Limitations of commonly used spectrophotometric assay methods for phosphoenolpyruvate carboxykinase activity in crude extracts of muscle

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Phosphoenolpyruvate carboxykinase activity in crude extracts of muscle has frequently been determined by using a continuous spectrophotometric method, which is shown to grossly overestimate enzyme activity. NADH oxidation attributed to phosphoenolpyruvate carboxykinase activity in the assay is due to lactate production. Under the normal assay conditions, Na^+ ions stimulate pyruvate kinase, providing pyruvate for lactate formation by lactate dehydrogenase and sufficiently to account for most of the observed NADH oxidation.

Evidence has been presented for the involvement of phosphoenolpyruvate carboxykinase (PEPCK) in the pathway of alanine formation from certain amino acids in rat skeletal muscle (Snell & Duff, 1977, 1979, 1981; Newsholme & Williams, 1978; Snell, 1980) and in glycogen formation from lactate in frog and rat muscle (Connett, 1979; Odedra & Palmer, 1981).

Two methods for assaying PEPCK activity in crude extracts of muscle have been employed, both of which supposedly follow the formation of oxaloacetate from phosphoenolpyruvate and are coupled to malate formation from oxaloacetate by added malate dehydrogenase. One method is a continuous spectrophotometric assay based on the decrease in absorbance at 340 nm due to the oxidation of NADH by the conversion of oxaloacetate, formed in the assay, into malate by malate dehydrogenase (Opie & Newsholme 1967; Crabtree *et al.*, 1972). The other method measures incorporation of $^{14}\text{CO}_2$ into oxaloacetate, which is trapped for counting of radioactivity as acid-stable malate (Nolte *et al.*, 1972; Snell & Duff, 1979), and is based on the assay method developed for liver PEPCK (Chang & Lane, 1966; Ballard & Hanson, 1967).

Although the reaction mixtures for the two methods are very similar, two important differences have been observed between PEPCK activities in crude extracts of muscle determined by the two techniques. Our previous study (Snell & Duff, 1979) showed that, although in agreement with values reported by Nolte *et al.* (1972), the $^{14}\text{CO}_2$ -incorporation assay method gave muscle PEPCK ac-

tivities approximately one order of magnitude lower than those obtained with the spectrophotometric assay method (Opie & Newsholme, 1967; Crabtree *et al.*, 1972; Newsholme & Williams, 1978; Newsholme *et al.*, 1979; McLane & Holloszy, 1979; Odedra & Palmer, 1981). In addition, the $^{14}\text{CO}_2$ -incorporation assay method indicated that red muscle had PEPCK activity similar to or slightly higher than that of white muscle (Nolte *et al.*, 1972), whereas the spectrophotometric assay showed substantially higher PEPCK activity in white muscle than in red muscle (McLane & Holloszy, 1979).

The present study investigates the basis for the differences between the two assay methods and reveals some limitations of the continuous spectrophotometric assay.

Experimental

Chemicals

$\text{NaH}^{14}\text{CO}_3$ was obtained from Amersham International (Amersham, Bucks., U.K.). Enzymes and coenzymes were from Boehringer Corporation (Lewes, Sussex, U.K.) and Sigma Chemical Co. (Poole, Dorset, U.K.). 2,5-Diphenyloxazole was obtained from Packard Instruments Co. (Downers Grove, IL, U.S.A.) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene from Fisons Scientific Apparatus (Loughborough, Leics., U.K.). Synperonic NX was supplied by Durham Chemicals Distributors (Birtley, Tyne and Wear, U.K.). Inorganic chemicals were from BDH Chemicals (Poole, Dorset, U.K.).

Animals

Male rats of the Wistar albino strain, bred in and

Abbreviation used: PEPCK, phosphoenolpyruvate carboxykinase (EC 4.1.1.32).

obtained from the Animal House of the University of Surrey, were fed *ad libitum* and used at a body weight of 100–120 g. Rats were killed by cervical dislocation, and diaphragm, soleus and/or extensor digitorum longus muscles were rapidly dissected out.

