Automated enzymatic fluorometric method for the determination of pyruvic and lactic acids in blood

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SYNOPSIS A simple automated fluorometric method for the estimation of lactate and pyruvate is described. Analyses can be performed at 60/hour and can conveniently determine pyruvate and lactate in the ranges 0-5 mg. and 0-30 mg/100 ml. The methods give a high degree of analytical precision and good yields from recovery experiments.

In studies in intermediary carbohydrate metabolism in this laboratory (Wynn and Doar, 1966) the need has arisen for the accurate and rapid determination of blood pyruvic and lactic acids. Enzymatic methods for estimating these intermediaries are well established (Bergmeyer, 1963) and commonly utilize a nicotinamide-adenine dinucleotide (N.A.D.) lactate dehydrogenase (L.D.H.; EC 1. 1. 27, L-lactate; N.A.D. oxidoreductase) coupled system with measurement spectrophotometrically of the increase or decrease of reduced N.A.D. in the reaction. Reduced N.A.D. has a characteristic native fluorescence, whereas the corresponding oxidized form has not (Lowry, Roberts, and Kappahn, 1957; Greengard, 1956): this distinction between the reduced and oxidized N.A.D. forms the basis of a simple procedure for estimation of pyruvic and lactic acids by measuring change in fluorescence of N.A.D.H₂ involved in the reversible lactate dehydrogenase catalysed reaction:
$$\text{pyruvate} + \text{N.A.D.H}_2 + \text{H}^+ \rightleftharpoons \text{lactate} + \text{N.A.D.}$$
(Neilands, 1955).

At pH 7.4 in the presence of excess N.A.D.H₂ the equilibrium strongly favours formation of lactate, the pyruvic acid being reduced almost quantitatively. At pH 9.6 the reverse reaction occurs, with formation of N.A.D.H₂, and the quantitative conversion of lactate to pyruvate. The reaction goes to completion at this pH if a ketone trapping agent such as hydrazine or semicarbazide is used to fix the pyruvate.

This paper describes a simple, speedy automated procedure for fluorometric assay of pyruvate and

lactate, which provides an alternative to other automated enzymatic methods, *e.g.* that of Antonis, Clark, and Pilkington (1966) for lactate and pyruvate, and of Hochella and Weinhouse (1965) for lactate.

MATERIALS

REAGENT SOLUTIONS

0.6 M Perchloric acid

0.4 M Triethanolamine hydrochloride buffer, pH 7.4 Triethanolamine hydrochloride, 74.4 g., and 3.7 g. E.D.T.A.-Na salt are dissolved in about 800 ml. distilled water. The solution is adjusted to pH 7.4 with saturated sodium hydroxide, and made up to 1 litre with distilled water.

0.5 M Glycine buffer, containing 0.2 M hydrazine, pH 9.6 Glycine, 37.5 g., 20.5 g. hydrazine hydrochloride, and 2 g. E.D.T.A.-Na salt are dissolved in distilled water, the pH is adjusted to 9.6 with saturated sodium hydroxide, and the solution made up to 1 litre with distilled water.

Pyruvate standard Lithium pyruvate, 37.5 mg. ($\equiv$ 100 mg/100 ml. pyruvic acid), is dissolved in 100 ml. distilled water. Dilutions are made to give a range of working standards 0.5 mg./100 ml.

Lactate standard Zinc sarcosylactate, 55 mg. ($\equiv$ 100 mg./100 ml. lactic acid), is dissolved in 100 ml. distilled water. Dilutions are made to give a range of working standards 0.30 mg./100 ml.

The buffer solutions are filtered before use through fritted glass filters of fine porosity. This is important as it removes dust and other possibly fluorescent particles.

Enzyme-coenzyme reagent The enzyme and coenzymes are obtained from Boehringer Corporation (London) Ltd. The L.D.H. is a crystalline suspension of heart muscle enzyme in 2.2 M ammonium sulphate and is used without further treatment. Its specific activity is about 360 units/milligram of protein.

FIG. 1. Flow diagram for measurement of pyruvate and lactate by an automated fluorometric method.

METHOD

PYRUVATE ESTIMATION For pyruvate estimation to 10 ml. of 0.1 M phosphate buffer, pH 7.4, are added 2.5 mg. N.A.D.H₂ together with 2.5 mg. (as protein) of lactate dehydrogenase.

LACTATE ESTIMATION For lactate estimation to 10 ml. of 0.1 M phosphate buffer, pH 7.4, are added 200 mg. N.A.D. together with 10 mg. (as protein) lactate dehydrogenase.

The enzyme-coenzyme solutions are made up freshly in sufficient quantities for each batch of analyses, and are kept refrigerated during assay by placing in a crushed ice bath, otherwise there is loss of activity of about 2% per hour. The solutions are made up in 10 ml. or multiples thereof, each 10 ml. being sufficient for analysis of approximately 100 samples.

INSTRUMENTATION A Technicon AutoAnalyzer sampler II and proportioning pump, a 32°C. heating bath containing a 40 foot delay coil, a Locarte single-sided fluorimeter mark V fitted with flow cell, and a 10 mV potentiometric recorder comprised the instrumental system. The fluorimeter was fitted with a 360 mμ primary filter and a 470 mμ secondary filter. No modification of any module is required, except that a 5k ohm resistance is required across the fluorimeter-recorder outlet.

