



Enthalpy–entropy compensation and the isokinetic temperature in enzyme catalysis

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Enthalpy–entropy compensation supposes that differences in activation enthalpy ΔH^\ddagger for different reactions (or, typically in biochemistry, the same reaction catalysed by enzymes obtained from different species) may be compensated for by differences in activation entropy ΔS^\ddagger . At the *isokinetic temperature* the compensation is exact, so that all samples have the same activity. These ideas have been controversial for several decades, but examples are still frequently reported as evidence of a real phenomenon, nearly all of the reports ignoring or discounting the possibility of a statistical artefact. Even for measurements in pure chemistry artefacts occur often, and they are almost inescapable in enzyme kinetics and other fields that involve biological macromolecules, on account of limited stability and the fact that kinetic equations are normally valid only over a restricted range of temperature. Here I review the current status and correct an error in a recent book chapter.

Keywords. Arrhenius equation; compensation; enthalpy; entropy; isokinetic temperature

1. Introduction

Correlation between the thermodynamic parameters of the reaction rates of a series of similar compounds was first reported in a study of the decomposition rates of various aliphatic alcohols. Constable (1925) found a straight-line relation between the logarithm of the frequency factor (corresponding approximately to the entropy of activation ΔS^\ddagger) and the energy of activation (corresponding approximately to the enthalpy of activation ΔH^\ddagger). However, he used a very wide range of temperature, 220–420°C, which corresponds to a ratio of absolute temperatures of 1.4 (= 693/493). In contrast, many modern studies of enzyme-catalysed reactions have a ratio of more than 10, implying a very long extrapolation to estimate ΔS^\ddagger . Constable commented that ‘the error to be expected is considerable’, a warning that is usually ignored today.

In the following decades there were many reports of enthalpy–entropy compensation, before and after Exner (1964) drew attention to the possibility that statistical artefacts might suggest compensation when none exists. His arguments have been discussed in numerous later papers (Leffler 1965; Exner 1972; Exner and Beranek 1973; Exner 1973; Krug *et al.* 1976a, b; Krug 1976; Exner 1997; Ouvrard *et al.* 2001; Cornish-Bowden 2002; Olsson *et al.* 2011; Piguet 2011; Barrie 2012; Cornish-Bowden 2012a; Bushuev *et al.* 2016; Perez-Benito and Mulero-Raichs 2016).

Recently a study of 473 papers on the removal of heavy metals or dyes from waste water that reported ΔG^0 , ΔH^0 and ΔS^0 estimates published between 2013 and 2016 led the authors to conclude that ‘all the studied papers overlooked the possible flaws in the thermodynamic parameters being reported’ (Chang *et al.* 2016).

Supplementing Exner’s work, Krug *et al.* (1976a, b) thoroughly analysed 47 reports of enthalpy–entropy compensation in ‘major reviews in the literature’, and reached conclusions concordant with Exner’s. Among the 47 examples they found only three for which the harmonic mean of the measurement temperatures was outside the 95% confidence interval for the isokinetic temperature, showing significant compensation that could not be attributed to statistical effects. Only a small number of their examples referred to proteins (myoglobin or haemoglobin), and none of these showed significant compensation. As enzymes can be studied over much smaller ranges of temperature for which the Arrhenius equation is obeyed than may be possible with experiments in pure chemistry, it would be very optimistic to think that the results for enzyme-catalysed reactions would be any better.

In some cases compensation may be genuine (but I am not aware of any such cases for enzyme reactions), especially for analysis of results from calorimetry rather than from Arrhenius or van’t Hoff plots, but it is still advisable to use some caution: Sharp (2001) made the important point,

discussed in some recent papers (Keszei 2016; Pan *et al.* 2016), that if ΔG^\ddagger is very small then $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$ approximates to the trivial equation $\Delta S^\ddagger = \Delta H^\ddagger/T$, from which a ‘correlation’ follows automatically, as indeed it does if ΔG^\ddagger is small and varies very little over the range of temperatures used. So even in calorimetric studies one should avoid concluding that there is significant correlation unless ΔG^\ddagger is sufficiently far from zero.

