A problem in the radiochemical assay of glucose-6-phosphatase in muscle

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A radiochemical assay for glucose-6-phosphatase, which depends on the absorption of radiolabelled substrate on to a nascent precipitate of BaSO_4 and $\text{Zn}(\text{OH})_2$, can give rise to erroneously high activities. Adsorption on to a $\text{Fe}(\text{OH})_3$ precipitate does not expose the incubation medium to extreme alkaline conditions and provides a satisfactory and simple assay. This method demonstrates a very low activity of the enzyme in mammalian muscle, in contrast with previous findings.

Glucose-6-phosphatase (EC 3.1.3.9) hydrolyses glucose 6-phosphate to form glucose and phosphate and is known to be involved in the formation of glucose by the gluconeogenic tissues liver and kidney (for review see Nordlie, 1971). Low activities of the enzyme have been reported in other tissues, but such activities are difficult to measure in crude extracts: sampling assays for glucose or phosphate production suffer from the problems of glucose formation from polysaccharides and oligosaccharides, or phosphate formation from the many organic phosphates present in the crude extract. Although such 'non-specific' activities can be measured in 'control' incubations, large inaccuracies result if the activity of the specific phosphatase is low. This is a problem for measuring low activities, not only of glucose-6-phosphatase, but also of other phosphatases such as glycerol-3-phosphatase (EC 3.1.3.21), which is known to be present in yeast (Tsuboi & Hudson, 1956). The finding that glycerol accumulates in over-wintering insects (see Storey & Storey, 1983) has led to the suggestion that glycerol-3-phosphatase may be present in these higher animals.

Kitcher *et al.* (1978) described a radiochemical assay for glucose-6-phosphatase which overcame many of the problems raised above. Tissue extracts are incubated with radiolabelled glucose 6-phosphate and the reaction is terminated by addition of $\text{Ba}(\text{OH})_2$ followed by an equimolar solution of ZnSO_4 . This not only precipitates protein, but the nascent precipitate of BaSO_4 and $\text{Zn}(\text{OH})_2$ adsorbs organic phosphates, including the radiolabelled glucose 6-phosphate. After centrifugation,

the radioactivity in the supernatant is used to indicate the rate of product formation. The method can be used for phosphatase enzymes in general, provided that the product contains no phosphate groups. This assay was used to carry out a comparative study of muscle glucose-6-phosphatase, and activities from 0.1 to $8.0 \mu\text{mol/min per g}$ were reported (Surholt & Newsholme, 1981). However, during a similar study on glycerol-3-phosphatase activities, problems with the radiochemical assay for glycerol-3-phosphatase in crude extracts of muscle tissue were discovered. Problems with the radiochemical assays for both enzymes, together with their solution, are described in the present paper.

Materials and methods

Chemicals

Radiochemicals were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Dowex-2 (chloride form; 8% cross-linked; 200–400 mesh) was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) and converted into the formate form by the method of Hammerstedt (1980). 2,5-Diphenyloxazole, 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and Triton X-100 were obtained from Fisons Scientific Apparatus (Loughborough, Leics., U.K.). All other biochemicals were obtained from Boehringer Corp. (Lewes, Sussex, U.K.). Inorganic chemicals of an analytical grade were obtained from BDH Chemicals (Poole, Dorset, U.K.).

Animals

Male rats of the Wistar albino strain were obtained from Bantin and Kingman (Hull, U.K.). Other animals were obtained from sources given

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previously (Alp *et al.*, 1976; Surholt & Newsholme, 1981).

