

## HARDEN AND YOUNG'S DISCOVERY OF FRUCTOSE 1,6-BISPHOSPHATE\*

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As in the case of so many discoveries, the new phenomenon was brought to light, apparently by chance, as the result of an investigation directed towards other ends, but fortunately fell under the eye of an observer possessed of the genius which enabled him to realise its importance and give to it the true interpretation.

HARDEN (1932)

Eduard Buchner's discovery that a cell-free extract of yeast was capable of fermenting sugar (Buchner, 1897) opened the door to much of the development of modern biochemistry. As discussed by Bohley and Fröhlich in this book (pp. 51–60), others before Buchner, such as Marie von Mannasein (1897), had made similar claims, but these were based on inadequate evidence and did not lead to the explosion of new research that followed from Buchner's work. In any investigation it is not sufficient to come to the right conclusions; one must base them on evidence firm enough to convince others. Among those who built on Buchner's work was Arthur Harden, who devoted a large part of his career to the study of alcoholic fermentation. Harden's assessment of Buchner's contribution is well expressed by the sentence quoted at the beginning of this chapter. After deciding to include

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\* The parts of this chapter concerned with the thermodynamic interpretation of the experiments of Harden and Young are based to a large extent on an article (Cornish-Bowden, 1981) originally published in *Biochemical Education*.

this quotation I was pleased to find that the same sentence had caught the eye of one of the giants of 20th century biochemistry, Fritz Lipmann (1971), who added his own comment that “What I delight in here is [Harden’s] preoccupation with the accidental”.

Lipmann went on to say that these remarks applied equally to Harden’s own “great discovery that phosphate takes part in the chemical reactions that convert glucose to ethanol and carbon dioxide”. This discovery derived, of course, from Buchner’s work, and was itself the beginning of the complete understanding of the reactions of fermentation and glycolysis. This discovery, and especially its implications for understanding the application of thermodynamic ideas to metabolism, will form the main theme of this chapter. First, however, I shall digress to discuss the importance of yeast fermentation in general, and Buchner’s work in particular, in the origins of our present ideas on enzyme kinetics in the early years of the 20th Century.

When I was reading the background literature for my first book about enzyme kinetics (Cornish-Bowden, 1976) I was working in the Department of Biochemistry at Birmingham, which had one of its roots in the British School of Malting and Brewing, and I was already conscious of the importance of brewing in the early development of biochemistry, and also of how this had been transformed by Buchner’s discovery. As early as 1892 Adrian Brown, Professor of Malting and Brewing at Birmingham, suggested that the kinetics of enzyme-catalysed reactions could be related to the occurrence of an enzyme–substrate complex along the reaction pathway (Brown, 1892), but his kinetic observations on live yeast appeared to conflict with those of O’Sullivan and Tompson on isolated preparations of invertase (1890), and did not establish a new view of enzyme catalysis.

Buchner’s discovery transformed Brown’s view of his own work, and ten years later he reexamined it using purified invertase. Finding that purified invertase showed the same sort of kinetic behaviour as live yeast (Brown, 1902), he explained the now very familiar idea of enzyme saturation at high substrate concentrations in essentially the same way as we would explain it now, saying that the need to pass through an enzyme–substrate complex placed a limit on how fast a reaction could go, because this complex could not break down infinitely fast to give products.

Victor Henri (1902, 1903) criticized Brown’s ideas, not because he thought they were fundamentally wrong, but because he objected to the notion of an enzyme–substrate complex with a fixed lifetime between its abrupt creation and decay. He reformulated the hypothe-

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sis in a way that was conceptually quite similar but much more in accord with contemporary ideas of chemical kinetics. He appears to have been the first to write down an equation equivalent to what is nowadays called the Michaelis–Menten equation.

Some modern authors see it as unjust that Leonor Michaelis and Maud Menten (1913) are normally credited with discoveries that could also be attributed to Brown or Henri. In reality, however, the earlier work was open to the same criticisms that Buchner made of von Manassein\*: Brown and Henri may have been essentially right, but they had no understanding of the need to control the pH or to take account of mutarotation or effects that might alter the enzyme activity during the course of a reaction followed for an hour or more, so it is difficult to have much confidence in the experimental observations on which they based their ideas. It remained for Michaelis and Menten to define experimental standards that are not very different from those that are used today, more than 80 years later.