SAMPLE PREPARATION (LONDON, FAWCETT, AND WYNN, 1962) About 4 ml. of blood is withdrawn rapidly and transferred to a tube containing heparin; then 3 ml. of blood is pipetted slowly, continuously mixing with a small rod, into 6 ml. of 0.6 M perchloric acid in a centrifuge tube surrounded by ice and water. Manipulations are adjusted so that the interval between the midpoint of withdrawing the blood and midpoint of adding it to the perchloric acid is as near as possible to one minute. The tube is sealed with parafilm, the contents mixed carefully by inversion, and subsequently kept at 4°C. Each tube is centrifuged at 3,000 r.p.m. for 5 minutes and the supernatant used for analysis.

PROCEDURE The manifold and flow diagram are depicted in Figure 1. The sample is aspirated at 0.23 ml./min., air at 2.5 ml., and buffer at 3.9 ml./min. and are joined by a cactus before entering a single mixing coil. The stream is joined by the enzyme-coenzyme reagent at 0.1 ml./min., and both mix in a single mixing coil before entering a 40 foot delay coil maintained at 32°C. The stream finally enters the fluorimeter flow cell where it is activated by light at 360 mμ and the increase, or decrease, of fluorescence is measured at 470 mμ.

The buffer used for pyruvate determination is the triethanolamine hydrochloride buffer (pH 7.4) and for the lactate determination, the glycine buffer (pH 9.6).

The lag, and thus approximate reaction time, of the system is about seven minutes. The samples are run at

60 per hour with distilled water as wash, and using a 1 : 2 sample wash ratio cam.

RESULTS

The analytical ranges of the method are 0.5 mg. and 0.30 mg. for pyruvic and lactic acids respectively, and both produce linear calibration curves.

The analytical reproducibility of the method is good, and was checked by performing duplicated analyses on 50 samples. For pyruvate the standard error of a single sample¹ was ± 0.004 , the mean difference between duplicates being 0.03 over the range 0.5 mg., and for lactate over the range 0.30 mg. the standard error of a single sample was ± 0.026 with a mean difference of 0.13.

Results obtained for pyruvate assay (100 samples) were compared with those obtained by a manual spectrophotometric enzymatic method (Landon *et al.*, 1962). The results were indistinguishable.

Recovery of lactate in 20 experiments averaged 97% (96-98%) over the range 0.30 mg./100 ml., and of pyruvate 99% (98-101%) over the analytical range of 0.5 mg./100 ml.

DISCUSSION

Both methods are highly specific for their respective substrates. Only L-(+)⁺ lactic acid (sarcolactic acid) reacts, D-lactate does not. In similar amounts α -hydroxybutyrate and β -chlorolactate could enhance analytical results but these substances are rare. Meister (1950) pointed out that muscle L.D.H. is not entirely specific, in that other α -keto and α , γ -diketo acids may react at around neutral pH; however, their reaction rates are so slow that their contribution can be regarded as insignificant.

Many workers have noted the rapid loss of pyruvate from blood samples on shedding (Huckabee, 1956; Bueding and Wortis, 1940; Gloster and Harris, 1962; Landon *et al.*, 1962) and have endeavoured to overcome this with immediate deproteinization. It has further been noted that using perchloric acid as deproteinizing agent there is incomplete recovery of added lactate or pyruvate, and trichloroacetic acid has been substituted because of better recovery yields. The technique of Landon *et al.* (1962) has overcome these difficulties using perchloric acid, and there are good recoveries of both lactate and pyruvate.

FIG. 2. Recordings showing calibration curves (mg./100 ml.); reproducibility of paired samples of (A) pyruvate, (B) lactate.

The pyruvate standard was made up from commercial lithium pyruvate. Stock solutions of several pyruvate salts were compared and all gave similar analytical results, and all were stable several months in the refrigerator. A stock solution of pyruvic acid is stable for only a few days as are also the dilute working standards. Therefore working standards must be made before each batch of analyses. L-(+)⁺ zinc sarcolactate was used for standardization of the lactate method rather than the D-L salt.

In calculating the concentration of the stock standards a factor of $\frac{9.0}{8.54} \times \frac{1}{3}$ must be applied to allow for dilution of blood in perchloric acid and for blood water. (As 3 ml. of blood contains about 2.54 ml. of water the volume of solution after deproteinization is 8.54 ml. rather than 9 ml.) In the case of pyruvate 3% is added to correct for loss of pyruvate (Huckabee, 1956) in the one-minute interval between shedding and deproteinization.

The pH of buffers, incubation coil temperature, reagent concentrations, and flow rates were determined by considerable preliminary trial. Initially, enzyme, coenzyme, and buffer were mixed as a single solution, but this resulted in rapid denaturation of enzyme. This was obviated by making up small quantities of concentrated enzyme-coen-

¹Calculated as the standard error of a single determination $e = \sqrt{\frac{\sum d^2}{2N}}$, where d is the difference between two single tests performed on each sample, and N is the number of double determinations.

enzyme solutions and refrigerating in ice during analysis. The incubation coil temperature of 32°C. was chosen as enzyme denaturation is much reduced compared with 37°C. at which temperature blocked lines are common.

As shown by the precision of the method reproducibility is good, for there is little or no cross-contamination of samples (Fig. 2). The proposed method gave pyruvate results that were indistinguishable from those obtained by a manual spectrophotometric method previously reported from this laboratory (Landon *et al.*, 1962).

The method has been in use in this department for over one year and has proved reliable, precise, and economical.

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