Preparation of homogenates

Freshly dissected muscles were homogenized, with a ground-glass homogenizer and pestle, in 9 vol. of ice-cold extraction medium consisting of 100 mM-Tris/HCl, 1 mM-EDTA, 5 mM-MgCl₂ and 20 mM-2-mercaptoethanol at a final pH of 7.5 (Crabtree *et al.*, 1972). The 10% (w/v) homogenates were centrifuged at 600 g for 10 min at 4°C and the supernatants were used for enzyme activity determinations.

Assay of enzyme activities

PEPCK was assayed by four methods.

Method A was based on the ¹⁴CO₂-incorporation method of Chang & Lane (1966). The assay medium contained 100 mM-imidazole/HCl buffer, pH 6.6, 3 mM-phosphoenolpyruvate (sodium salt), 2 mM-IDP (sodium salt), 2 mM-MnCl₂, 2 mM-dithiothreitol, 2.5 mM-NADH (sodium salt), 50 mM-NaH¹⁴CO₃ (40 mCi/mol), 2.5 µg of antimycin A and 12 units (µmol/min) of malate dehydrogenase (in glycerol) in a final volume of 1 ml. Phosphoenolpyruvate was omitted from controls. The assay was terminated after 15 min by adding 1 ml of 2 M-HCl. CO₂ was bubbled through the assay solution for 10 min to drive off ¹⁴CO₂ not incorporated into acid-stable products. A 0.5 ml sample was removed, 4 ml of scintillation 'cocktail' [5 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene per litre of toluene/Synperonic NX (2:1, v/v)] was added, and the radioactivity of the mixture was counted in an LKB 1210 Ultrabeta liquid-scintillation counter. Units of enzyme activity for this method are defined as µmol of ¹⁴CO₂ fixed/min.

Method B was the continuous spectrophotometric assay as described by Opie & Newsholme (1967). The assay medium consisted of 66 mM-Tris/HCl buffer, pH 7.5, 1 mM-MnCl₂, 1 mM-phosphoenolpyruvate (sodium salt), 0.16 mM-NADH (sodium salt), 1.54 mM-IDP (sodium salt), 17 mM-NaHCO₃ and 12 units of malate dehydrogenase (in glycerol). NaHCO₃ was omitted from controls.

Method C was also a continuous spectrophotometric assay in the direction of oxaloacetate formation and was based on the method described by Bentle & Lardy (1976). The assay medium contained 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH 6.5, 1 mM-MnCl₂, 2 mM-phosphoenolpyruvate (sodium salt), 0.25 mM-NADH (sodium salt), 1 mM-IDP (sodium salt), 50 mM-NaHCO₃, 1 mM-dithiothreitol,

2.5 µg of antimycin A and 7.5 units of malate dehydrogenase (in glycerol). Phosphoenolpyruvate was omitted from controls.

Method D measured the PEPCK reaction in the direction of phosphoenolpyruvate formation and was also as described by Bentle & Lardy (1976).

Pyruvate kinase was assayed as described by Zammit *et al.* (1978) and lactate dehydrogenase by the method of Kornberg (1955).

A Gilford 250 spectrophotometer was used for spectrophotometric measurements, and all enzyme assays were performed at 30°C.

Lactate production during the course of PEPCK assays was also measured. Spectrophotometric assays of PEPCK by methods B and C were allowed to proceed for various times. Reactions were stopped by the addition of 0.1 ml of ice-cold 20% (w/v) HClO₄ to each cuvette. HClO₄ was also added to both control and test assay mixtures before the addition of crude muscle extracts to yield zero-time control values for initial lactate concentrations. Acidified samples were centrifuged at 4°C. Supernatants were removed, neutralized with KOH and re-centrifuged, and lactate was determined in the neutralized supernatant (Hohorst, 1963).

