible rate equations always contain more parameters than the corresponding irreversible equations, sometimes many more, and in practice this often means that they contain parameters that are not experimentally measured, forcing the modeller to make guesses of dubious validity. When we wished to use a reversible rate equation for pyruvate kinase in a model of glycolysis in *T. brucei* [1,24] we had to face the reality that no experimental data were available for the kinetics of the reverse reaction, forcing us to assume reasonable values that were consistent with the equilibrium constant and with the kinetics of the forward reaction. Even the argument based on consideration of computing power is not entirely convincing, because regardless of the amount of power available the total size of the model accessible to investigation will be decreased by making the individual equations more complicated than they need to be.

Actually, although the examples we have cited represent the dichotomy that existed in the literature between 1964 and 2000-either fully reversible on the one hand, or irreversible and product-insensitive on the other-this is a false dichotomy, because it ignores an intermediate possibility, one that is both real and realistic.

Products affect rates in a fully reversible rate equation in two different and separable ways: they not only cause a negative term to be present in the numerator of the rate expression, allowing the reaction to proceed in reverse under appropriate thermodynamic conditions; they also cause one or more positive terms to appear in the denominator, producing product inhibition even in irreversible conditions.

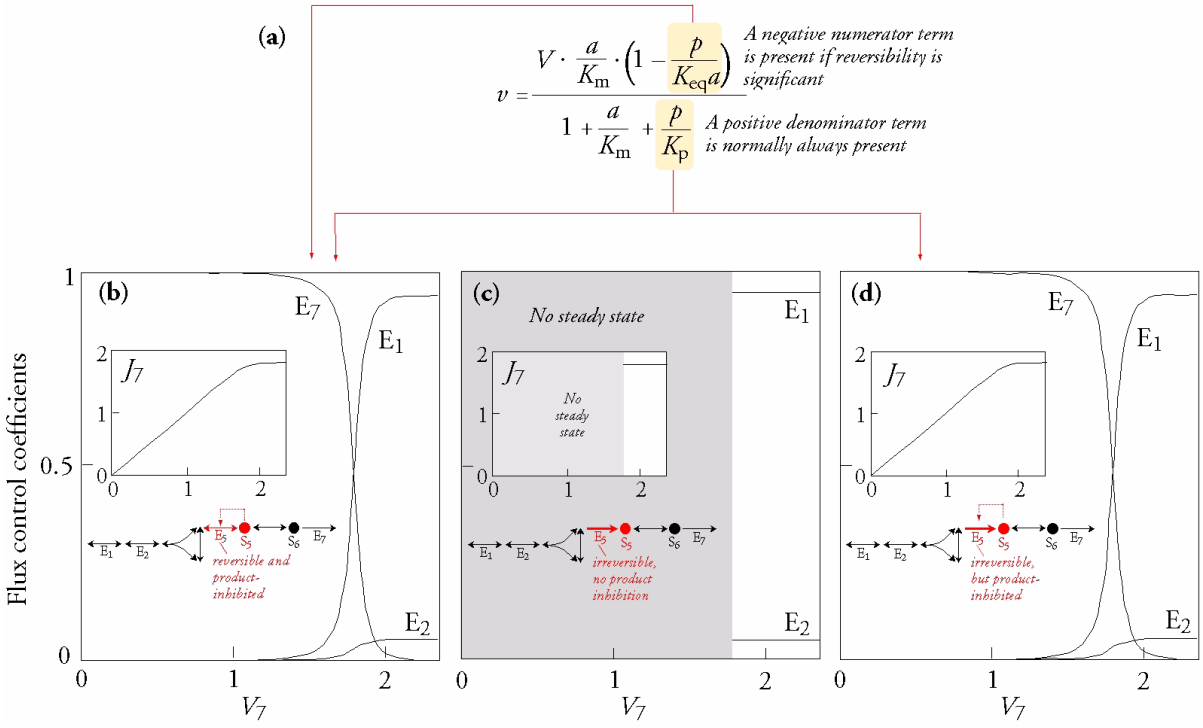

Figure 2. Effects of a product on the rate of an enzyme-catalysed reaction. **(a)** If a product is present in the reaction mixture in sufficient concentrations, it has two different inhibitory effects on the rate of the forward reaction: it leads not only to a negative term in the numerator due to the reversibility of any chemical reaction, but also, and independently, to a positive term in the denominator due to the tendency of almost any product to bind to the substrate-binding site of an enzyme. These properties are conceptually separate, and although the second must normally be present if the first is, it can also be, and often is, important when the first is negligible. The remainder of the Figure illustrates how these considerations act on the behaviour of a metabolic model: **(b)** with both terms present but small in the case of the numerator term; **(c)** with both absent; and **(d)** with only the denominator term present. The part of the model concerned with the action of the product is shown in red. Notice that Panels **(b)** and **(d)** are indistinguishable from one another but very different from Panel **(c)**, so ignoring the denominator term on the mistaken assumption that it can be neglected when the reverse reaction is negligible can lead to gross errors in the behaviour of models. The original article [25] describes the model used for the simulations in detail, and also discusses an additional affect, feedback inhibition by end-product, that we do not consider here.