Many authors have taken the experimentally observed correlation to be real, and have searched for physical properties to explain it. Lumry and Rajender (1970), for example, were aware of the arguments of Exner (1964) and had an acute understanding of statistical analysis in other contexts (Johansen and Lumry 1961), but after a very thorough review of the literature they concluded that compensation effects were caused by ‘a ubiquitous property of water’ (Lumry and Rajender 1971). In a later paper they repeated that the occurrence of isokinetic temperatures around 280 K (7°C) appeared ‘to be a ubiquitous consequence of the properties of liquid water’. They argued that ‘the plotting procedure he [Exner] proposes overemphasizes experimental errors’. Nearly half a century later it still seems to me that Exner was essentially correct.

Nonetheless, Olsson *et al.* (2011) report several examples for which compensation deduced from calorimetric measurements of protein–ligand interactions (not enzyme kinetics) appears to be genuine. They also note that Cooper *et al.* (2001) pointed out that the range of ΔG values that are accurately measurable by the most common direct calorimetric method is limited by the necessity to obtain an analysable sigmoidal titration curve within the constraints of protein solubility and instrument sensitivity. As a consequence, the genuine compensation is normally much less precise than the spurious ones that emerge from van’t Hoff or Arrhenius plots. They add that they agree with my view ‘that genuine but imperfect correlations are biologically more interesting than meaningless perfect ones’ (Cornish-Bowden 2002).

In an earlier paper in this Journal (Cornish-Bowden 2002) I stated that reports of enthalpy–entropy compensation had become relatively rare in the literature, apart from occasional revivals. This statement has proved to be a very poor prediction of the present reality, as about 140 papers dealing with the topic are published each year. Even in 2001 it expressed the reality rather badly, as the modern enthusiasm for enthalpy–entropy compensation started in about 1990 and has increased, almost continuously, ever since.

Of the papers reporting or discussing enthalpy–entropy compensation that have appeared in recent years, many simply report results without referring to any doubts about whether they are meaningful. A few authors, such as Moss (2017), mention the existence of an ‘opposing view’, but suggest no reasons for rejecting it. Relatively few papers (for example Olsson *et al.* 2011; Piguat 2011; Bushuev *et al.*

2016) offer any serious discussion of the point raised by Exner (1964) half a century ago.

2. Experimental example

I shall use the same example as in my earlier paper (Cornish-Bowden 2002) to illustrate the ideas discussed, the thermodynamic parameters of ATPases from various fishes (Johnston and Goldspink 1975). It might be better to use a more recent example, but I have not been able to find recent papers that include sufficient numerical data to allow plots to be drawn. Moreover, although the ATPase experiments were reported in 1975 they were used much more recently to illustrate enthalpy–entropy compensation in an authoritative book (Gutfreund 1995).

As a different example, Singh *et al.* (2014) present plots (their figure 6) that show that ΔG^\ddagger is essentially constant when $T\Delta S^\ddagger$ changes. They interpret this as evidence of compensation, though it follows from the analysis by several authors mentioned earlier (Sharp 2001; Keszei 2016; Pan *et al.* 2016) that in reality it argues against significant compensation.

3. Isokinetic temperature

The slope of a plot of ΔH^\ddagger against ΔS^\ddagger (or ΔH^0 against ΔS^0 in the case of a van’t Hoff plot) has the dimensions of temperature, and is known as the *isokinetic temperature* or as the *compensation temperature*. It is the temperature at which the enthalpy and entropy terms cancel out, so that all entities (enzymes in the usual biochemical case) have the same activity. An example is shown in figure 1, which gives a slope of 290 K, corresponding to 17°C, just within the range of temperatures (0.5–18°C) used for the experimental measurements.

This may seem a rather indirect way of measuring the isokinetic temperature, which could in principle be determined without any calculation of thermodynamic parameters, and without any underlying theory, by determining the temperature at which all of the kinetic activities are equal. The reason why that is hardly ever done may be seen from figure 2, in which $\Delta G^\ddagger/RT$ is plotted against $1000/T$ for the data of table 1 of Johnston and Goldspink (1975). Although three of the lines cross close to a common point corresponding to 8°C there is no general tendency for the lines to cross at a common point, and no suggestion at all of a common intersection point corresponding to 17°C. Figure 6 of Exner (1997) shows a similar pair of plots, in which the plot of ΔH^\ddagger against ΔS^\ddagger shows an apparently well-defined isokinetic temperature, whereas the plot of $\log k$ against $1000/T$ shows no meaningful relationship. Similarly, figure 5 of Krug *et al.* (1976b) resembles figure 2. On the other hand, their figure 4 illustrates an example (hydrolysis of ethyl benzoate) in which there does