Results

Table 1 shows the PEPCK activity of crude extracts of diaphragm, soleus and extensor digitorum longus muscles determined by the four assay methods. The continuous spectrophotometric assays (methods B and C) gave apparent PEPCK activities that were in agreement with those obtained by other workers (Opie & Newsholme, 1967; Crabtree *et al.*, 1972; Newsholme & Williams, 1978; McLane & Holloszy, 1979; Odedra & Palmer, 1981) but that were at least 10 times greater than the activities obtained by using the ¹⁴CO₂-incorporation method (method A). Assay of muscle PEPCK activity in the direction of phosphoenolpyruvate formation (method D) showed negligible activity for all three muscles studied. However, it should be noted that the PEPCK activity reported with the use of the ¹⁴CO₂-incorporation assay (method A) would be below the level of detection of activity by the phosphoenolpyruvate-formation method (method D).

The specificity of the ¹⁴CO₂-incorporation assay method for PEPCK activity was tested by using 3 mM-sodium pyruvate in place of sodium phosphoenolpyruvate. Less than 5% of the ¹⁴CO₂ incorporated with phosphoenolpyruvate as substrate was found with pyruvate under method A assay conditions.

Spectrophotometric PEPCK assays by method B were performed with NaH¹⁴CO₃ in place of unlabelled NaHCO₃ and the incorporation of ¹⁴CO₂

Table 1. Activities of PEPCK determined by four different methods, of pyruvate kinase and of lactate dehydrogenase in crude extracts of diaphragm, soleus and extensor digitorum longus muscles

Activities were measured as described in the Experimental section. Values represent the means of measurements from different rats and are given $\pm$ S.E.M. with the numbers of observations in parentheses. N.D., Activity not detectable (see the text).

Muscle type	Enzyme activities ($\mu\text{mol}/\text{min}$ per g of muscle at 30°C)					
	PEPCK				Pyruvate kinase	Lactate dehydrogenase
	Method A	Method B	Method C	Method D		
Diaphragm	0.0246 ± 0.0021 (5)	0.36 ± 0.06 (4)	1.03 ± 0.15 (4)	N.D. (3)	104.6 ± 0.7 (3)	219.4 ± 19.3 (3)
Soleus	0.0253 ± 0.0015 (5)	0.30 ± 0.06 (7)	0.64 ± 0.03 (4)	N.D. (3)	68.1 ± 9.5 (4)	159.4 ± 13.2 (3)
Extensor digitorum longus	0.0211 ± 0.0013 (5)	0.62 ± 0.08 (6)	2.96 ± 0.18 (5)	N.D. (3)	215.6 ± 13.6 (4)	321.5 ± 22.2 (3)

Table 2. Effect of substituting NaCl for NaHCO_3 on NADH oxidation in assays for apparent PEPCK activity in crude extracts of extensor digitorum longus muscle

NADH oxidation was measured by PEPCK assay method B as described in the Experimental section.

$[\text{NaHCO}_3]$ (mm)	NADH oxidation ($\mu\text{mol}/\text{min}$ per g of muscle at 30°C)	$[\text{NaCl}]$ (mm)	NADH oxidation ($\mu\text{mol}/\text{min}$ per g of muscle at 30°C)
0	0.65	0	0.65
8.5	0.91	8.5	1.09
17	1.25	17	1.27
34	1.53	34	1.46
51	1.69	51	1.67

into acid-stable products under these assay conditions was compared with the formation of malate as judged by NADH oxidation: soleus, $0.0188 \mu\text{mol}$ of $^{14}\text{CO}_2$ incorporated/min per g of muscle and $0.18 \mu\text{mol}$ of NADH oxidized/min per g of muscle; extensor digitorum longus, $0.0216 \mu\text{mol}$ of $^{14}\text{CO}_2$ incorporated/min per g of muscle and $0.52 \mu\text{mol}$ of NADH oxidized/min per g of muscle. Product formation measured by radioactivity incorporated did not correspond to that measured by the decrease in [NADH] during the assay.

