BEHAVIOUR OF ENZYMES AT HIGH CONCENTRATION. USE OF PERMEABILISED CELLS IN THE STUDY OF ENZYME ACTIVITY AND ITS REGULATION

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1. Introduction

The multiplicity of regulatory phenomena revealed in recent years in the course of *in vitro* studies of enzyme behaviour has given rise to some concern over the validity of extrapolation to the *in vivo* situation. In particular, Srere [1] has emphasised that the intracellular concentrations of enzymes may be several orders of magnitude higher than those normally employed in enzyme assays and both he and Frieden and Colman [2] have drawn attention to the fact that these differences in concentration may give rise to different apparent behaviour. The implications of high enzyme concentration for metabolic regulation have been further explored by Sols and Marco [3].

The extreme sensitivity of most assay procedures makes it impossible to measure reaction rates at high enzyme concentration unless concentrations of substrates well below K_m values are used. For kinetic studies at high enzyme concentration it has therefore been necessary to apply the stopped flow technique in order to measure reaction rates over a very short time period.

In this communication I wish to suggest that the use of 'permeabilised' cells may offer a very much simpler means of overcoming the problem of high enzyme concentration and may even go some way beyond this in simulating the *in vivo* condition for studies of the behaviour and regulation of enzymes. Some experiments are reported on the kinetic behaviour of D-lactate dehydrogenase (EC 1.1.1.28) from *Klebsiella* (*Aerobacter*) *aerogenes* and on the regulatory sensitivity of citrate synthase (EC 4.1.3.7) from *Pseudomonas aeruginosa*.

2. Experimental

K. aerogenes (N.C.T.C. 10006) was grown in a medium containing essential salts and 50 mM glucose under essentially anaerobic conditions in a full stoppered 250 ml bottle at 37°. The cells were harvested when the A_{680} was approx. 0.6, washed and resuspended in 5 ml of 0.1 M Tris-HCl, pH 8. *Ps. aeruginosa* (N.C.I.B. 8295) was grown aerobically in 200 ml of nutrient broth at 37°. The cells were harvested when the A_{680} was approx. 1.2, washed and resuspended as above.

Cell-free extracts were prepared by sonication with an M.S.E. 100 W disintegrator operated at full power for 2 min, after which the preparations were centrifuged at 25,000 *g* for 10 min and the supernatant solutions decanted and used without further treatment.

Toluene treatment of bacterial cells was performed in the following way. One ml of resuspended cells was warmed to 37°, treated with 0.05 ml of toluene, vigorously mixed and left at 37° for 10 min. The cells were then collected by centrifugation, washed with 2 ml of buffer, recentrifuged and finally suspended in 2 ml of 0.1 M Tris-HCl, pH 8, and kept in ice-water. Enzyme activities in cells so treated were shown to be completely retained within the cells. The toluene-treatment procedure introduced a two-fold dilution

of the cells compared with the suspension subjected to sonication. When this was allowed for, the levels of lactate dehydrogenase and citrate synthase per unit volume of permeabilised cell suspension and sonic extract were found to be essentially similar.

Citrate synthase was assayed polarographically at 25° by following the rate of formation of coenzyme A with a dropping mercury electrode at −0.2 V relative to a saturated calomel anode [4]. Reaction mixtures contained 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 0.2 mM oxaloacetate, 0.16 mM acetyl coenzyme A and 5 μl of toluenised cells or 2 μl of sonic extract in a total volume of 1.0 ml. Lactate dehydrogenase was assayed spectrophotometrically at 25° by following the rate of oxidation of NADH at 340 nm. Reaction mixtures contained 0.1 M Tris-HCl, pH 8.0, 0.1 mM NADH, various concentrations of pyruvate and 20 μl of toluenised cells or 10 μl of sonic extract in a total volume of 1.0 ml. Some assays for lactate dehydrogenase were also carried out polarographically [5] by measuring the rate of formation of NAD with a dropping mercury electrode at −1.0 V. The results thereby obtained confirmed those given by the spectrophotometric assay.

