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Continuous Optical Assay of Acylphosphatase with Benzoylphosphate as Substrate[1]

Acylphosphatase determination has hitherto been commonly carried out by colorimetric estimation of residual substrate after suitable incubation times[2–4]: the employed substrate has usually been acetylphosphate, whose hydrolysis is followed by the hydroxylamine method of LIPMANN and TUTTLE[5]. This report describes an optical method for a continuous assay of acylphosphatase activity by using as substrate benzoylphosphate. The preparation and properties of this aromatic acylphosphate have been described elsewhere[6].

Materials and methods. Acylphosphatase was prepared from horse muscle as described by GUERRITORE et al.[7]: the final preparation had a specific activity, expressed as μmoles of split acetylphosphate/min/mg of protein, of 380 at 25 °C and pH 5.3, with an overall purification of 2300.

Lithium benzoylphosphate was synthesized by following with slight modifications the procedure of LIPMANN and TUTTLE[8] for the preparation of silver acylphosphates and then converting the silver salt into lithium salt by exchange with LiCl. The final preparation had a 98% content of hydroxylamine-reactive carboxyl-phosphate bonds, and a benzoic acid/P mole ratio of 1:1[6].

Optical measurements,including the test of acylphosphatase activity, were carried out with a Beckman DK 1A recording spectrophotometer, equipped with a thermoregulated cell holder.

Optical properties of benzoylphosphate and its use for acylphosphatase assay. The UV-absorption spectra in the region 260–300 nm of benzoylphosphate before and after complete hydrolysis by acylphosphatase are shown in Figure 1: a clear difference is visible between the 2 spectra, benzoylphosphate having in the scanned wavelength range higher extinction values than free benzoate. The measurement of the absorption change in this region

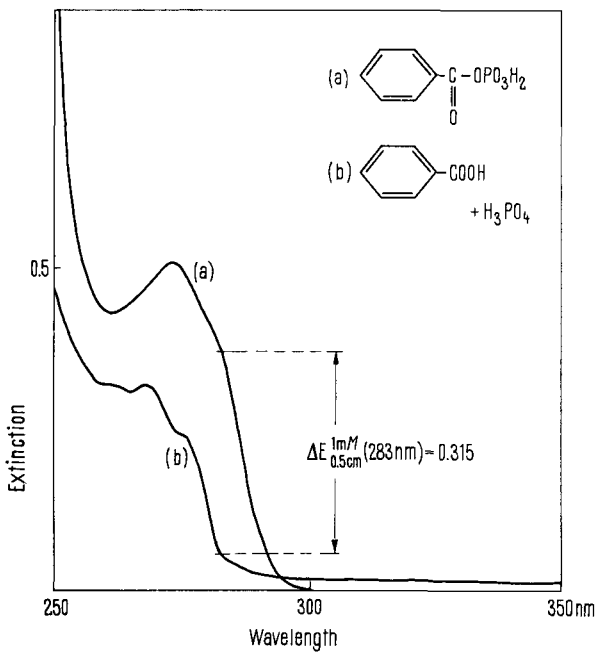

Fig. 1. Absorption spectra of benzoylphosphate before and after enzymic hydrolysis. The progress of the carboxyl-phosphate bond cleavage was checked by the hydroxylamine-ferric chloride test. (a) Spectrum before hydrolysis; (b) spectrum after complete hydrolysis; 25 °C, pH 5.3, 0.5 cm cell. Benzoylphosphate concentration: 1 m*M*.

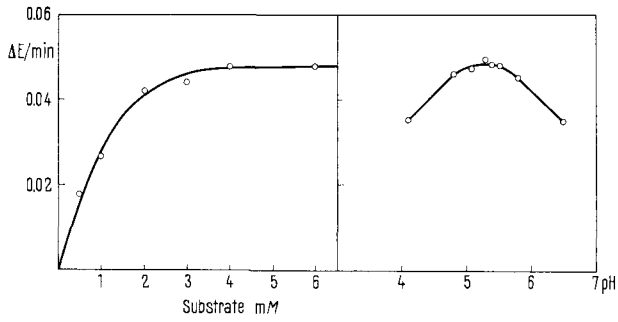

Fig. 2. Effect of substrate concentration (left side) and pH (right side) on the benzoylphosphate splitting by acylphosphatase. Benzoylphosphate hydrolysis was followed by optical measurement under the test conditions described in the text. The final preparation of purified enzyme was opportunely diluted before addition in the spectrophotometer cell. Assay starts with enzyme, 15 μl, 283 nm, 0.5 cm cell, 25 °C, final volume 1.5 ml.