NaCl was substituted for NaHCO_3 in assay method B and the results with extracts of extensor digitorum longus muscle are shown in Table 2. Soleus-muscle extracts assayed by method B in the presence of 17 mM-NaHCO_3 and of 17 mM-NaCl gave NADH oxidation rates of 0.37 ± 0.09 ($n = 3$) and 0.41 ± 0.09 ($n = 3$) $\mu\text{mol}/\text{min}$ per g of muscle respectively. Together these findings indicated that $[\text{Na}^+]$ and not $[\text{HCO}_3^-]$ was responsible for the stimulation of NADH oxidation found with increasing $[\text{NaHCO}_3]$. The question arose as to what in the crude muscle extracts was causing the Na^+ -stimulated NADH oxidation, which was not dependent on

HCO_3^- . Examination of the components of the assay mixtures of methods B and C suggested that lactate formation from phosphoenolpyruvate, by the joint action of pyruvate kinase and lactate dehydrogenase, could be responsible. Lactate produced during assays of apparent PEPCK activity by methods B and C was measured (Table 3). It was found that lactate production could indeed account for the oxidation of NADH that had previously been attributed to malate formation by apparent PEPCK activity. As for apparent PEPCK activity, lactate production was dependent on $[\text{Na}^+]$ and was greater in extensor-digitorum-longus-muscle extracts than in soleus-muscle extracts (Table 3).

Pyruvate kinase activities in crude extracts of muscle were measured under various conditions by omitting and/or substituting various components of the assay system of Zammit *et al.* (1978), in order to obtain an indication of the pyruvate kinase activity of crude muscle extracts under the conditions used in the assays of apparent PEPCK activity by methods B and C (Table 4). K^+ , Mg^{2+} and NH_4^+ are all known to stimulate muscle pyruvate kinase (Bücher & Pfeleiderer, 1955; Zammit *et al.*, 1978), but none of

Table 3. *Lactate production during continuous spectrophotometric PEPCK assays with crude extracts of muscle and the effect of $[Na^+]$ on lactate production*

Lactate production under the conditions of the PEPCK assay method indicated was measured as described in the Experimental section. Apparent PEPCK activity as measured by NADH oxidation is compared with lactate production in each assay. N.M., Activity not measured.

Muscle type	$[NaHCO_3]$ (mM)	Assay method	PEPCK activity (μ mol/min per g of muscle at 30°C)	Lactate production (μ mol/min per g of muscle at 30°C)
Diaphragm	50	C	0.92	0.81
Soleus	50	C	0.66	0.66
Soleus	50	C	0.61	0.87
Extensor digitorum longus	50	C	3.38	3.54
Extensor digitorum longus	50	C	3.20	3.40
Soleus	17	B	0.15	0.08
Extensor digitorum longus	17	B	0.47	0.57
Extensor digitorum longus	0	C	N.M.	0.36
Extensor digitorum longus	10	C	N.M.	2.18
Extensor digitorum longus	20	C	N.M.	2.42
Extensor digitorum longus	50	C	N.M.	3.23
Extensor digitorum longus	100	C	N.M.	4.68

Table 4. *Pyruvate kinase activities of crude extracts of (a) extensor digitorum longus and (b) soleus muscles assayed under various conditions*

'Normal' activity denotes that the assay mixture contained metal ion, cofactor and substrate concentrations as described by Zammit *et al.* (1978): 160 mM-triethanolamine/HCl buffer, 10 mM-MgCl₂, 80 mM-KCl, 0.17 mM-NADH (sodium salt), 5 mM-ADP (sodium salt), 2 mM-phosphoenolpyruvate (sodium salt) and 25 μ g of lactate dehydrogenase [5 μ l containing 16 μ mol of (NH₄)₂SO₄]. 'Dialysed LDH' indicates that lactate dehydrogenase which had been extensively dialysed against distilled water to remove NH₄⁺, replaced the normal commercial preparation of lactate dehydrogenase in (NH₄)₂SO₄ solution in the assay mixture. 'PEPCK conditions' indicates that 1.54 mM-IDP replaced 5 mM-ADP, 1 mM-phosphoenolpyruvate replaced 2 mM-phosphoenolpyruvate and dialysed lactate dehydrogenase replaced lactate dehydrogenase in (NH₄)₂SO₄ solution in the assay mixture, and that 1 mM-MnCl₂ was added but that MgCl₂ and KCl were omitted. 'PEPCK conditions-LDH' indicates that the assay conditions were as described for 'PEPCK conditions' except that lactate dehydrogenase in (NH₄)₂SO₄ solution replaced the dialysed lactate dehydrogenase preparation.

