



## An improved spectrophotometric assay of pyruvate dehydrogenase in lactate dehydrogenase contaminated mitochondrial preparations from human skeletal muscle

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### Abstract

In mitochondria-enriched preparations of human skeletal muscle, the measurement of pyruvate dehydrogenase activity, as determined by conventional spectrophotometric assay of NADH accumulation, is underestimated due to the oxidizing activity of the contaminating lactate dehydrogenase. Using a model reaction system consisting of varying mixtures of purified lactate and pyruvate dehydrogenases, we found that the presence of oxamate, a competitive inhibitor of the lactate dehydrogenase, allowed the measurement of a linear rate of pyruvate dehydrogenase activity without interference from lactate dehydrogenase. In the presence of 25 mM oxamate, this holds true up to a ratio of 30:1 for lactate to pyruvate dehydrogenases, respectively. A similar result was obtained when using human skeletal muscle mitochondria contaminated by lactate dehydrogenase. Rates of pyruvate dehydrogenase activity ranging from 50 to 120 nmol/min/mg protein could be routinely measured in such mitochondrial fractions. We concluded that the use of oxamate allows a spectrophotometric assay for pyruvate dehydrogenase activity to be utilized when screening for pyruvate dehydrogenase deficiency in mitochondria-enriched preparations of human skeletal muscle.

**Keywords:** Pyruvate dehydrogenase; Human mitochondria; Lactate dehydrogenase; Enzyme assay; Oxamate

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*Abbreviations:* BSA, bovine serum albumin; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; RC, respiratory chain.

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## 1. Introduction

Mitochondrial respiratory chain (RC) and pyruvate dehydrogenase (PDH) defects are known to cause human diseases which, as with Leigh disease, are sometimes difficult to differentiate clinically [1,2]. Consequently, a concomitant determination of both RC and PDH activities during screening procedures for such inherited metabolic disorders is often of importance for clinicians.

Most measurements of RC activities can be carried out by means of polarographic and spectrophotometric studies of mitochondria-enriched fractions from human tissues [3]. Theoretically, PDH activity could also be estimated using a simple spectrophotometric assay, by following the accumulation of NADH in the presence of pyruvate [4]. However, this simple assay is generally hampered by the presence of lactate dehydrogenase (LDH) contaminating to various extents most human tissue extracts. This enzyme tends, in the presence of pyruvate, to reoxidize the NADH produced by the PDH (see scheme of Fig. 1). As a consequence, the rather tedious, time consuming and radioactive assay of PDH activity generally has to be used in screening procedures [5,6].

We report here the successful use of oxamate, an LDH competitive inhibitor [7] to confidently measure PDH activity in mitochondria-enriched fractions from human skeletal muscle.

## 2. Material and methods

### 2.1. Mitochondria preparation

Mitochondria were isolated by differential centrifugation from minute amounts (100 mg) of skeletal muscle obtained at surgery as previously described [3].

### 2.2. Enzyme assays

LDH (EC 1.1.1.27) was spectrophotometrically measured [8] at 37°C by following the oxidation of NADH in 1 ml of medium A consisting of 0.3 M mannitol, 10 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.8) and 0.1% Triton X-100. The reaction

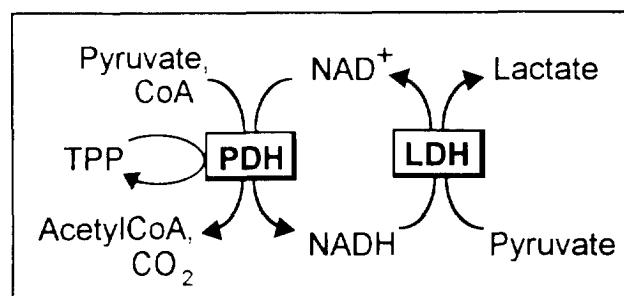


Fig. 1. The opposite redox reactions driven by the pyruvate dehydrogenase and the lactic dehydrogenase.

was started by adding pyruvate. Pyruvate dehydrogenase activity was spectrophotometrically measured by following  $\text{NAD}^+$  reduction in 1 ml of medium A to which was added 0.5 mM  $\text{NAD}^+$ , 200  $\mu\text{M}$  CoA, 1 mM cysteine, 200  $\mu\text{M}$  thiamine pyrophosphate. The reaction was started by adding 0.4 mM pyruvate. Enzyme kinetic parameters were determined by fitting reaction rates to a Michaelis Menten model using a microcomputer [9].

### 2.3. Protein determination and chemicals

Protein was determined by the method of Bradford [10] using BSA as a standard. Purified rabbit muscle L-LDH (Type II), porcine heart PDH and all other chemicals (analytical reagent grade) were from the Sigma Chemical Company.