Incidentally, if there has been any undue neglect of Brown's and Henri's contributions in the subsequent development of enzymology, Michaelis and Menten cannot be blamed for it; they were careful to give proper credit to their predecessors, particularly Henri:

The investigations of Henri are especially important because starting from reasonable ideas on the nature of enzyme action he arrives at a mathematical formulation of the course of enzyme action which fits the facts quite well. In this work we also start from these ideas of Henri. We undertook to re-investigate the whole work because Henri did not take into account two influences which are of very great importance and the neglect of which in Henri's work now appears so serious that a new investigation is worth while. The first is the effect of hydrogen ion concentration ( $[H^+]$ ), the second the effect of mutarotation.

MICHAELIS and MENTEN (1913), trans. BOYDE (1980)

Returning now to the main subject of this chapter, the experiments of Harden and Young (1906, 1908, 1911), my interest in these was stimulated by a casual remark of Dan Koshland's when I was making a return visit to his laboratory in the summer of 1974. Before

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\* See the chapter of Bohley and Fröhlich (pp. 51–60)

that, I had vaguely heard of Harden and Young, and knew that the Biochemical Society sponsored a series of conferences with the title Harden Conferences, but, like most biochemists educated since 1960, especially those with a background more in chemistry than in biochemistry, I knew next to nothing of what they had achieved. (My ignorance in this matter is hardly unique: a few days before writing these words I was talking with a distinguished biochemist — in the field of carbohydrate metabolism, no less — who was shortly to attend his first Harden Conference, and he had no idea what Harden’s contribution to biochemistry had been).

Even in the 1970s not all of the standard textbooks were very illuminating, and those of today are certainly no more so. For example, White, Handler and Smith (1968) limit themselves to an index entry “Harden–Young ester (*see* Fructose 1,6-diphosphate)”, which is suggestive but no more. Modern students of glycolysis can be thankful, incidentally, that the practice of naming metabolites after their discoverers has not survived, as they would have to contend not only with Harden–Young ester, but with others as well, such as Robinson ester (glucose 6-phosphate) and Neuberger ester (fructose 6-phosphate).

Lehninger (1975) is much more helpful, both because he identifies the Harden–Young experiments as the first major new steps made in the understanding of alcoholic fermentation after Buchner opened up this field, and because he makes it clear that they led to the discovery of a major metabolic intermediate, fructose 1,6-bisphosphate, and of the coenzymes NAD, ATP and ADP.

In fact, Harden and Young’s experiments were not only crucial in the development of our understanding of the glycolytic pathway; they also provide some of the most instructive examples for teaching biochemical equilibria to modern students. Dan Koshland’s remark mentioned earlier was that a thorough study of the experiments would give students more understanding of thermodynamics than a course of thermodynamics taught in the conventional way. By saying this he stimulated me to read the relevant papers and find out from them what Harden and Young had done. I thus confirmed that they do indeed provide an effective basis for teaching some of the crucial points about biochemical equilibria necessary for understanding why metabolic pathways behave as they do.

The experiments were published in a series of papers (more than the three cited here) entitled *The alcoholic ferment of yeast-juice*, and like most papers of their time they included much more detail than

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the modern reader is likely to find interesting. A more convenient, but equally authoritative source, for reading about them, therefore, is likely to be Harden's book *Alcoholic Fermentation* (Harden, 1932).

As Scopes also deals with the Harden–Young experiments in his chapter in this volume (pp. 151–158), I shall concentrate here on the thermodynamic aspect that drew me to them in the first place. Understanding them is easier for us than it was for Harden and Young, because of all of the knowledge about the chemical details of glycolysis that has been gained since their time. In modern terminology, the crucial observations were as follows:

1. In the presence of an ample supply of inorganic phosphate, alcoholic fermentation of glucose by yeast extract proceeds until all of the glucose is consumed (Harden and Young, 1906).
2. Under inorganic phosphate limitation fermentation becomes very slow and fructose 1,6-bisphosphate accumulates (Harden and Young, 1908).
3. Addition of a small amount of inorganic phosphate to yeast extract fermenting under phosphate limitation causes a rapid increase in the rate of fermentation, during which 1 mol CO<sub>2</sub> is produced per mol phosphate added, after which very slow fermentation resumes (Harden and Young, 1908).
4. In the absence of inorganic phosphate, addition of a trace of arsenate causes a rapid increase in fermentation, which may continue until all of the glucose is consumed, and analysis shows no perceptible change in the concentration of arsenate at any time (Harden and Young, 1911).