It follows that although reversibility implies the presence of both kinds of term in almost all cases, irreversibility does not exclude the possibility of product inhibition, and hence a kinetically significant positive denominator term. This point, obvious once pointed out though ignored in the literature for many years, resolves all of the difficulties that we are aware of with pyruvate kinase in metabolic models [25].

In particular, it explains an observation that was initially very puzzling: introducing reversibility of the pyruvate kinase reaction into a model of glycolysis in *T. brucei* [22] caused a far more dramatic redistribution of control than one would have guessed from the small degree of reversibility [24]. Study of more simple models with nearly irreversible reactions in them showed that introducing positive denominator terms into a rate equation could lead to significant changes in behaviour, whereas introducing negative numerator terms that would be extremely small in any reasonable conditions never produced significant effects [25]. There is, of course, nothing surprising in this result; all that is surprising is that it was ignored for so long. As a general conclusion, it appears clear that one can safely ignore reversibility (negative numerator terms) when modelling nearly irreversible reactions, but it will not be safe to ignore product inhibition (positive denominator terms) unless there is very good experimental evidence that product inhibition is truly negligible over the whole range of conditions to be simulated.

An important point to consider is the possible difference between isoenzymes, which despite catalysing the same reaction, and thus having the same thermodynamic constraints, may vary considerably in the importance of reversibility and product sensitivity in the reaction, because of differences in kinetic parameters. This is well illustrated by the mammalian hexokinases [26]. Product inhibition of the liver isoenzyme, hexokinase D, is always negligible, as the inhibition constant for the product glucose 6-phosphate is far above any conceivable physiological concentrations, but inhibition of the other isoenzymes by glucose 6-phosphate is by no means negligible. Furthermore, although the equilibrium constant of the reaction, phosphorylation of glucose by MgATP, is not high enough to make the negative term in the numerator necessarily negligible, this term can certainly be neglected for hexokinase D because the concentration of glucose 6-phosphate needed to half-saturate the enzyme is very high.

In all of this discussion we are, of course, concerned with reactions that are essentially irreversible in the physiological direction. A reaction like that of aspartokinase, considered earlier, must of course be treated as reversible unless the rest of the model ensures that one or more product concentrations are truly negligible, because although they have large equilibrium constants these do not favour the direction in which the reaction is proceeding.

DISCUSSION

Thermodynamic ideas remain the basis of much of physical chemistry and of biochemistry. Avoiding the simple errors that we discussed at the beginning of this article is not difficult, but it does require more than a superficial understanding of what is being measured and what the numbers mean. The growing interest in systems biology implies a growing need to incorporate information about the kinetics and thermodynamics of enzyme reactions into large-scale models of cells. This certainly involves avoiding simple errors, of course, but it also demands intelligent decisions to be made to deal with the lack of much of the experimental information that one would ideally want to incorporate in a model. At present kinetic information is so sparse for some organisms that one is forced to develop methods that allow predictions based solely on the stoichiometric structure of a system [27,28], but this will certainly improve in the future if the enzymes concerned are studied directly. Even systems, for which a large amount of experimental kinetic information exists, such as the glycolytic pathway in *T. brucei* [22], have some gaps that need to be filled with reasonable guesses. In such cases knowing that nearly all reactions (including those that are virtually irreversible) are sensitive to their products can allow models that avoid being excessively simple, and hence capable of making invalid predictions, while also avoiding being excessively complicated, requiring values to be unnecessarily guessed and equations to be unnecessarily complicated. A simple rule in such cases is that it is safe to omit the term in the numerator that represents a reverse reaction that occurs to a negligible extent, but it is not safe to omit the terms in the denominator that take account of product inhibition. If any such simple rule is used, however, one must take account of the very different conditions that exist in different tissues and in different metabolic states, as well as the different kinetic parameters of isoenzymes that catalyse the same reaction.

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