

GLYCOLYSIS IN CELL-FREE SYSTEMS

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Eduard Buchner's discovery of cell-free metabolism 100 years ago was a landmark in biochemistry; it can be said to be the start of detailed metabolic studies. The fact that a soluble fraction of yeast, which he called "zymase", was able to produce ethanol from glucose was revolutionary, and finally put paid to the idea that living cells were essential for fermentation (Buchner, 1897). Later, this protein-containing extract was recognized as being a complex mixture containing enzymes responsible for the process we now call glycolysis, and over the next half-century all the metabolites and enzymes of glycolysis were described. This classic biochemistry of using a cell-free extract to catalyse a biological process has formed the basis of experiments that have occupied many of my research efforts over the past 30 years.

One feature of Buchner's experiments was that cell-free systems never produced anything like the conversion of sugars to alcohol that whole yeast cells can achieve. Even his very concentrated extract, equivalent to a thick slurry of yeast cells, produced carbon dioxide and alcohol at a rather slow rate, which we can retrospectively estimate as less than 1% of the potential enzyme activity. Typically in the production of wine, the starting liquor contains about 20% by weight of sugars, which are converted completely to ethanol, CO₂, yeast biomass and the all-important minor flavour components. But with the early experiments with yeast extracts, only a small amount of the added sugar could be converted to ethanol before the process stopped after a day or two. One of the principal reasons is that the control of glucose entry to the glycolytic pathway is, at least in part, provided by the yeast cell membrane, which of course is lost in making the cell-free extracts. We can now appreciate that the restrained metabolism was due mainly to uncontrolled phosphorylation of glucose, and the

misbalance of metabolites that this caused. Although they were not able to state the same in as many words, Harden and Young in 1906 effectively recognized the same thing when they discovered the accumulation of hexose phosphates in fermenting yeast extracts (Harden and Young, 1908).

My own investigations into cell-free glycolysis commenced not with yeast, but with muscle extracts. In fact our first experiments were with minced muscle preparations which contained all the cellular components, but in which the cells were disrupted so as to release the cytoplasmic enzymes, enabling addition of various compounds to study their effects (Newbold and Scopes, 1971ab). This work was done as part of an applied study for the meat industry: what happens when "muscle" becomes "meat", in the 24 hours *post mortem* in an abattoir? Later this system was simplified, first using only the soluble components, and then to a purified glycolytic system isolated from interfering reactions (Scopes, 1973ab).

When muscle becomes meat, the intracellular glycogen is broken down and metabolized by the glycolytic pathway. As the dead muscle is anaerobic, the end product pyruvate is diverted to lactic acid, and the pH of the meat falls from 7.2 to about 5.5; typically about 60 mM lactic acid is produced, and most of the glycogen is used up. Many workers associated with the meat industry were interested in this *post mortem* glycolysis process because on occasions something goes wrong; the pH falls too rapidly, or perhaps not at all, with detrimental effects on the meat quality. In the 1960s, much effort was spent looking at individual enzymes, especially lactate dehydrogenase (the isoenzymes of which had recently been discovered), which was naively assumed to be an important enzyme in the acidification. In fact, the lactate dehydrogenase reaction actually consumes acid; proton release as a result of anaerobic glycolysis is much more complex than the final step of lactic acid production. I remember how difficult it was to convince people of the idea that the rate of glycolysis is controlled, not by lactate dehydrogenase activity, nor even by phosphofructokinase or phosphorylase, but ultimately by the net ATPase activity in the cell. Many learned papers were published at this time describing the control of glycolysis (e.g. at phosphofructokinase) without one mention of the obvious fact that glycolysis makes ATP, and that without a simultaneous breakdown of ATP it would soon stop. Eventually meat researchers (and investigators at a more fundamental level) moved on to studying the ATPase systems which were principally at the root of their problems.

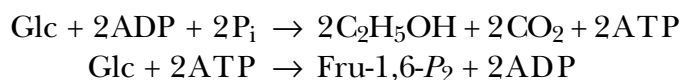
I was fortunate in having a relatively free hand to study muscle

glycolysis in detail when I was at the Agricultural Research Institute's Meat Research Laboratories just outside Bristol, U.K., around 1970. By purifying all of the necessary enzymes, and reconstituting the complete glycolytic system, it was possible to demonstrate some of these factors, especially the ATPase control (Scopes, 1973b). A handy ATPase was the "apyrase" from potatoes (Traverso-Cori *et al.*, 1965), which we purified to a specific activity of over $1000 \mu\text{mol min}^{-1} \text{mg}^{-1}$ — this would be a fatal enzyme to express in a recombinant form! Why potatoes have it is still a mystery. Adding no ATPase, glycolysis was able to phosphorylate the creatine we added (creatine/phosphocreatine being the high energy phosphate buffer essential in muscle tissue) to about 90% phosphocreatine, then all activity stopped. There was plenty of glycolytic potential, but no ADP to set it going. One puzzle was that even when the phosphorylase that we used was partly in the a form, and excess phosphate was present, added glycogen was not further degraded, and there was almost no accumulation of intermediates such as glucose 6-phosphate. This we later determined to be due to a property of muscle phosphorylase a that has been little recognized, namely that it is inhibited by phosphocreatine only at high enzyme concentration, such as occurs *in vivo* (Eagle and Scopes, 1981).

