SC 2167

Glucose 6-phosphate formation as a measurement of oxidative phosphorylation in liver mitochondria

The measurement of ATP formed by oxidative phosphorylation in mitochondria from animal tissues is often based on the uptake of inorganic phosphate or the measurement of glucose 6-phosphate formed in the presence of hexokinase (EC 2.7.1.1), Mg^{2+} and D-glucose[1]. The purpose of this paper is to point out some difficulties which have arisen when glucose 6-phosphate is used as an index of ATP formed by oxidative phosphorylation in liver mitochondria, and to suggest an alternative method which may overcome these difficulties.

SLATER[2] has pointed out that in heart sarcosomes, the total hexose monophosphate formed must be corrected for that formed in the absence of added substrate by the adenylate kinase (EC 2.7.4.3) reaction, and for that formed by oxidative, cyanide-sensitive phosphorylation from "endogenous" substrate. The magnitude of this correction is variable, but appreciable, and could be reduced, but not abolished, by the addition of 40 mM KF and AMP as inhibitors of the adenylate kinase reaction.

In liver mitochondria 15 mM KF is often added in an attempt to inhibit the adenylate kinase reaction[3]. However, in our hands, up to 60 mM KF did not inhibit glucose 6-phosphate formation in the absence of added substrate. For this reason, P/O ratios will differ according to whether a correction for this formation is applied. A typical experiment with rat-liver mitochondria is shown in Table I.

In the absence of added substrate up to 2.5 μmoles of glucose 6-phosphate per mg of mitochondrial protein could be formed after periods of 5–15 min incubation. Its formation was unaffected by repeated washings of the mitochondria. If phosphate, Mg^{2+}, or D-glucose were omitted, lower, but appreciable, quantities of glucose 6-phosphate were still formed. Cyanide (1 mM), 1 μg of antimycin A, and 2 mM iodoacetamide, iodoacetate, malonate or Amytal did not inhibit the reaction. The reaction mixture lost approx. 20% of its ability to form glucose 6-phosphate after 3 min at 50°, and nearly 90% after 1 min at 90°. Immersion in boiling water destroyed the activity. Pyruvate, dihydroxyacetone phosphate, glyceraldehyde phosphate, fructose phosphate, and fructose 1,6-diphosphate[9] could not be demonstrated in the reaction mixture, nor did the reagents contain measurable quantities of glucose oxidase-positive material.

Maximal formation of glucose 6-phosphate without added substrate occurred in

TABLE I

OXYGEN UPTAKE AND GLUCOSE 6-PHOSPHATE FORMATION IN LIVER MITOCHONDRIA

Mitochondria[4] (2 mg protein/ml of incubation mixture) were incubated in the medium described by HATEFI[5]. Each ml contained 5 μmoles ADP and 10 mg hexokinase (Sigma Type IV). Oxygen was measured manometrically at 25° with the Kirk Erei ultramicrorespirometer[6,7], and glucose 6-phosphate was determined enzymically[8].

	Complete system	*No succinate*	*Net*
Oxygen uptake (μatoms/mg protein/20 min)	2.27	Trace	2.27
Glucose 6-phosphate formed (μmoles/mg protein/20 min)	5.64	5.06	0.58
Glucose 6-phosphate:O	2.48	—	0.26

the presence of 5–10 μmoles of ADP per mg of mitochondrial protein. Higher concentrations of ADP resulted in a lower yield of glucose 6-phosphate, possibly because of inhibition of the hexokinase reaction, and significant amounts of AMP were only recovered at near optimal concentrations of ADP. The stoichiometric sum of glucose 6-phosphate formed (assumed to be equivalent to ATP produced) and AMP recovered was always less than 80 % of twice the amount of ADP used. The addition of 2.5 μmoles of AMP per mg of mitochondrial protein inhibited glucose 6-phosphate formation by about 50 %. It, therefore, appears that with the concentrations of ADP commonly used, the adenylate kinase reaction makes a significant contribution to the total quantity of ATP formed, in addition to lesser contributions by "endogenous" substrates[10,11].

