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The Apparent Inhibition of Phosphofructokinase by Reduced Nicotinamide–Adenine Dinucleotide: a Problem of Coupled-Enzyme Assays

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Phosphofructokinase (EC 2.7.1.11) is known to be a regulatory enzyme for glycolysis in many tissues, and its properties are important as they provide a basis for the theory of metabolic control of the pathway in these tissues (for review see Newsholme & Gevers, 1967). The properties of the enzyme have been investigated *in vitro* by methods that involve following the formation of products by using coupled-enzyme assays: either fructose 1,6-diphosphate or ADP formation is followed by coupling to aldolase, triose phosphate isomerase and glycerol phosphate dehydrogenase or to pyruvate kinase and lactate dehydrogenase respectively, and the oxidation of NADH is measured at 340nm. Such assays provide specific and precise means of measuring enzyme activities particularly when they are used with a recording spectrophotometer. However, a major disadvantage of such methods is that in order to measure the total activity of the test enzyme the activities of the coupling enzymes should be about 100-fold higher than that of the test enzyme; if the activities of the coupling enzymes are less than optimum then the measured activity of the test enzyme will be less than the total activity (for full discussion see Bergmeyer, 1965). Consequently an inhibitor of a coupling enzyme could lead to a decrease in the rate of oxidation (or reduction) of the nicotinamide nucleotide, and this could be interpreted as inhibition of the test enzyme. Moreover this will occur despite the fact that the activity of the coupling enzyme is very much higher than that of the test enzyme (Bergmeyer, 1965).

It has been reported that NADH is an inhibitor of phosphofructokinase and that this inhibition might be important in the metabolic control of glycolysis (Weber, Lea, Convery & Stamm, 1967; Brock, 1969). However, no satisfactory evidence was presented to rule out the possibility that this effect of NADH was due to inhibition of the coupling enzyme. Brock (1969) reported that phosphofructokinase was inhibited by NADH in both coupled-enzyme assay techniques (for fructose diphosphate and ADP) and claimed that the inhibition therefore could not be explained by effects on the coupling enzymes. However, both glycerol 1-phosphate dehydrogenase (Black, 1966; see also Table 1) and lactate dehydrogenase (Thiers & Vallee, 1958; see also Table 1) are inhibited by NADH at concentrations similar to those reported to inhibit phosphofructokinase.

Because of the possible importance of this inhibition of phosphofructokinase in understanding the control of glycolysis in muscle it was decided to reinvestigate this NADH effect, and to carry out systematic control experiments to differentiate between inhibition of phosphofructokinase and inhibition of the coupling enzymes.

All chemicals and enzymes, including rabbit muscle phosphofructokinase, were obtained from Boehringer Corp., London W.5, U.K., with the exception of KCl, $MgCl_2$ and EDTA, which were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Phosphofructokinase activity was assayed by a coupled-enzyme technique and by a direct sampling technique. The coupled-enzyme method was based on that described by Mansour (1965) as modified by Opie & Newsholme (1967); the medium contained 50mM-tris–HCl, 6.7mM-$MgCl_2$, 1mM-fructose 6-phosphate, 0.5mM-ATP, 1mM-2-mercaptoethanol, 50mM-KCl, 1mM-AMP and various concentrations of NADH, at pH 8.0. Aldolase (80μg), triose phosphate isomerase (5.5μg) and glycerophosphate dehydrogenase (4.5μg) were added to 2ml of medium in the cuvette, and the reaction was initiated by addition of phosphofructokinase (0.1μg) and followed at 340nm in Gilford model 240 recording spectrophotometer at 25°C. In the sampling assay phosphofructokinase (1μg) was incubated in the same medium as above except that the coupling enzymes were omitted. After incubation for 5min at 25°C (preliminary experiments had established that the assay was linear for at least 10min) the reaction was stopped by addition of $HClO_4$, the solution neutralized with K_2CO_3 and fructose diphosphate measured by the method of Bücher & Hohorst (1965) with the Gilford spectrophotometer. Aldolase was assayed by a coupled-enzyme assay with the same medium as that

Table 1. *Effect of NADH on the activities of lactate dehydrogenase, glycerol 1-phosphate dehydrogenase, aldolase and phosphofructokinase*

Enzyme activities are assayed as described in the text and are presented as μmol of substrate transformed/min per mg of enzyme.