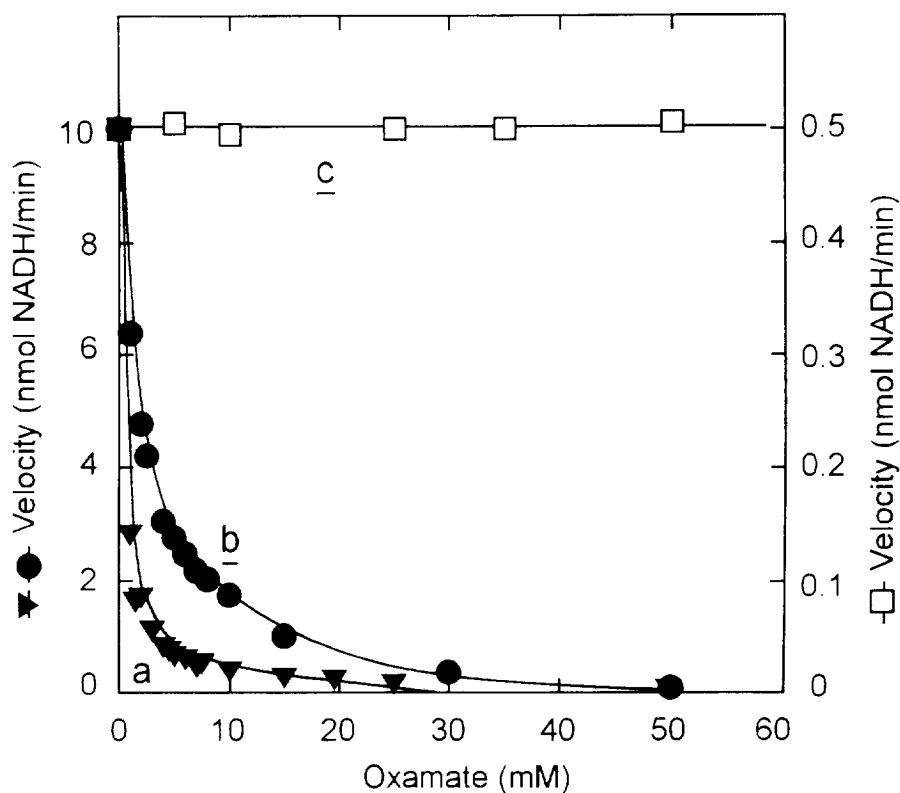


Fig. 2. The effect of oxamate on the activity of lactate and pyruvate dehydrogenases. Oxamate isotherm carried out with purified lactate dehydrogenase in the presence of 0.4 mM pyruvate and 0.032 units LDH (line a) or 4 mM pyruvate and 0.012 units LDH (line b), or with purified pyruvate dehydrogenase (0.00053 units) in the presence of 0.4 mM pyruvate (line c). Other experimental conditions were as described under Materials and methods.

### 3. Results and discussion

Oxamate has been shown to act as a potent competitive inhibitor of the LDH [7]. Under our experimental conditions, the  $K_i \pm 1$  S.D. for oxamate of the purified rabbit muscle enzyme was determined to be  $0.83 \pm 0.24$  mM when tested at 4 mM pyruvate concentration (Fig. 2, trace b) and  $0.69 \pm 0.22$  mM, when the enzyme was assayed at 0.4 mM pyruvate (Fig. 2, trace a). Under these conditions, no effect of oxamate on the purified porcine heart pyruvate dehydrogenase could be detected up to 40 mM oxamate (Fig. 2, trace c). It therefore appeared that oxamate led to a specific inhibition of the LDH reaction, when used at a concentration below 40 mM.

Taking into account the fact that oxamate acted as a pyruvate competitive inhibitor of the LDH reaction, it appeared judicious to use the lowest possible concentration of pyruvate for the PDH assay in order to obtain a maximal inhibition of any