Assay of enzyme activities

Glucose-6-phosphatase was assayed as described by Surholt & Newsholme (1981), except that three different methods for terminating the reaction were employed. (i) The reaction was terminated by addition of $\text{Ba}(\text{OH})_2$ and ZnSO_4 as described by Surholt & Newsholme (1981). (ii) The reaction was terminated by addition of a solution of 0.06 M FeCl_3 adjusted to pH 6 with NaOH. This produces a precipitate of $\text{Fe}(\text{OH})_3$, which not only precipitates protein but also adsorbs glucose 6-phosphate in a similar manner to the BaSO_4 and $\text{Zn}(\text{OH})_2$ precipitate; however, it does not subject the assay medium to an extreme alkaline pH. (iii) The reaction was terminated by addition of HClO_4 (25%, w/v) to a final concentration of 4%. The protein precipitate was removed by centrifugation (8500 g for 3 min) and the supernatant neutralized with 2 M-KOH containing 0.5 M-triethanolamine buffer. Glucose was then separated from other compounds on a Dowex-2 (formate form) column (Hammerstedt, 1980). In some cases, glucose was further identified either by paper chromatography (Surholt & Newsholme, 1983) or enzymic conversion into glucose 6-phosphate and re-chromatography (Hammerstedt, 1980).

Glycerol-3-phosphatase was assayed as described by Surholt & Newsholme (1981), and the reaction was terminated by method (i) or (ii).

Results and discussion

Glycerol-3-phosphatase activity

Homogenization of insect flight muscle and assay of glycerol-3-phosphatase by a method similar to that described by Kitcher *et al.* (1978) and Surholt & Newsholme (1981) for glucose-6-phosphatase gives an activity of 2–10 $\mu\text{mol/min per g}$ of fresh muscle, depending on the insect used. However, such activities were almost completely inhibited by respiratory-chain inhibitors such as cyanide and antimycin A. Furthermore, if the assay was terminated with FeCl_3 and NaOH, as described in the Materials and methods section, the activity was very low and similar to that observed above in the presence of cyanide. It is therefore suggested that in insect flight muscle the activity of a glycerol-3-phosphatase is very low and probably not detectable above that of non-specific phosphatase. The apparent activity of this phosphatase obtained with $\text{Ba}(\text{OH})_2$ and ZnSO_4 is probably due to the high activity of mitochondrial glycerol phosphate dehydrogenase, which converts radiolabelled glycerol phosphate into dihydroxyacetone phosphate. The latter may be hydrolysed

by the alkaline $\text{Ba}(\text{OH})_2$, and the dihydroxy[^{14}C]-acetone would not be adsorbed on the nascent precipitate and would thus appear in the supernatant used for measurement of radioactivity. Inhibition of the respiratory chain with cyanide or antimycin A would explain the inhibition of the apparent phosphatase, since the mitochondrial glycerol phosphate dehydrogenase is a flavoprotein and requires electron transfer for activity. The use of FeCl_3 to stop the reaction should provide a simple assay for further work on this enzyme.

Glucose-6-phosphatase activity

The activity of glycerol-3-phosphatase revealed a major problem with the assay for phosphatases using $\text{Ba}(\text{OH})_2$ and ZnSO_4 to terminate the reaction. This prompted a re-investigation of the glucose-6-phosphatase assay. A crude extract of muscle contains not only possible phosphatases but also enzymes that can metabolize glucose 6-phosphate to compounds that will not be adsorbed on the nascent precipitates, or to compounds readily hydrolysed by the alkaline conditions created on addition of $\text{Ba}(\text{OH})_2$ to the incubation mixture. For example, glucose 6-phosphate could be converted into triose phosphates, via the enzymes phosphoglucosomerase, 6-phosphofructokinase and aldolase, and triose phosphates are readily hydrolysed to trioses by alkaline conditions (Newsholme & Randle, 1962). These two possibilities should be differentiated by the precipitation with ferric hydroxide or HClO_4 and separation of glucose by column chromatography.