A direct demonstration that the amount and/or state of glycolytic enzyme activity is not important in determining the rate of glycolysis was provided in an experiment in which we had a constant buffer and a fixed amount of ATPase, but varied the glycolytic enzyme content (Scopes, 1973b). The rate of pH fall parallels the rate of glycolysis. Using glycolytic enzyme concentrations varying as much as 100-fold (though all in the same proportions), it was shown that the rate of pH fall was identical in each case, and was exactly the rate expected from the amount of ATPase added. Of course, the rate was slow compared with the potential at the highest enzyme concentration, but it demonstrated clearly the absolute rate control by ATPase activity. This control is mediated by the levels of adenine nucleotides, which in turn activate or inhibit phosphorylase b and phosphofructokinase so that there is little accumulation of glycolytic intermediates.

The experiments with muscle were convenient in that the simulated metabolism being observed was entirely intracellular: the substrate was glycogen and the product lactate. When we moved on to the much-studied yeast system, there were more complications. This is because the substrate was glucose, and this is normally controlled in its entry into the yeast cell. Adding glucose to a reconstituted yeast glycolytic system resulted in uncontrolled phosphorylation of glu-

cose, and an accumulation of hexose phosphates (Welch and Scopes, 1985). This effectively repeated the experiments of Harden and Young nearly 80 years earlier, which had enabled them to propose hexose bisphosphate as a glycolytic intermediate (Harden and Young, 1908), although it was 20 more years before this compound was fully identified as fructose 1,6-bisphosphate (Harden, 1932). What was happening can be simply explained. Hexokinase is present at quite high levels in a yeast extract, and it has no significant allosteric controls. So added glucose is quickly phosphorylated, and with rapidly accumulating glucose/fructose 6-phosphate levels, inhibition of phosphofructokinase is also relieved, allowing fructose bisphosphate to build up. This also pushes the complete glycolysis through, with the ADP formed at the two hexose kinases being resynthesized by phosphoglycerate kinase and pyruvate kinase. But the resynthesis makes four ATP molecules, compared with the two that have been used by the hexose kinases. In the absence of any ATPase activity (in the experiments of Buchner and of Harden and Young most of the ATPase had been removed with the particulate material of the cell), this newly formed ATP is used in still further phosphorylation of glucose. This results in a net accumulation of phosphorylated intermediates. These are mainly hexose phosphates, and the phosphate esters are formed at the expense of inorganic phosphate, which declines rapidly. When the inorganic phosphate approaches zero, no more activity at glyceraldehyde phosphate dehydrogenase can occur, so no more ATP can be made. A final loss of ATP then occurs, and metabolism comes to a virtual stop because there is neither inorganic phosphate nor any ATP left. Harden and Young were able to restart glycolysis by adding excess phosphate (Harden and Young, 1906), all of which became incorporated in hexose phosphates. The net reaction results in one molecule each of carbon dioxide and ethanol produced for every phosphate ion taken up, a stoichiometry that they had recognized 80 years before our studies:



[In these equations the species shown as fructose 1,6-bisphosphate includes the amounts of triose phosphates in equilibrium with it in the reactions catalysed by the aldolase and triose phosphate isomerase reactions, as discussed by Cornish-Bowden elsewhere (pp. 135–148) in

this volume.]

Harden and Young also discovered that replacing phosphate with arsenate, rather than poisoning the system as might have been expected, actually stimulated glycolysis (Harden and Young, 1911). They correctly concluded that the arsenate was taking the place of phosphate, and that an unstable arseno-ester was breaking down, the net effect being the maintenance of sufficient inorganic phosphate required for the overall fermentation process. We now interpret the effect of arsenate as a pseudo-ATPase activity which not only bypasses the sequestration of inorganic phosphate, but also eliminates any net synthesis of ATP. This is due to the rapid spontaneous breakdown of 1-arseno-3-phosphoglycerate formed by glyceraldehyde phosphate dehydrogenase, which we have estimated to occur with a half-life of no more than a few milliseconds (Welch and Scopes, 1985).