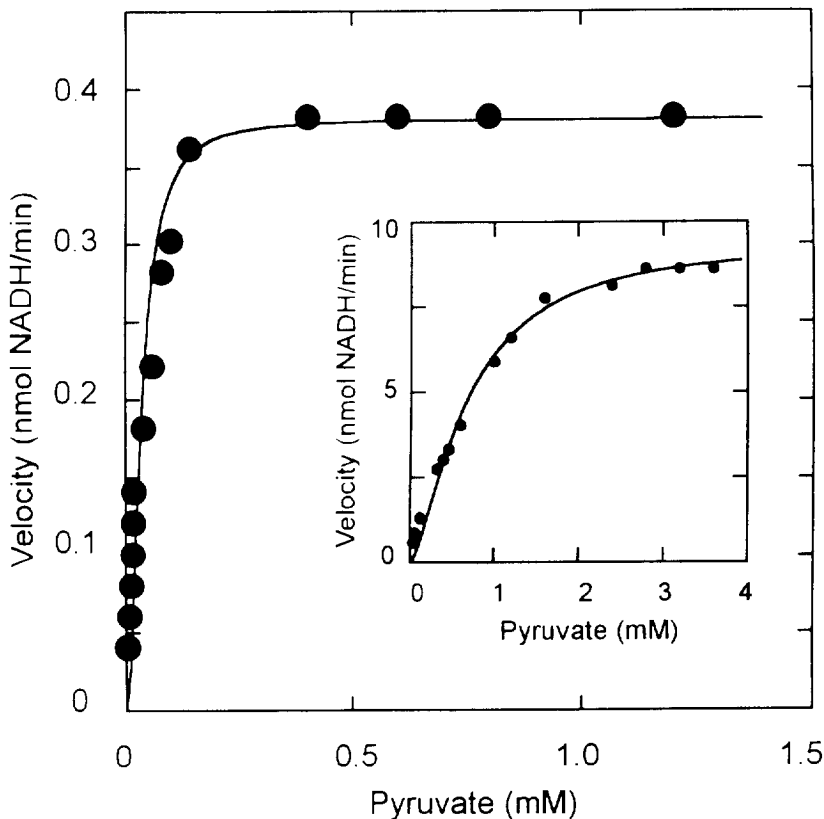


Fig. 3. Activity of the pyruvate and lactate dehydrogenases as a function of pyruvate concentration. Pyruvate isotherm measured with purified pyruvate dehydrogenase (0.00042 units). Inset: Pyruvate isotherm measured with purified lactate dehydrogenase (0.01 units). Experimental conditions were as described under Materials and methods.

contaminating LDH. We therefore subsequently determined the saturating concentration of pyruvate for the purified porcine heart PDH reaction (Fig. 3). Maximal activity was obtained for a pyruvate concentration of 0.4 mM, not significantly different from the saturating concentration previously reported for the human enzyme [11]. Under our experimental conditions, this concentration of pyruvate was not saturating for the purified rabbit muscle LDH reaction (Fig. 3, inset).