Furthermore, recoveries of added glucose 6-phosphate (1.5 mM) were often as low as 80 % and could be as low as 40 % if succinate was added. The poor recoveries may be due to the presence of glucose-6-phosphatase (EC 3.1.3.9), as pointed out previously[12], and taken into account in the experiments of VESTER AND STADIE[13]. Repeated washings of the mitochondria or the omission of Mg^{2+}, phosphate, or D-glucose did not improve recoveries. Omission of hexokinase, which was found to be free of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity, reduced the loss of glucose 6-phosphate by up to 50 %. Approx. 20 % of the added glucose 6-phosphate was not recovered when 4 mM KF was added to the complete system, from which both mitochondria and substrate had been omitted.

While loss of glucose 6-phosphate by glucose-6-phosphatase or other reactions

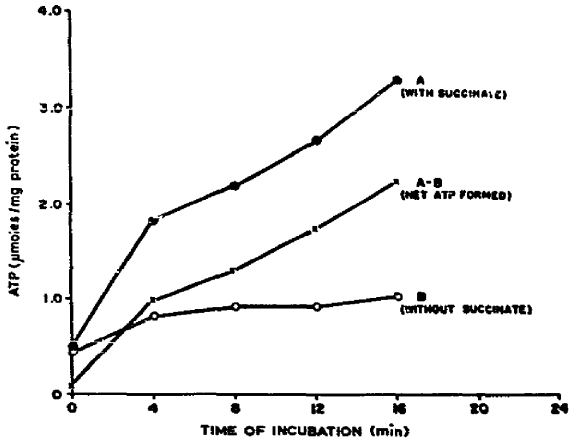

Fig. 1. 1.0 ml of the incubation mixture contained 80 μmoles KCl, 15 μmoles triethanolamine phosphate (pH 7.0), 1 mg mitochondrial protein, 15 μmoles succinate, and 20 μmoles ADP. (Addition of 5 mM Mg^{2+} inhibited the reaction and was, therefore, omitted.) The mixture was incubated at 25° with gentle shaking of the flasks open to the atmosphere. Identical mixtures without added substrate were run simultaneously. At the end of the incubation period the flasks were placed in boiling water for 4 min. (This method of inactivation was found preferable to the use of $HClO_4$, because 0.3 M $HClO_4$ failed to stop the reaction promptly, while 0.6 M $HClO_4$ resulted in some loss of added ATP.) The mixture was diluted to a convenient volume with water and centrifuged. ATP was measured in the supernatant with phosphoglycerate and phosphoglycerate kinase, measuring the diphosphoglycerate formed with glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) and DPNH. (Reagents from California Corporation.) The difference between ATP formed in the presence and absence of substrate was taken as the amount of ATP formed by oxidative phosphorylation. Recoveries of added ATP averaged 98 %.

may be different in an experiment in which oxidative phosphorylation is measured as distinct from a recovery experiment, the unpredictable extent of this loss, nevertheless, adds additional uncertainty to the determination of ATP by the glucose-hexokinase trap procedure, as compared, for example, to the polarographic method of CHANCE AND WILLIAMS[14] or the assay of ATP with phosphoglycerate kinase (EC 2.7.2.3). A typical experiment employing this assay is shown in Fig. 1.

In five consecutive experiments with succinate as substrate, ATP measured by this method was comparable with the rate of ADP phosphorylated as measured polarographically (Table II).

TABLE II

COMPARISON OF TECHNIQUES

Oxygen electrode (ADP used)	*ATP assay (ATP formed)*
(μmoles/mg protein/20 min)	
2.20	2.27
1.26	1.68
2.60	2.22
4.52	3.57
1.85	1.92
Mean: 2.49	2.33

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