Assay conditions and additions	Enzyme activity	
	(μ mol/min per g of muscle at 30°C)	(% of 'normal' activity)
<i>(a) Extensor digitorum longus</i>		
Normal	209.6	100
+17 mM-NaHCO ₃	199.2	95.0
PEPCK conditions-LDH	17.9	8.5
Dialysed LDH	342.0	163
PEPCK conditions	0.31	0.15
PEPCK conditions + 17 mM-NaCl	0.78	0.37
PEPCK conditions + 50 mM-NaCl	1.35	0.64
PEPCK conditions + 100 mM-NaCl	1.90	0.90
PEPCK conditions + 150 mM-NaCl	2.37	1.13
PEPCK conditions + 100 mM-NaHCO ₃	2.08	0.99
<i>(b) Soleus</i>		
Normal	56.3	100
Dialysed LDH	63.8	113.4
PEPCK conditions	0.05	0.09
PEPCK conditions + 17 mM-NaCl	0.22	0.40
PEPCK conditions + 50 mM-NaCl	0.35	0.62
PEPCK conditions + 100 mM-NaCl	0.59	1.06
PEPCK conditions + 150 mM-NaCl	0.48	0.86
PEPCK conditions + 100 mM-NaHCO ₃	0.51	0.91

these ions was present in the spectrophotometric assays for PEPCK activity by methods B and C. NH_4^+ is present in the commercial lactate dehydrogenase preparation normally used in the pyruvate kinase assay. A sample of the commercial preparation was therefore extensively dialysed against distilled water to remove NH_4^+ . Cofactor and metal ion conditions similar to those used in the assay of PEPCK activity by methods B and C gave low pyruvate kinase activity in crude muscle extracts compared with the maximum value obtained. Although Na^+ was slightly inhibitory to pyruvate kinase activity when K^+ was present, in the absence of K^+ , Mg^{2+} and NH_4^+ (as in the assay of PEPCK activity by methods B and C) Na^+ stimulated muscle pyruvate kinase activity (Table 4).

Discussion

Our findings have shown that the continuous spectrophotometric methods for assay of PEPCK activity in crude extracts of muscle principally measure NADH oxidation due to lactate, and not malate, formation. The crude extracts of muscle include the enzymes pyruvate kinase and lactate dehydrogenase. Under the conditions of PEPCK assay by methods B and C, NADH oxidation, previously attributed to PEPCK activity, is due to lactate production by lactate dehydrogenase from pyruvate formed from phosphoenolpyruvate by a low activity of pyruvate kinase. Compared with the activity of muscle pyruvate kinase assayed under optimal conditions, Na^+ -stimulated pyruvate kinase activity in the absence of K^+ , Mg^{2+} and NH_4^+ ions reached a maximum value of 1.2% of optimal. This is in close agreement with the findings obtained by Kachmar & Boyer (1953), who found that, although Na^+ inhibited rabbit muscle pyruvate kinase in the presence of K^+ , Na^+ stimulated pyruvate kinase activity in the absence of K^+ , although never to greater than 1.5% of the maximum activity obtained. Although the Na^+ stimulation of pyruvate kinase activity is relatively small, it is sufficient to account for the NADH oxidation previously attributed to PEPCK activity determined by methods B and C. Table 1 shows that, in crude extracts of diaphragm, soleus and extensor digitorum longus muscles, apparent PEPCK activity as determined by method B was substantially less than 1% of the corresponding pyruvate kinase maximum activity.