appear to be a well-defined isokinetic temperature, probably because this was an example from pure chemistry: however many difficulties may arise for recognizing compensation in simple chemical examples, they are likely to be much worse with examples in enzyme kinetics, which suffer far more from lack of stability and reproducibility, and in which the Arrhenius equation is typically obeyed only over a restricted range of temperatures. All of these papers should be carefully studied from the point of view of enthalpy–entropy compensation. In addition, Exner (1997) examined other kinds of experiment for which incorrect statistical analysis can lead to erroneous conclusions.

If the isokinetic temperature cannot be measured directly when the range of temperatures is too small (figure 2), why should it be possible to obtain a reliable value by an indirect method (figure 1)? It seems that the proper lesson to be drawn from figure 2 is that there is no meaningful isokinetic temperature for the fish ATPases. A different reason for scepticism (Cornish-Bowden 2002, 2016) is that if the supposed isokinetic temperature of about 17°C is an ‘adaptation’, the result of natural selection (Gutfreund 1995) then there needs to be a plausible hypothesis for the advantage to

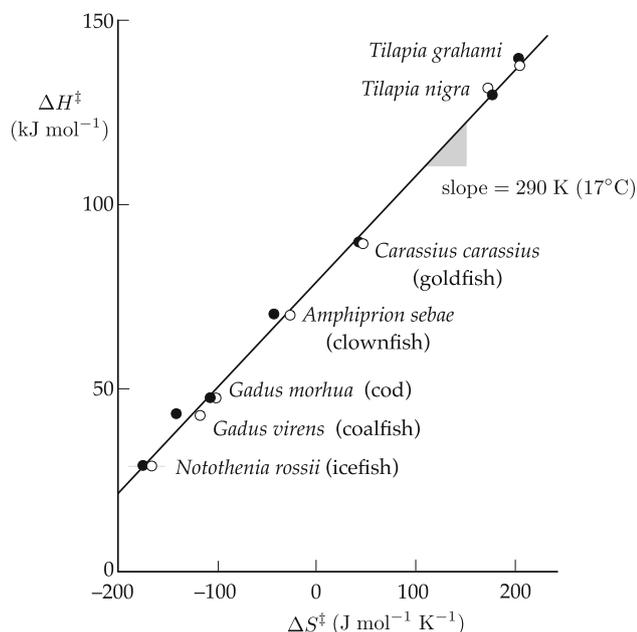


Figure 1. Compensation plot for ATPases from various fishes. This is drawn from the thermodynamic parameters at 0.5 and 18°C given in table 1 of Johnston and Goldspink (1975). The points fit the line well, but not as well as that in figure 1 of their paper. Although it is not explained in their paper, one may guess that the original plot was drawn from averages of measurements at more than two temperatures. This is far from being an ideal example to use as an illustration, but unfortunately more recent publications do not include enough experimental details to make a similar analysis. Moreover, this example was used to illustrate compensation in a textbook by a distinguished author (Gutfreund 1995).

Tilapia grahami and *Notothenia rossii* of having the same ATPase activity at 17°C, far too cold for one to survive, and far too hot for the other.

With a larger range of experimental temperatures, as used, for example, by Bushuev *et al.* (2016), a plot of $\ln k$ against $1/T$ may produce a far more convincing intersection point than can be seen in figure 2: compare, for example, the plots for the hysteresis loop width for an iron(II) complex (figures 5 and 6 of Bushuev *et al.* 2016). The temperatures ranged from 162 to 227°C (435–500 K), and the data were from chemical measurements, and thus much less sensitive to experimental variation than biological data. Even in this case we see families of intersection points rather than unique points.

4. Detecting real compensation

Exner (1997) described how to detect whether there is a genuine compensation. Variation of ΔG^\ddagger with ΔH^\ddagger should reveal whether the compensation is real. In most (but not all) experimental studies ΔG^\ddagger proves not to vary significantly with ΔH^\ddagger , as seen for the ATPase data in figure 3.