3. Results and discussion

The lactate dehydrogenase of *K. aerogenes* was first chosen for examination because of the report by Sawula and Suzuki [6]. These workers employed stopped flow spectrophotometry to assay the purified enzyme at low and high concentration. They observed a sigmoid dependence on pyruvate at low enzyme concentration but a hyperbolic dependence at higher enzyme concentration and concluded that normal Michaelis-Menten kinetics are exhibited at the intracellular concentration. As discussed below, the use of permeabilised cells permits the examination of enzymes precisely at their intracellular concentrations. Fig. 1 shows the dependences of lactate dehydrogenase rate on pyruvate concentration for both toluenised cells and sonic extract, and these are seen to be essentially similar. It thus appears that the sigmoid dependence exhibited at low concentrations of enzyme is retained at the relatively high enzyme concentration present intracellularly. The discrepancy between these results

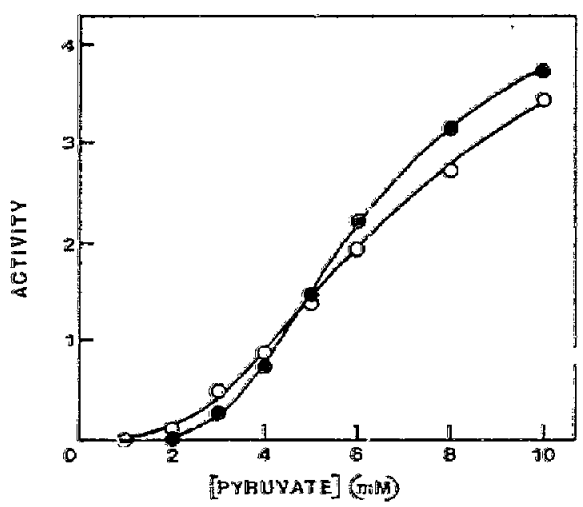

Fig. 1. Dependence of *K. aerogenes* lactate dehydrogenase activity on pyruvate concentration. (o—o—o) Sonic extract; (•—•—•), toluenised cells. Activities are in arbitrary units per 1 ml of initial cell suspension (prior to permeabilisation or sonication).

and those of Sawula and Suzuki [6] suggests that consideration of enzyme concentration alone is insufficient to simulate the *in vivo* behaviour. It appears that the toluenised cell may preserve other factors contributing to the behaviour of the enzyme which are lost on extraction. Experiments performed at high concentrations of purified enzymes may thus not necessarily reflect the *in vivo* properties.

In previous studies we have shown that NADH is an inhibitor of citrate synthase from Gram-negative bacteria [7, 8]. In view of the instances of observed differences between regulatory behaviour at low and high enzyme concentration cited below it was desirable to examine the effect of NADH on citrate synthase in toluenised cells. To do this, advantage has been taken of the absence of NAD-linked malate dehydrogenase in *Ps. aeruginosa*. Most cells contain high levels of this enzyme which, in the presence of NADH, acts to remove oxaloacetate and can thereby interfere with the examination of the effect of NADH on citrate synthase.

Fig. 2 shows that the citrate synthase in both toluenised cells and sonic extracts of *Ps. aeruginosa* is inhibited by NADH and that the dependences of inhibition on NADH concentration are very similar. This result suggests that citrate synthase, at *in vivo*

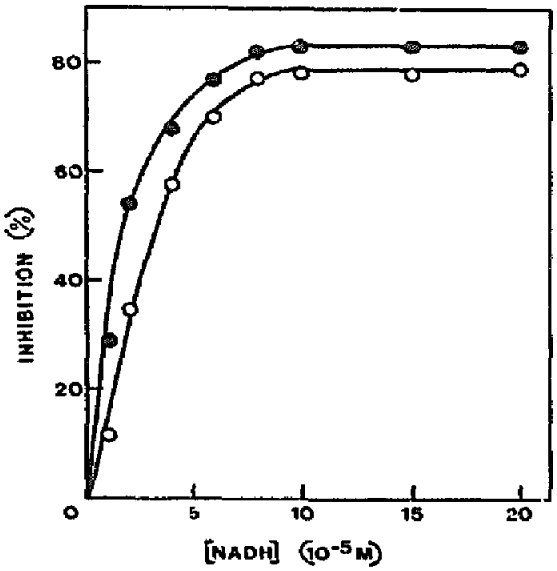

Fig. 2. Inhibition of *Ps aeruginosa* citrate synthase by NADH (o—o—o) Sonic extract, (•—•—•), toluenised cells.

concentration, retains its sensitivity to NADH inhibition and supports the likelihood that such inhibition is physiologically significant both in this organism and in other Gram-negative bacteria.