The activities of glucose-6-phosphatase indicated by the three methods, $\text{BaSO}_4/\text{Zn}(\text{OH})_2$ precipitation, $\text{Fe}(\text{OH})_3$ precipitation and column chromatography, for a variety of muscles are presented in Table 1. In general, the activities obtained by using $\text{Ba}(\text{OH})_2/\text{ZnSO}_4$ precipitation were higher than those with $\text{Fe}(\text{OH})_3$ or column separation, but activities with the latter two techniques were similar (Table 1). This suggests that, in muscle extracts, a metabolite is produced from glucose 6-phosphate which is hydrolysed by alkaline conditions. The ability to hydrolyse glucose 6-phosphate does not necessarily indicate a specific phosphatase for the hydrolysis of glucose 6-phosphate (i.e. glucose-6-phosphatase), and this has been tested by adding a high concentration of another organic phosphate (glycerol 2-phosphate) to the assay as a competitive inhibitor. There is little difference in the activities, which suggests that a non-specific phosphatase is not important in the hydrolysis of glucose 6-phosphate in the muscles studied.

This work suggests that the activities of glucose-6-phosphatase reported in some muscles by Surholt & Newsholme (1981) are too high and, in

Table 1. *Glucose-6-phosphatase activity in different muscles as measured by different methods*

The activity was measured by incubation with [^{14}C]glucose 6-phosphate under different conditions or with different means of terminating the reaction, as follows: A, protein precipitation with $\text{Ba}(\text{OH})_2/\text{ZnSO}_4$ (results from Surholt & Newsholme, 1981); B, protein precipitation with $\text{Fe}(\text{OH})_3$; C, incubation medium contains additionally 10 mM-2-glycerophosphate, and precipitation with $\text{Fe}(\text{OH})_3$; D, precipitation with HClO_4 and separation of glucose by column chromatography. Results are presented as means $\pm$ S.E.M., with numbers of separate animals or pools of muscle from several animals used given in parentheses.

Animal	Muscle	Glucose-6-phosphatase activity ($\mu\text{mol}/\text{min}$ per g wet wt. of muscle)			
		A	B	C	D
Cockroach (<i>Periplaneta americana</i>)	Flight	—	0.35 ± 0.04 (4)	0.35 ± 0.05	0.34 ± 0.03
	Coxal	—	0.39 ± 0.05 (5)	0.45 ± 0.04	0.35 ± 0.07
Bumble bee (<i>Bombus</i> sp.)	Flight	5.0	2.0 ± 0.25 (6)	1.8 ± 0.30	1.9 ± 0.28
Honey bee (<i>Apis mellifera</i>)	Flight	3.5	2.3 ± 0.48 (3)	2.0 ± 0.30	1.5 ± 0.11
Wasp (<i>Vespa vulgaris</i>)	Flight	8.0	4.3 ± 0.75 (4)	3.2 ± 0.78	3.3 ± 0.93
Pigeon (<i>Columba livia</i>)	Pectoral	1.8	0.63 ± 0.05 (5)	0.53 ± 0.06	0.47 ± 0.08
Domestic fowl (<i>Gallus gallus</i>)	Pectoral	0.3	—	0.11 ± 0.02 (4)	0.14 ± 0.02
Sparrow (<i>Passer domesticus</i>)	Pectoral	1.6	—	0.4 ± 0.07 (4)	0.40 ± 0.06
Rabbit (<i>Oryctolagus cuniculus</i>)	Semitendinosus	3.4	0.18 ± 0.04 (4)	0.17 ± 0.04	0.25 ± 0.04
Laboratory rat (<i>Rattus norvegicus</i>)	Heart	0.30	—	—	<0.10 (5)
	Soleus	0.17 ± 0.04 (4)	—	—	<0.10 (4)
	Quadriceps (red)	1.8	0.20 ± 0.04 (5)	—	0.15 ± 0.03
	Quadriceps (white)	1.5	0.24 ± 0.08 (5)	—	0.13 ± 0.02
	Epitrochlearis	0.70	0.17 ± 0.07 (5)	—	0.11 ± 0.02

particular, the indication that mammalian muscle contains significant activities of a specific glucose-6-phosphatase is probably erroneous. However, the presence of this enzyme in pectoral muscle of the pigeon and sparrow and insect flight muscle has been confirmed. These muscles exhibit marked increases in the rate of glucose utilization during flight, which suggests a role for glucose-6-phosphatase in the regulation of glycolysis in these muscles.

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