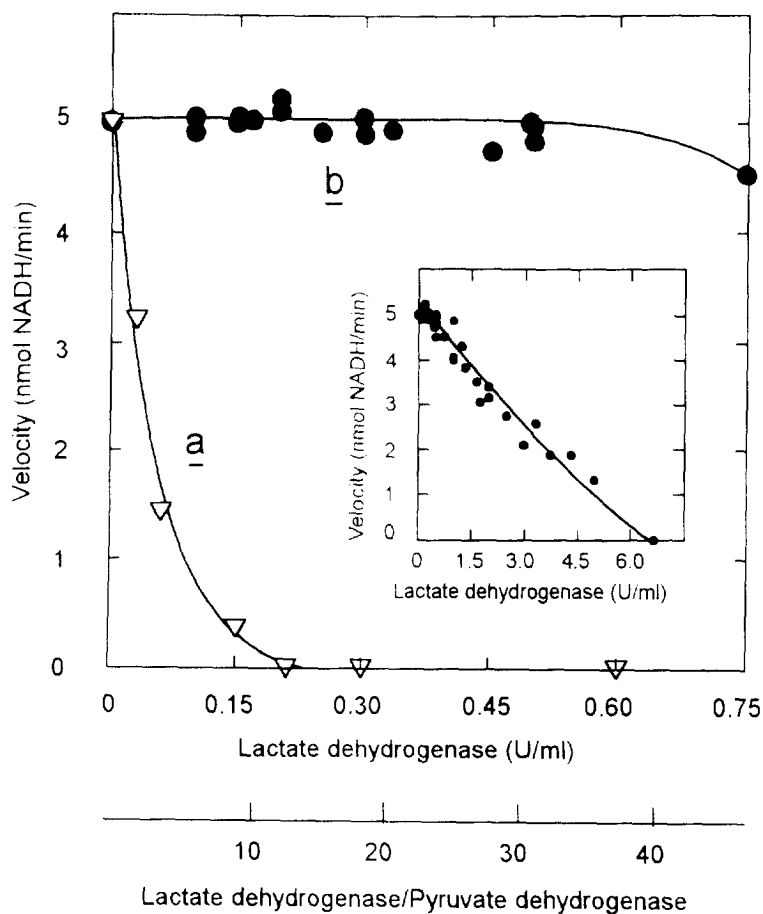


Fig. 4. The effect of oxamate on the pyruvate dehydrogenase measurement in the presence of lactate dehydrogenase. The reduction of  $\text{NAD}^+$  by the purified pyruvate dehydrogenase (0.0053 units) on adding increasing amounts of purified lactate dehydrogenase was spectrophotometrically followed in the absence (line a) or in the presence (line b) of 25 mM oxamate. Lactate dehydrogenase/pyruvate dehydrogenase ratios refer to ratios of activities actually measured at 0.4 mM pyruvate for both PDH and LDH. Inset: Effect of higher amounts lactate dehydrogenase on the NADH accumulation due to pyruvate dehydrogenase activity in the presence of 25 mM oxamate. Pyruvate concentration was 0.4 mM. Other experimental conditions were as described under Materials and methods.

Further, the effect of oxamate on the purified porcine heart PDH determination in the presence of varying amounts of purified rabbit muscle LDH was studied (Fig. 4). In the absence of oxamate (trace a), the reaction of the PDH was rapidly undetectable by a spectrophotometric study of the NADH accumulation. Under our experimental conditions, the activity of 0.0053 units (5 nmol NADH actually produced/min) of PDH was virtually undetectable when 0.15 units of LDH were present in the assay medium (LDH/PDH: 15). In the presence of 25 mM oxamate (Fig. 4, inset), 6 units of LDH were required in order to fully mask the PDH reaction (LDH/PDH: 450). Moreover, in the presence of 25 mM oxamate, the activity of the PDH was fully measurable up to a LDH/PDH ratio of 30 (Fig. 4, trace b).

As these amounts of LDH exceeded by far the amount generally contaminating mitochondria-enriched fractions of human skeletal muscle, we next studied the effect of oxamate on the spectrophotometric determination of PDH activity in such mitochondria-enriched fractions (Fig. 5). In the absence of oxamate, PDH activity

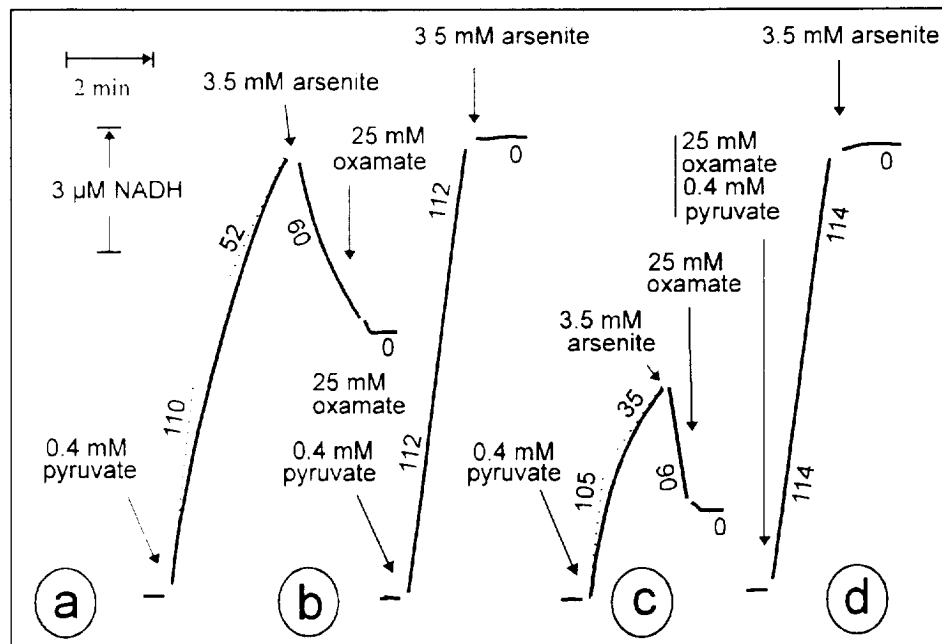


Fig. 5. The effect of oxamate on the measurement of the pyruvate dehydrogenase in the mitochondria-enriched fraction from human skeletal muscle. Measurement of the pyruvate dehydrogenase activity of the mitochondria-enriched fraction from human skeletal muscle in the absence (trace a) or in the presence (trace b) of 25 mM oxamate (contaminating lactate dehydrogenase activity: 540 nmol/min per mg protein; final LDH activity in the 1 ml cuvette: 24 nmol/min; LDH/PDH: 4.8). Similar experiments were carried out on a mitochondria-enriched preparation adding with purified lactate dehydrogenase (final LDH activity in the 1 ml cuvette: 124 nmol/min; LDH/PDH: 28) in the absence (trace c) and in the presence (trace d) of 25 mM oxamate. Numbers along the traces are nmol/min per mg protein. For each assay, 44  $\mu$ g mitochondrial protein were used. Experimental conditions were as described under Materials and methods.