To understand the first three of these observations it is sufficient to know the glycolytic reactions and their standard Gibbs energies  $\Delta G^{0'}$  shown in Table 1. To understand the fourth it is also necessary to know that glyceraldehyde 3-phosphate dehydrogenase will accept arsenate as a substrate instead of inorganic phosphate, but that the 1-arseno-3-phosphoglycerate presumed to be produced is unstable and is spontaneously hydrolysed to 3-phosphoglycerate and arsenate.

If we ignore for the moment the experiment with arsenate, it is clear that the reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase cannot proceed in the absence of inorganic phosphate and that consequently the whole process must cease. But why should

fructose 1,6-bisphosphate accumulate rather than any of the other four intermediates that occur before the blocked reaction? To understand this we need to examine the  $\Delta G^{0'}$  values shown in Table 1.

A naive (and wrong) interpretation of  $\Delta G^{0'}$  values commonly encountered states that large negative values indicate that the forward reaction is strongly favoured, whereas large positive values indicate that the reverse reaction is strongly favoured. (To make matters more precise I should define “large” as “larger than about 11 kJ/mol” and “strongly favoured” as “favoured by at least a factor of 100”). This seems to explain the result in the absence of inorganic phosphate,

Table 1. Thermodynamic characteristics of the early reactions of fermentation

Enzyme	Reaction	$\Delta G^{0'}$ kJ/mol	$K_c$
Hexokinase	$\text{Glc} + \text{ATP} = \text{Glc6P} + \text{ADP}$	-16.7	850
Hexose- <i>P</i> isomerase	$\text{Glc6P} = \text{Fru6P}$	+1.7	0.50
Phosphofructokinase	$\text{Fru6P} + \text{ATP} = \text{Fru1,6P}_2$	-14.2	310
Aldolase	$\text{Fru1,6P}_2 = \text{DHAP} + \text{Gla3P}$	+23.8	0.000067 M
Triose- <i>P</i> isomerase	$\text{DHAP} = \text{Gla3P}$	+7.5	0.048
Glyceraldehyde 3 <i>P</i> dehydrogenase	$\text{NAD}_{\text{ox}} + \text{Gla3P} + \text{P}_i$ $= \text{NAD}_{\text{red}} + 1,3\text{P}_2\text{Glo}$	+6.3	0.079 M <sup>-1</sup>
3 <i>P</i> -Glycerate kinase	$1,3\text{P}_2\text{Glo} + \text{ADP}$ $= 3\text{PGlo} + \text{ATP}$	-18.8	2000

because it indicates that the hexokinase and phosphofructokinase equilibria favour the forward reactions (which is true), that the hexose phosphate isomerase equilibrium does not strongly favour either direction (which is also true), but the aldolase equilibrium strongly favours the reverse reaction, i.e. accumulation of fructose 1,6-bisphosphate. This “explanation” creates a worse problem than the one it solves, however, because it suggests that the aldolase reaction should never proceed forwards at all, whether inorganic phosphate is available or not — yet fermentation does proceed readily when phosphate is available.

To understand properly what is happening we must examine the relationship between  $\Delta G^{0'}$  and the equilibrium constant  $K$  for a

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reaction:

$$\Delta G^{0'} = -RT \ln K \approx -5.7 \log K \text{ (in kJ/mol) at } 25^\circ\text{C}$$

where  $R = 8.314 \text{ J mol}^{-1} \text{ K}^{-1}$  is the gas constant and  $T$  is the absolute temperature. Thus at  $25^\circ\text{C}$  each  $-5.7 \text{ kJ/mol}$  in the value of  $\Delta G^{0'}$  corresponds to a factor of 10 in the equilibrium constant in favour of product formation. If we apply this relationship for the first three reactions of glycolysis we have:  $K = 850$  for hexokinase,  $K = 0.50$  for hexose phosphate isomerase, and  $K = 310$  for phosphofructokinase, i.e. hexokinase and phosphofructokinase catalyse reactions with equilibria that strongly favour the forward reaction, whereas hexose phosphate isomerase catalyses a reaction that is easily reversible, in good agreement with the interpretation of the standard Gibbs energies given above. For aldolase, however, we find that  $K = 6.7 \times 10^{-5}$ , which seems to suggest that the reaction will not readily proceed forwards, even though we know that it does. What has gone wrong? Why should a calculation that works with the first three enzymes not give a sensible result for aldolase?