[1] This research is a contribution from the 'Impresa Enzimologia' Group of the Italian Consiglio Nazionale delle Ricerche.

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gives, therefore, a sensitive measure of the hydrolytic reaction: at 283 nm the extinction change is maximum, and this wavelength was chosen for a quantitative optical test of acylphosphatase activity with benzoylphosphate as substrate. It must be pointed out that this wavelength corresponds to a steep descending zone of the absorption curve: a careful calibration of the spectrophotometer is therefore needed in order to minimize errors. It is however recommended, before starting a series of measurements, to check directly the working conditions with standard solutions of benzoylphosphate and benzoic acid.

Optimal conditions for the test were investigated with the muscle enzyme at 283 nm and 25 °C: the effects of substrate concentration and pH upon enzyme activity are shown in Figure 2. On the basis of these data the test composition for a standard assay of acylphosphatase in a 'split beam' recording spectrophotometer can be the following:

	Volume	Final concentration
Sample cell (light path 0.5 cm)	ml	m*M*
200 m*M* acetate buffer, pH 5.3	0.750	100
15 m*M* Li benzoylphosphate	0.500	5
Enzyme solution	0.010 or more	
Water to	1.500	
Reference cell (light path 0.5 cm)		
200 m*M* acetate buffer, pH 5.3	0.750	100
15 m*M* Li benzoylphosphate	0.300	3
Water to	1.500	

The chart of an assay carried out in this way with 2 amounts of enzyme is shown in Figure 3: for a reaction time of 4 min, corresponding to a hydrolysis extent of 7 and 14%, there is good agreement with apparent zero-order kinetics and a good proportionality between the amount of enzyme and the rate of reaction.

According to the measured extinction change coefficient of 0.630 mM^{-1} cm^{-1} (see Figure 1), in the above test conditions the μmoles of substrate split per minute are obtained by multiplying the ΔE/min by 4.8.

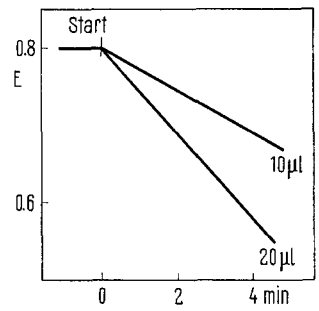

Fig. 3. Continuous record of an optical assay of acylphosphatase activity at 25 °C. The assay conditions are indicated in the text. The final preparation of purified enzyme was opportunely diluted before addition in the spectrophotometer cell: assay starts with enzyme, 10 and 20 μl, as indicated in the figure. 283 nm, 0.5 cm cell, final volume 1.5 ml, chart speed 12.5 mm/min.

Discussion. By the use of an aromatic acylphosphate as substrate the well-known advantages of the continuous optical tests are made available for acylphosphatase estimation.

The described optical test has shown itself accurate, simple and rapid: it is reliable even with crude tissue extracts, provided that not too strong an interference is caused by the presence of materials, like proteins or phenols, absorbing around 280 nm[9].

Riassunto. Viene descritto un metodo ottico continuo per la determinazione dell'acilfosfatasi, usando come substrato il benzoilfosfato di litio. Il metodo si basa sulla differenza di estinzione tra benzoilfosfato e benzoato.

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Istituto di Chimica biologica dell'Università di Firenze (Italy), April 20, 1966.

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The Activities of Citrate Cleavage Enzyme, Acetyl-CoA Synthetase and Lipoprotein Lipase in White and Brown Adipose Tissue and the Liver of the Rat during Development

The activity of citrate cleavage enzyme (E.C. 4.1.3.6), and to a lesser extent also that of acetyl-CoA synthetase (E.C. 6.2.1.1), correlates well with the nutritional state of the animal and available evidence suggests that particularly the former participates in fatty acid (FA) synthesis[1].

In suckling rats FA synthesis in the liver is low[2,3] and indirect evidence indicates that the same applies to adipose tissue[4], which shows low activity of hormone sensitive lipase, thought to be responsible for FA mobilization. It has been suggested that the high fat diet (milk) consumed by infant rats may be responsible for many of these metabolic conditions[5]. If that is true then one would expect low activities of citrate cleavage enzyme and acetyl-CoA synthetase and high activity of lipoprotein lipase, at least in adipose tissue, since this latter enzyme is said to be active in the transfer of lipids into adipose tissue[6].

Citrate cleavage enzyme and acetyl-CoA synthetase activities were assayed in high-speed supernatants (57,000 *g*, MSE centrifuge) of liver and white and brown

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