Na^+ stimulation of muscle pyruvate kinase also probably accounts for the previous findings that crude extracts of red muscle have lower apparent PEPCK activity than do extracts of white muscle (Opie & Newsholme, 1967; McLane & Holloszy, 1979), because red muscle contains less pyruvate kinase activity than does white muscle. Table 1 confirms that extracts of muscle display spectro-

photometrically determined apparent PEPCK activity and pyruvate kinase activity in the order: soleus < diaphragm < extensor digitorum longus.

The continuous spectrophotometric method for measuring PEPCK activity in crude extracts of muscle has been widely used (Opie & Newsholme, 1967; Crabtree *et al.*, 1972; Zammit & Newsholme, 1976, 1978; Newsholme & Williams, 1978; Newsholme *et al.* 1979; McLane & Holloszy, 1979; Odedra & Palmer, 1981). However, our findings that the assay conditions employed largely measure Na^+ -stimulated pyruvate kinase activity indicate that this method is unsuitable for determination of PEPCK activity in muscle preparations that have not been extensively purified to remove pyruvate kinase and lactate dehydrogenase activities, although it may still prove to be suitable for extracts of muscles that lack lactate dehydrogenase (Zammit & Newsholme, 1976).

An alternative continuous spectrophotometric PEPCK assay method has been described (Petrescu *et al.*, 1979) in which pyruvate kinase activity is limited by employing dGDP in place of IDP. This method was used to measure PEPCK activity spectrophotometrically in guinea-pig liver preparations (where pyruvate kinase activity exceeds that of PEPCK by 5–15-fold) (Petrescu *et al.*, 1979). Rat muscle preparations, however, contain much lower PEPCK activities than does liver, and have pyruvate kinase activities that exceed those of PEPCK (measured by method A) by approx. 2500–10000-fold. As dGDP still permits slight activity of rabbit muscle pyruvate kinase (Petrescu *et al.*, 1979), a large error in the estimation of muscle PEPCK activity is still found with this modified spectrophotometric method (D. A. Duff, unpublished work). PEPCK activity in crude extracts of muscle is best determined by the $^{14}\text{CO}_2$ -incorporation method, in our opinion.

The activities of PEPCK as measured by the $^{14}\text{CO}_2$ -incorporation assay method give values in skeletal muscle that are orders of magnitude lower than those measured by the spectrophotometric methods (see Table 1). In view of the proposed role for the enzyme in muscle alanine synthesis *de novo* (see Snell, 1980; Snell & Duff, 1981), it is important to assess the activity in relation to observed rates of alanine synthesis. The PEPCK activities reported in Table 1 (with method A) and by Snell & Duff (1979) for diaphragm muscle in fed and starved rats are more than sufficient to account for the valine-stimulated increase in alanine formation observed in diaphragm incubations *in vitro* (Snell & Duff, 1977, 1979). The same considerations apply to extensor digitorum longus (Snell & Duff, 1981) and soleus (K. Snell & D. A. Duff, unpublished work) muscles. The pathway of pyruvate formation for muscle alanine synthesis involving PEPCK also requires the

participation of pyruvate kinase (Snell, 1980; Snell & Duff, 1981), and this activity will be greatly in excess in relation to rates of alanine synthesis (Table 1). Other evidence (Snell & Duff, 1981; K. Snell & D. A. Duff, unpublished work) supports a role for PEPCK in muscle alanine synthesis in preference to other possible enzymic routes. On the other hand, in relation to the proposed involvement of PEPCK in a route for muscle glycogen formation from lactate (Connett, 1979; Odedra & Palmer, 1981), the PEPCK activity as determined by the $^{14}\text{CO}_2$ -incorporation assay is not sufficient to account for the rates of glycogen accumulation, with lactate as sole precursor, observed by McLane & Holloszy (1979).

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