The explanation lies in the fact that if we calculate an equilibrium constant  $K$  from the definition of  $\Delta G^{0'}$  above we must get a dimensionless result, because the expression contains  $\ln K$  (or  $\log K$ ), and only dimensionless numbers have logarithms. Each  $K$  given above must therefore be a dimensionless number. This presents no problem for the first three, because each refers to a reaction in which the number of reactants is the same as the number of products, and so we should never expect them to have dimensions. For aldolase, however, we might expect to interpret the equilibrium constant as

$$K_c = \frac{[\text{G3P}]_{\text{eqm}} [\text{DHAP}]_{\text{eqm}}}{[\text{FBP}]_{\text{eqm}}}$$

in which the subscripts eqm indicate concentrations at equilibrium. Now this quantity  $K_c$  cannot be the same as  $K$ , because it is not a dimensionless number but a concentration. We therefore need to ask what is the relationship between  $K_c$  and  $K$ .

The convention that we use to make  $K$  dimensionless so that we can take its logarithm and relate it to the thermodynamic quantity  $\Delta G^{0'}$  is to say that we are not defining  $K$  in terms of real concentrations measured in mol/l, but concentrations relative to a set of standards, thus:

$$K = \frac{\frac{[\text{G3P}]_{\text{eqm}} [\text{DHAP}]_{\text{eqm}}}{[\text{G3P}]^0 [\text{DHAP}]^0}}{\frac{[\text{FBP}]_{\text{eqm}}}{[\text{FBP}]^0}}$$

where the superscripts (<sup>0</sup>) indicate these standards. Most of the time we regard it as intolerably cumbersome and pedantic to define an equilibrium constant in this sort of way, but it is important to realize that the standard concentrations are implied even if they are not written down. In principle we could choose any values we liked for the standard concentrations, and they could be different for each chemical species if we wished. But a chaotic set of standards would be very difficult to remember and so, with a very few exceptions such as the proton (see below) and water, we adopt the same standard concentration of 1 M for every species. Thus we have, for the aldolase equilibrium,

$$K = \frac{K_c}{1 \text{ M}}$$

or, from the value we calculated for  $K$ ,

$$K_c = 6.7 \times 10^{-5} \text{ M}$$

The appearance of units in this equation provides the key to understanding why aldolase needs to be treated differently from the first three glycolytic enzymes: it is certainly true that a mixture of the three reactants in their standard concentration of 1 M will tend to react in the reverse direction, i.e. from triose to hexose. But why should a biochemist care what happens at 1 M? Much more interesting is what happens at a physiologically realistic concentration, such as 50  $\mu\text{M}$ . If we put both  $[\text{G3P}]$  and  $[\text{DHAP}]$  to this value (ignoring for the moment the complication that in the presence of triose phosphate isomerase they will equilibrate to unequal concentrations) we can readily calculate the concentration of fructose 1,6-bisphosphate at equilibrium with them as

$$[\text{FBP}] = \frac{5 \times 10^{-5} \times 5 \times 10^{-5}}{6.7 \times 10^{-5}} = 3.7 \times 10^{-5} \text{ M}$$

or 37  $\mu\text{M}$ , a little smaller than the concentrations of the two triose phosphates, despite the large positive value of  $\Delta G^0$ .

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As this example illustrates, the naive use of  $\Delta G^{0'}$  referred to above to predict which direction of reaction is favoured is worse than naive; it is wrong, and can give wrong results in virtually any circumstances, because it is rare in practice that the question one is asking is the one that the sign of  $\Delta G^{0'}$  answers, i.e. in which direction will the reaction proceed under standard conditions? To determine the direction in which any particular (non-standard) mixture of reactants will be able to react the proper quantity to consider is not  $\Delta G^{0'}$  but  $\Delta G$ , i.e. not the standard Gibbs energy but the Gibbs energy, which is defined as

$$\Delta G = \Delta G^{0'} + RT \ln \left( \frac{\frac{[\text{G3P}]}{[\text{G3P}]^0} \frac{[\text{DHAP}]}{[\text{DHAP}]^0}}{\frac{[\text{FBP}]}{[\text{FBP}]^0}} \right)$$