In order to establish a steady long-term cell-free yeast glycolysis, it was clear that we must either balance the ATPase activity accurately with a controlled glucose input or find an alternative way of breaking the ATP down. The arsenate experiments (Harden and Young, 1911) were the clue. Using arsenate, it was not necessary to add any actual ATPase enzyme. It was possible to get a prolonged fermentation, with a complete conversion of glucose to ethanol. We managed to achieve a conversion of 1 M glucose to 2 M ethanol plus CO₂ in 7 hours (Welch and Scopes, 1985). The fermentation was not “natural”, in that it ran at a very low ATP concentration due to the efficiency with which the system composed of glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase system breaks down ATP in the presence of arsenate. But the steady-state level of ATP of little more than 0.1 mM was sufficient to keep the glycolysis ticking over. (This is probably the sort of concentration of ATP that existed in Buchner’s experiments). A similar experiment using an extract of the ethanologenic bacterium *Zymomonas mobilis* resulted in the complete conversion of 2 M glucose to 3.6 M ethanol, a final ethanol concentration of nearly 20% by volume, higher than any natural fermentation can achieve (Scopes and Griffiths-Smith, 1986).

The glycolytic pathway, specifically the Embden–Meyerhof pathway, has been central to many biochemical studies. The first metabolic pathway to be studied, it was fully elucidated in terms of the actual reactions being catalysed by the 1940s, but since then each individual enzyme has separately been studied in great detail, and the control systems investigated *ad infinitum*. It is possible to simulate the process in a computer, but a satisfactory simulation of glycolysis

in silico has not yet been carried out, because the exact parameters for each enzyme are not really known in sufficient detail under physiological conditions. It is always possible to adjust the parameters so as to get the right answers, but this is hardly convincing. One problem is that conventional Michaelis–Menten kinetics have to be modified to take into account the fact that in many cases the enzyme concentration is of the same order as that of its substrates, or even higher. The initial assumption in deriving the Michaelis–Menten equation, that substrate concentration is far greater than enzyme concentration, is not valid. Many of the metabolites in glycolysing cells at any given instant are mostly bound to enzymes rather than free in the cytoplasm. A second problem is that the concentration of enzymes *in vivo* may be as much as 1000-fold higher than in *in vitro* kinetic studies, and extrapolation of *in vitro* kinetics over three orders of magnitude is not always valid, as we have shown for phosphorylase and the effects of phosphocreatine (Eagle and Scopes, 1981). Finally, experiments reported in the literature to determine kinetic parameters are rarely carried out at physiological pH, ionic strength, or even temperature, so each value must be considered with caution.

Our reconstituted systems, which were operated in as close as possible to physiological conditions, with 40 mg/ml total glycolytic enzyme protein, showed that for muscle glycolysis, concentrations of intermediates were always very small (as found in direct measurements of living tissue). This reflected the tight controls on phosphorylase and phosphofructokinase activities by the adenine nucleotide system, which itself is tightly controlled by the high-energy phosphate buffering system constituted by adenylate kinase and creatine kinase. But the yeast system was very unstable, due to a lack of a high-energy phosphate buffering system comparable to that in muscle, which made it difficult to balance ATP breakdown with glucose phosphorylation. As a consequence there were wide fluctuations in concentrations of various intermediates and of the adenine nucleotide levels, which occasionally oscillated in a regular fashion.

Oscillations of metabolites in yeast have been recognized as possibly a natural phenomenon, which can be much increased *in vitro* (Hess and Boiteux, 1971). They are a natural consequence of feed-back and feed-forward allosteric controls on the key enzymes phosphofructokinase and pyruvate kinase by metabolites such as fructose 1,6-bisphosphate, and by AMP, ADP and ATP concentrations. Most of this work was done before the discovery of fructose 2,6-bisphosphate, an important additional controlling metabolite; it has since been shown that inclusion of this effector amplifies the oscilla-

tory effect (Yuan *et al.*, 1990). The studies generally used trehalose as substrate, a natural storage sugar in yeast which slowly hydrolyses to supply the glucose for glycolysis.

One of the most beneficial aspects of using a cell-free metabolic system is that one can add, or subtract an enzyme at will, and adjust conditions such as pH, ionic strength, etc., things that are either difficult or impossible to do with living cells. Many researchers are currently adding (or subtracting) enzymes by molecular biological methods, a process coming under the general title of metabolic engineering. By using a cell-free system it is possible to demonstrate whether or not the planned metabolic alteration will indeed do what it is intended. For example, we have recently shown that addition of the *Zymomonas mobilis* enzymes alcohol dehydrogenase and pyruvate decarboxylase to an extract of a thermophilic organism was able to direct the fermentation towards ethanol even at 60° (the limit of the foreign enzymes' stability). This was done before a suitable transformation system had been developed, and demonstrated that it is worth continuing with the attempt to incorporate the genes encoding these enzymes in this thermophile, as a potential ethanol-producer.

There have been many studies of metabolic pathways other than glycolysis using cell-free extracts of relevant tissues. These sorts of experiments are links between on the one hand whole organism metabolism, and on the other, studies on isolated enzymes. These links enable a fuller understanding of the overall processes than either of the other studies can provide themselves. Thus although the original experiments of Buchner have led in many directions to provide in-depth understanding of many complex biological problems, there is still useful work to be done with experiments very much in the same category as those he carried out 100 years ago.

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