5. Other related cases of spurious correlation

Well known to people who have studied the analysis of enzyme kinetic data, but largely ignored, even today, by people who use double-reciprocal plots to analyse experiments, estimates of the Michaelis–Menten parameters V_{\max} and K_m are very highly correlated (Cornish-Bowden 2012b). The correlation arises from a very long extrapolation of data in a restricted range, just as with enthalpy–entropy compensation. This was noted by Krug *et al.* (1976a):

Correlated parameter estimates are obtained in other chemical investigations as well. In enzyme studies, for example, the Michaelis–Menten parameters \hat{v} and \hat{K}_m are highly correlated when estimated from a Lineweaver–Burk plot when the range of inverse substrate concentration is small.

Exner (1997) made a similar point.

6. Dependence of the isokinetic temperature on the measurement temperatures

I claimed in a book chapter (Cornish-Bowden 2012a) to have proved that the value of the isokinetic temperature was independent of the measurements, depending only on the temperatures at which they were made. The ‘proof’ was invalid, however, as it only showed that the slope of a plot of ΔH^\ddagger against ΔS^\ddagger for a single process is independent of the measurements, but that does not mean that the slope of a plot of ΔH^\ddagger against ΔS^\ddagger for a series of *different* processes, i.e. the

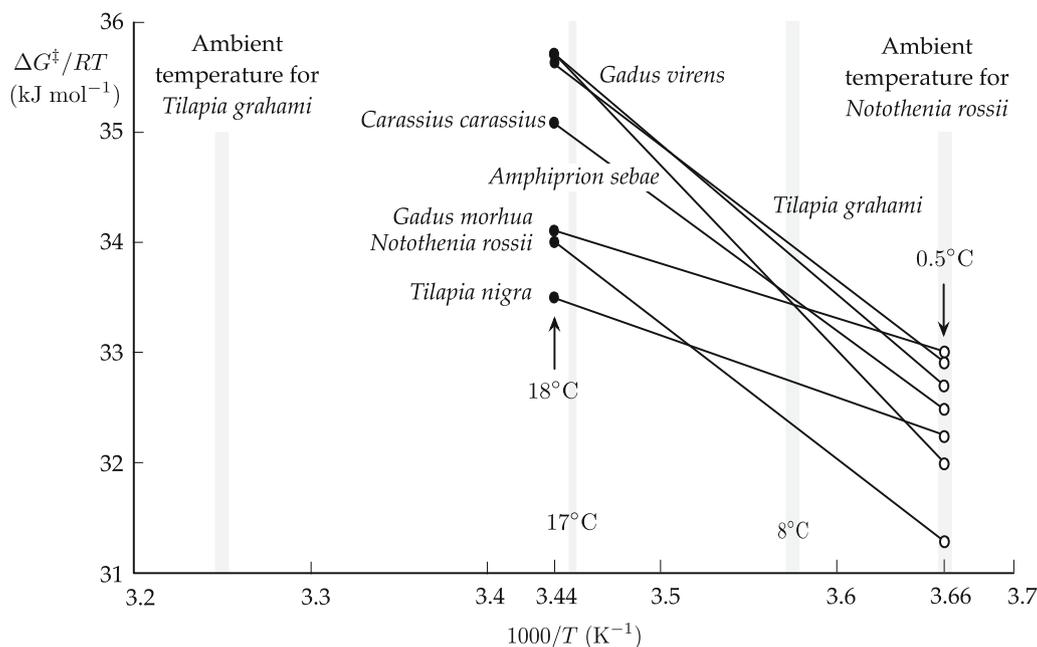


Figure 2. Attempt to measure the isokinetic temperature directly. Values of $\Delta G^\ddagger/RT$ were taken from table 1 of Johnston and Goldspink (1975), which unfortunately reported $\Delta G^\ddagger/RT$ at only two temperatures, though measurements were clearly made at several others. These were used in preference to the values of V_{\max} given in the same table, as the values of V_{\max} vary haphazardly, and probably therefore contain errors: for example, the calculated thermodynamic parameters change monotonically down the table, whereas the V_{\max} values do not, not even approximately. It is also hard to explain why *Tilapia grahami* and *Tilapia nigra* had similar V_{\max} values at 0.5°C (0.017 and 0.016 $\mu\text{mol P}_i \text{mg}^{-1} \text{min}^{-1}$), and very different ones at 18°C (0.045 and 0.69 $\mu\text{mol P}_i \text{mg}^{-1} \text{min}^{-1}$). The entire experiment was done at temperatures where *Tilapia grahami* could not survive. Two other fishes live at temperatures (not marked on the figure) outside the range of measurement: *Tilapia nigra* (23–31°C) and *Amphiprion sebae* (23–25°C). *Carassius carassius* lives over a wide range of temperatures in the wild, but the domesticated fish used in the study was from an aquarium at 26°C, so it would also find 17°C rather cold.

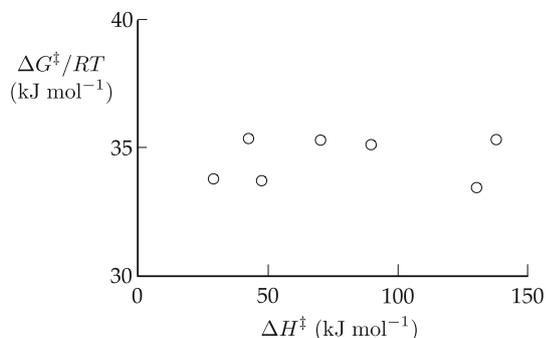


Figure 3. Exner plot for the data of figures 1, 2. $\Delta G^\ddagger/RT$ is plotted against ΔH^\ddagger . A definite trend would be evident if there were a real compensation effect.