was found to be somewhat variable from one preparation to another, but NADH accumulation consistently slowed down as the reaction proceeded (Fig. 5, trace a). Adding arsenite, an inhibitor of the dihydrolipoamide dehydrogenase of the PDH complex [12], caused a rapid reoxidation of the accumulated NADH that could be inhibited by the addition of oxamate. When oxamate was initially added (Fig. 5, trace b), a linear rate of PDH reaction could be measured which was fully inhibited by arsenite. Under these conditions, no reoxidation of the accumulated NADH took place. Values ranging from 50 to 120 nmol/min per mg protein were measured using mitochondria-enriched preparations from eight control skeletal muscle samples. In the next experiment, additional LDH (3 units of purified rabbit muscle LDH) was added to the mitochondrial preparation (LDH/PDH activity ratio: 30). Under these conditions, the measurable reaction of the PDH rapidly slowed down (Fig. 5, trace c). However, in the presence of initially added oxamate (Fig. 5, trace d), despite the presence of a high amount of LDH, the reaction of the PDH proceeded linearly with a rate similar to that measured in only the presence of the endogenous contaminating LDH (Fig. 5, trace b).

#### 4. Conclusions

The above data established that, in the presence of oxamate, a standard spectrophotometric assay for PDH can be used, allowing a confident estimation of PDH activity in skeletal muscle mitochondrial preparations. In the presence of the inhibitor, the actual amount of LDH generally found in the mitochondria-enriched pellet obtained by differential centrifugation appeared too low to interfere in the measurement of the PDH reaction.

Compared with the conventional method using radioactive-labelled pyruvate to determine PDH activity, the spectrophotometric measurement presents several major advantages. Avoiding the use of radioactive material, it appears rapid and quite sensitive, being easily included in the screening procedure for mitochondrial disorders. Finally, it allows the measurement of the whole PDH complex, giving an easy access to its kinetic parameters.

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#### References

- [1] Robinson BH. Lactic acidemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The metabolic basis of inherited disease*, 6th ed. New York: McGraw-Hill, 1989;869–888.
- [2] Willems JL, Monnens LAM, Trijbels JMF et al. Leigh's encephalomyopathy in a patient with cytochrome *c* oxidase deficiency of muscle tissue. *Paediatrics* 1977;60:850–857.
- [3] Rustin P, Chretien D, Bourgeron T, Rötig A, Saudubray JM, Münnich A. Biochemical and molecular investigations in respiratory chain deficiencies. *Clin Chim Acta* 1994;228:35–51.
- [4] Robinson JB, Brent LG, Sumegi B, Srere PA. An enzymatic approach to the study of the Krebs

- tricarboxylic acid cycle. In: Darley-Usmar VM, Rickwood D, Wilson MT, eds. *Mitochondria: a practical approach*. Oxford: IRL Press, 1987;153–170.
- [5] Blass JP, Avigan J, Uhlendorf BWA. A defect of pyruvate decarboxylase in a child with an intermittent movement disorder. *J Clin Invest* 1970;49:423–432.
- [6] Sheu KFR, Hu CWC, Utter MF. Pyruvate dehydrogenase activity in normal and deficient fibroblasts. *J Clin Invest* 1981;67:1463–1471.
- [7] McPherson A Jr. Binding of oxamate to the apoenzyme of dogfish M4 lactate dehydrogenase. *J Mol Biol* 1973;76:528–531.
- [8] Bergmeyer HU. *Methods of enzymatic analysis*. New York: Academic Press, 1963;1–1063.
- [9] Rustin P, Meyer CR, Wedding RT. Identification of substrate and effector binding sites of phosphoenolpyruvate carboxylase from *Crassula argentea*. A possible role of phosphoenolpyruvate as substrate and activator. *J Biol Chem* 1988;263:17611–17614.
- [10] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;248–254.
- [11] Clot JP, Benelli C, Fouque F, Bernard R, Durand D, Postel-Vinay MC. Pyruvate dehydrogenase activity is stimulated by growth hormone (GH) in human mononuclear cells: a new tool to measure GH responsiveness in man. *J Clin Endocrinol Metab* 1992;74:1258–1262.
- [12] Lenartowicz E. A complex effect of arsenite on the formation of alpha-ketoglutarate in rat liver mitochondria. *Arch Biochem Biophys* 1990;283:388–396.