(As in the definition of  $K$  above, we obtain a dimensionless number before taking logarithms by dividing each concentration by the corresponding standard. In practice we usually omit the standard concentrations from such expressions, which is not strictly correct but acceptable provided we remember that it is these ratios that we mean when we write the concentrations. No problems arise if all concentrations are measured in M, but if other units are used the concentrations must always be converted to M before calculating  $\Delta G$ .) The numerical value of  $\Delta G$  directly specifies how far the system is from equilibrium, and its sign specifies the direction unambiguously: if  $\Delta G$  is negative the reaction must proceed forwards to reach equilibrium; if it is positive it must proceed backwards; if it is zero the system is at equilibrium. If we put all three of the concentrations in the aldolase equilibrium to 50  $\mu\text{M}$ , we have

$$\Delta G = 23.8 + 5.71 \log (5 \times 10^{-5}) = -0.72 \text{ kJ/mol}$$

This small negative value shows that such a mixture is close to equilibrium but that the reaction needs to proceed forwards to a small degree to reach equilibrium, a result in good agreement with our calculation above that the equilibrium concentration of fructose 1,6-bisphosphate is 37  $\mu\text{M}$  when the two triose phosphates are present at concentrations of 50  $\mu\text{M}$ .

This calculation, then, allows us to understand why fermentation can proceed under normal conditions, and it also indicates that the

forward reaction is only weakly favoured, so that the enzymes that remove the products must be capable of high activity, because only if the products are maintained at very low concentrations can the forward reaction continue. This explains, incidentally, why triose phosphate isomerase has a very high specific activity in numerous tissues.

Nonetheless, even if this analysis explains why the aldolase reaction can proceed forwards in normal conditions of fermentation, it does not immediately explain why it is fructose 1,6-bisphosphate that accumulates when the supply of inorganic phosphate is cut off (rather than, for example, dihydroxyacetone phosphate or glyceraldehyde 3-phosphate). However, when the reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase is blocked, the pool of *all three* aldolase reactants must increase, so that 50  $\mu\text{M}$  ceases to be a realistic concentration to consider. Even at 50  $\mu\text{M}$   $\Delta G$  is only just negative, and as the concentrations increase, it must become positive and then increase steadily, and the more the concentration increases the more the reverse reaction is favoured. Thus it is the accumulation of fructose 1,6-bisphosphate that is noticeable, not that of the two triose phosphates, though they certainly accumulate as well.

Actually the situation in the cell is a little more complicated than I have indicated, because dihydroxyacetone phosphate and glyceraldehyde 3-phosphate are not at equal concentrations at equilibrium, as they are interconvertible by a reaction catalysed by triose phosphate dehydrogenase, which has  $\Delta G^{0'} = 7.5 \text{ kJ/mol}$ . This corresponds to the ratio  $[\text{G3P}]/[\text{DHAP}] = 0.048$  at equilibrium and so if we consider a concentration of 50  $\mu\text{M}$  for glyceraldehyde 3-phosphate we must assume a concentration of 1.04 mM for dihydroxyacetone phosphate before calculating the equilibrium concentration of fructose 1,6-bisphosphate as described above. When the concentration of glyceraldehyde 3-phosphate is 20  $\mu\text{M}$  (a realistic value under ordinary conditions *in vivo*), the equilibrium concentration of fructose 1,6-bisphosphate is 0.12 mM, and although this is greater than the concentration of glyceraldehyde 3-phosphate it is still not the predominant species. But if the concentrations of the triose phosphates are increased 10-fold there is a 100-fold increase in the concentration of fructose 1,6-bisphosphate and it becomes the predominant component of the equilibrium mixture. This is effectively what happens in the fermentation by yeast extract when the supply of inorganic phosphate is cut off and the reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase is blocked.

We have seen from this discussion that the quantity to be

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examined is not  $\Delta G^{0'}$  but  $\Delta G$  if we want to know which direction of reaction will be possible under any set of conditions. Why, then, do we seem to get the right answer if we look at  $\Delta G^{0'}$  for the first three reactions of glycolysis? This is because we have, by implication, assumed that we are dealing with equal (though not necessarily standard) concentrations of reactants and products, e.g. for hexose phosphate isomerase:

$$\Delta G = \Delta G^{0'} + 5.7 \log \left( \frac{[\text{F3P}]}{[\text{G6P}]} \right) = \Delta G^{0'} = +1.7 \text{ kJ/mol if } [\text{F3P}] = [\text{G6P}]$$