isokinetic temperature, is independent of the measurements. In reality, it is certainly possible for this slope to vary with the measurements.

The result is, however, approximately correct in most cases even if the reasoning used to reach it was not. Krug *et al.* (1976a) gave the following expression for the correlation coefficient ρ between ΔH^\ddagger and ΔS^\ddagger in the absence of any extrathermodynamic effect:

$$\rho = \frac{\text{Cov}(\Delta H^\ddagger, \Delta S^\ddagger)}{\sqrt{V(\Delta H^\ddagger)V(\Delta S^\ddagger)}} = \frac{\sum 1/T}{\sqrt{n \sum (1/T^2)}}$$

in which V refers to the variance of the estimate in parentheses (usually symbolized as σ^2), and n is the number of temperatures considered. They did not define ‘extrathermodynamic effect’, treating it as a standard well understood term, as it apparently was at the time they were writing, but it is clear from the rest of their paper that they meant compensation beyond what can be explained as a statistical artefact. They commented as follows: ‘Notice that this correlation coefficient depends only on the choice of experimental temperatures.’

7. Discussion

Examples of enthalpy–entropy compensation are reported with increasing frequency, with more than 60 publications in the first half of 2017. Only about 11% of these were in journals of biochemistry or biophysics, about 55% were in journals of chemistry or physics, and a substantial

proportion, about 33%, were in journals such as *Industrial Crops Production* and *Dyes and Pigments* with no strong foundation in chemistry. The problem of severe spurious correlation between estimates of ΔH^\ddagger and ΔS^\ddagger due to trivial statistical effects, to which Exner (1964) drew attention half a century ago, remains relevant to modern research. Moreover, despite some controversy, Exner's point remains valid today.

The correlation between estimates of ΔH^\ddagger and ΔS^\ddagger (or between estimates of ΔH^0 and ΔS^0) appears to be meaningless in most experiments, though there are some exceptions, as Exner himself recognized (Exner 1997; Ouvrard *et al.* 2001), and as more recent authors have discussed (Olsson *et al.* 2011; Piguet 2011; Bushuev *et al.* 2016). Moreover, the concept of an isokinetic temperature at which all of the reactions considered proceed at the same rate also has problems. In principle it can be measured directly without thermodynamic parameters, but when this is done (for example, in figure 2) the lines are usually so scattered that no isokinetic temperature can be found. For that reason, most experimenters prefer to estimate it indirectly from a compensation plot. However, that does not eliminate the problem; it just makes it less obvious.

Apart from these general considerations, I have taken the opportunity in section 6 to correct an error made in another publication (Cornish-Bowden 2012a).

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