Concn. of NADH (mM)	Lactate dehydrogenase activity	Glycerol 1-phosphate dehydrogenase activity	Phosphofructokinase activity as measured by coupled-enzyme assay	% inhibition of phosphofructokinase by NADH above 0.1 mM	Aldolase activity	% inhibition of aldolase by NADH above 0.1 mM	Phosphofructokinase activity as measured by sampling assay
0	—	—	—	—	—	—	38.2
0.05	—	42	—	—	—	—	—
0.1	150	—	37.9	—	11.2	—	38.6
0.2	186	—	35.2	7	10.6	5	36.2
0.3	—	27	31.0	18	10.2	9	36.0
0.4	—	—	24.9	34	8.6	23	41.2
0.5	103	—	22.5	41	5.7	49	36.0
0.6	58	4.5	16.1	58	3.1	72	35.8
0.7	20	1.6	4.2	89	1.4	88	36.8

for phosphofructokinase except that the latter enzyme was omitted, fructose diphosphate replaced fructose 6-phosphate and the reaction was initiated with 0.3 μg of aldolase; in particular the amounts of triose phosphate isomerase and glycerol phosphate dehydrogenase were the same as in the phosphofructokinase assay. Glycerol 1-phosphate dehydrogenase (0.2 μg) and lactate dehydrogenase (0.25 μg) were similarly assayed by following the oxidation of NADH in solutions containing 20 mM-imidazole and 30 μM-dihydroxyacetone phosphate at pH 7.0 and 100 mM-tris–HCl and 125 μM-pyruvate at pH 7.5 respectively.

The results in Table 1 show that with the coupled-enzyme assay the activity of phosphofructokinase is apparently inhibited by NADH with 50% inhibition at approx. 0.5 mM-NADH. However, the activity of aldolase, which was assayed by exactly the same method as phosphofructokinase, was shown to be inhibited by NADH and 50% inhibition was obtained at approx. 0.5 mM-NADH. This result suggested that NADH does not affect phosphofructokinase but inhibits one of the coupling enzymes. This was confirmed when the activity of phosphofructokinase was measured by a sampling technique. In this case no coupling enzymes were required as the amount of product accumulating after a certain period of incubation (the reaction is stopped with a protein precipitant) is used to assay the enzyme activity. The results of this experiment are also shown in Table 1: increasing the concentration of NADH from 0 to 0.7 mM had no effect on the enzyme activity.

Thus it is concluded that phosphofructokinase is not inhibited by NADH (up to 0.7 mM) and that the effect observed in the coupled-enzyme assay is due to inhibition of one of the coupling enzymes, probably glycerol phosphate dehydrogenase. It is important to note that when a quantity of fructose 1,6-diphosphate (0.05 μmol) was added to the cuvette during the coupled-enzyme assay of phosphofructokinase activity there was an immediate and rapid oxidation of NADH, but there was no detectable difference in the response whether NADH was 0.1 or 0.7 mM. Presumably the activities of the coupling enzymes are so high that when presented with a large concentration of substrate the rate of conversion into the final product (i.e. glycerol phosphate) is so rapid that no inhibition can be detected. Thus the addition of product of the test enzyme to the cuvette and observation on the effect of an inhibitor (or activator) on the response of the coupling enzymes, a control procedure that has been used for many enzymes, is not a satisfactory method for distinguishing between an effect on the test enzyme or an effect on the coupling enzymes.

The results of the present paper suggest two satisfactory control procedures: first, the test

enzyme can be investigated individually by means of a sampling technique that does not involve the use of coupling enzymes; secondly, if a reaction is available that can lead to the formation of the same product at a rate similar to that of the test enzyme this will provide a control system somewhat similar to that of the aldolase experiment in the present paper.

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