This value, though small, is positive, which means that if the two concentrations really were equal in the cell fermentation could not proceed. Thus under cellular conditions  $\Delta G$  cannot be exactly equal to  $\Delta G^{0'}$ . In fact, under glycolytic conditions in the human erythrocyte Minakami and Yoshikawa (1965) found  $[\text{G6P}] = 83 \mu\text{M}$  and  $[\text{F6P}] = 14 \mu\text{M}$ , so

$$\Delta G = \Delta G^{0'} + 5.7 \log \left( \frac{14}{83} \right) = 1.7 - 4.4 = -2.7 \text{ kJ/mol}$$

Although this correction is not large it is crucial because it shows that under glycolytic conditions  $\Delta G$  is negative, as it must be if glycolysis is to be possible.

Most of the other glycolytic reactions are free from the sort of complication that had to be considered for aldolase, because they have equal numbers of molecules participating in the forward and reverse directions. Glyceraldehyde 3-phosphate dehydrogenase does need to be considered, however: if we include  $\text{H}^+$  as a product we do have equal numbers of reactant and product molecules, but if we omit  $\text{H}^+$  from consideration we have an excess of reactants over products. In principle, we could treat  $\text{H}^+$  just like any other reactant, and that is what chemists typically do. But a standard state of 1 M for  $\text{H}^+$  (corresponding to pH 0) is extremely inconvenient for most biochemical purposes, and biochemists customarily use a standard concentration of 0.1  $\mu\text{M}$  for  $\text{H}^+$  (corresponding to pH 7); they write  $\Delta G^{0'}$  (rather than  $\Delta G^0$ ) to indicate this, and they work with buffered solutions and consequently do not have to worry about changes in the  $\text{H}^+$  concentration as a reaction proceeds. If this convention is followed consistently much of the confusion that can result from applying values defined at pH 0 to cellular conditions can easily be avoided, but unfortunately the practice in the biochemical literature has not always been as

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consistent as one might wish (Alberty and Cornish-Bowden, 1993).

Applying the convention, we can ignore  $H^+$  as a reactant and treat the reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase as one in which there are three reactants but only two products. The practical equilibrium constant is consequently a reciprocal concentration,

$$K_c = 7.9 \times 10^{-2} \text{ M}^{-1}$$

not a pure number,

$$K = 7.9 \times 10^{-2}$$

The effect of dilution thus works in the opposite direction for this reaction from the way it works for the aldolase reaction: the aldolase equilibrium is by no means as unfavourable to the forward reaction as it appears at first sight; the glyceraldehyde 3-phosphate dehydrogenase equilibrium is much *more* unfavourable to the forward direction than it appears at first sight. How, then, is it able to proceed when cells undergo glycolysis or fermentation? The explanation is partly that the ratio  $[NAD_{ox}]/[NADH_{red}]$  is typically maintained at a value much greater than unity, and partly that the reaction is followed in glycolysis by a reaction with  $\Delta G^{0'} = -18.8 \text{ kJ/mol}$ , a large negative value that ensures that the concentration of 1,3-bisphosphoglycerate is very low.

If we put  $[NAD_{ox}]/[NADH_{red}] = 240$ ,  $[G3P] = 19 \mu\text{M}$ ,  $[BPG] = 0.6 \mu\text{M}$  and  $[P_i] = 1 \mu\text{M}$  (as given by Minakami and Yoshikawa, 1965), we obtain

$$\Delta G = 6.3 + 5.7 \log \left( \frac{0.6 \times 10^{-6}}{240 \times 0.001 \times 19 \times 10^{-6}} \right) = +1.3 \text{ kJ/mol}$$

As this value is positive it cannot be quite right, but it is close enough to zero for us to believe that the discrepancy can be accounted for by errors in measuring the concentrations of the various metabolites in the cell.

It remains to consider the effect of arsenate discovered by Harden and Young. As I have suggested, this is a consequence of the ability of glyceraldehyde 3-phosphate dehydrogenase to accept arsenate and the extreme lability of the product, 1-arseno-3-phosphoglycerate. This presumed species is hydrolysed to 3-phosphoglycerate and arsenate as soon as it is formed. The former continues through the later stages of

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fermentation whereas the arsenate is immediately available for a further cycle of the glyceraldehyde 3-phosphate dehydrogenase reaction. Thus even though fermentation requires reagent quantities of inorganic phosphate, it can proceed in the presence of catalytic amounts of arsenate.

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