Citrate synthase from *Escherichia coli* and other Gram-negative facultative anaerobes has been shown to be sensitive to inhibition by α-oxoglutarate [9, 10]. Examination of toluenised cells of these organisms has shown that this inhibition is also retained at high enzyme concentration.

The permeabilisation of bacterial cells, brought about by treatment with toluene, has long been used to measure enzyme activities in cells which are otherwise impermeable to the substrates or products. Perhaps the best-known example is in the assay of β-galactosidase using *o*-nitrophenyl-β-galactoside [11], though the method has been adopted with other enzymes, e.g. acid and alkaline phosphatases [12], arabinose isomerase [13], galactokinase [14] and serine dehydratase [15]. By obviating the necessity for an extraction procedure the permeabilisation technique lends speed and convenience to the measurement of enzyme activities. It has also found application in the investigation of DNA synthesis [16] and in the study of the phosphotransferase system associated with carbohydrate transport in *E. coli* [17, 18] where disruption of cells would lead to destruction of the activity.

Alternative procedures leading to the breakdown of permeability barriers have also been employed. These include the use of other organic solvents [19], EDTA [20–22] and sucrose–EDTA [23].

The first published report of a comparative study of an enzyme in extracts and toluene-treated cells appears to be that by Bridgeland and Jones [15] on the L-serine dehydratase of *Arthrobacter globiformis*. These authors observed a lag in pyruvate formation, a cation requirement and a sigmoid serine saturation curve in cell-free extracts but not in the toluenised cells. Toluenised *E. coli* cells have also been used by Ashworth [24] who demonstrated the regulation of isocitrate lyase by phosphoenolpyruvate in both toluenised cells and cell-free enzyme preparations and the present author has shown that the sigmoid aspartate saturation curve characteristic of *E. coli* aspartate transcarbamylase is also exhibited by toluenised cells. Liersch and Preiss [25] showed that ADP-glucose pyrophosphorylase may be studied with permeabilised *E. coli* and, as the present paper was being completed, Reeves and Sols [26] reported studies of the regulation of phosphofructokinase in toluenised *E. coli* and also recognised the approximation to physiological conditions offered by such cells.

However, despite these few reports, the permeabilisation technique has not been extensively applied to the detailed investigation of the catalytic and regulatory behaviour of enzymes. Yet it offers some distinct advantages over cell-free or purified preparations. The permeabilisation treatment leads to the equilibration and free movement of low molecular weight metabolites into and out of the cells, while the macromolecular constituents are retained intracellularly. Studies on *E. coli* [27] have shown that treatment with toluene leaves the cells intact. As a result, enzymes will be present in permeabilised cells at concentrations essentially similar to those in normal living cells. In addition, the presence of the whole range of protein and other macromolecules, all at their *in vivo* concentrations, is likely to result in the preservation of at least some molecular interactions characteristic of living cells but not present in cell-free extracts. The behaviour of enzymes in permeabilised cells may thus more accurately reflect their normal *in vivo* behaviour insofar as this may be affected by high concentration and by interactions with other macromolecular constituents.

A suspension of permeabilised cells offers enzymes at high concentrations within cells which are themselves dispersed homogeneously throughout the solution. The *overall* concentration of enzyme is therefore very much lower and can be adjusted to any desired level while leaving the *effective* concentration high. Thus, unlike cell-free preparations, permeabilised cells may be used to examine enzyme behaviour at *in vivo* concentration by conventional assay procedures without recourse to more complex stopped flow techniques.

Several cases of differences between the kinetic and regulatory behaviour of enzymes at high and low concentrations have already been reported [2,6,28–31]. Such observations emphasise the desirability of coupling normal enzymic catalytic studies with an examination of the behaviour at elevated enzyme concentration. Any differences observed merit further examination for their metabolic implications. The simple technique of using permeabilised cells should, at least for bacterial systems, permit such studies to be carried out very easily and, as the results presented here show, may reveal enzymic behaviour different from that shown by high concentrations of 'extracted' enzymes.

Finally, the examination of toluenised cells may present problems of turbidity interference with spectrophotometric assays. The polarographic procedures being developed in this laboratory for the assay of a variety of enzymes [4, 5] offer the distinct advantage of being unaffected by even quite dense suspensions. Such procedures may assist the systematic examination of the behaviour of a wide range of enzymes in permeabilised cells.

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