REVISTA ESPAÑOLA DE FISIOLOGIA, 32, 281-288. 1976

Measurement of Enzymatic Activities in Turbid Suspensions by Double Beam Spectrophotometry *

R. García-Cañero and M. Gosálvez

Bioquímica Experimental
Clínica Puerta de Hierro
Facultad de Medicina
Universidad Autónoma
Madrid

(Received on March 3, 1976)

R. GARCIA-CAÑERO and M. GOZALVEZ. *Measurement of Enzymatic Activities in Turbid Suspensions by Double Beam Spectrophotometry.* Rev. esp. Fisiol., 32, 281-288. 1976.

A method for the determination of enzyme activities in turbid suspension is described, based on the use of a double beam or double wavelength spectrophotometer in one or two cell mode. It measures the disappearance of NADH, coupled to the activity of the enzyme, at a 340 wavelength or at a 340 minus 374 mμ wavelength pair. The method has been applied to the determination of pyruvate kinase activity, in the presence of isolated mitochondria, and other possible applications are discussed.

The measurement of the activity of several enzymes is based on the coupling of the enzyme to a series of enzymatic reactions which end in the reduction of NAD or the oxidation of NADH (2). These methods are based on the spectrophotometric measureemnt at 340 mμ of the appearance or disappearance of NADH in transparent solutions which are provided with all the chain of reactans and triggered with the addition of the enzyme (2).

New developments in the field of metabolic control have prompted studies of the competition between soluble enzymes and isolated organelles (9) and studies on the measurement of enzymes *in situ* in «permeabilized» cells (6, 8). For these studies the availability of a method for measuring the activity of enzymes present in solutions within cell suspensions or in suspensions of cell organelles which have a high turbidity would be highly desirable.

We describe here a method designed to measure in turbid suspensions, enzyme activities by coupling them to NADH disappearance and by using a double beam spectrophotometer set at 340 mμ, or a double wavelength spectrophotometer set at 340-374 mμ. The method has been

* This work was supported by Grant 12-1012-74 from the «Instituto Nacional de Previsión».

successfully applied to the study of the competition between mitochondria and pyruvate kinase (9) and could represent a new tool in future studies of metabolic control when applied to other enzymes and turbid systems.

Materials and Methods

Lactic acid was determined by the method described by HOHORST (10) in the supernatant of samples treated with 12 % perchloric acid and neutralized with 6N KCO_3H. Pyruvate kinase was assayed as described by BEIGENHERZ *et al.* (11), using the assay medium described below at 22° C, pH 7.2. A unit of enzyme activity was defined as the amount of enzyme which produces a μmole of pyruvate per minute at 22° C, pH 7.2, in this medium.

Rat liver mitochondria were isolated essentially by the method of SCHNEIDER (11). The respiration and coupling of the mitochondria were measured as described by ESTABROOK (5). The assay medium for the incubation of mitochondria together with the enzymes was composed of 250 mM sucrose, 10 mM Tris HCl, 7 mM MgCl, 5 mM Tris Pi, 20 mM KCl (pH 7.2, 22° C). Enzymes were purchased from Boehringer. A.G. Substrates and cofactors were purchased from Sigma Co. Mitochondria protein was measured as described by GORNALL *et al.* (7).

Double beam-double wavelength spectrophotometer. Although the double beam-double wavelength spectrophotometer was described by CHANCE (3), not many laboratories are well acquainted with double wavelength spectrophotometry. Also, double beam spectrophotometers exist widely while double wavelength spectrophotometers are scarcer and higher priced.

The spectrophotometer used throughout all of the study was the Hitachi-Perkin Elmer 356 double-beam-double wavelength spectrophotometer and was used in the double-beam and the double mode, depending on the case. In essence, this recording, ultraviolet-visible, spectrophotometer, splits the light radiating from the light source in two parts which are dispersed by means of a monochromator which includes two independently moveable gratings, thus giving birth to two beams of light having wavelengths λ_1 and λ_2 respectively. After being modulated with a chopper, (60 cycles) the two beams pass independently through graduating diaphragms (optical attenuators λ_1 and λ_2) and are both focused in a location where the cell is placed (double wavelength-one cell mode) or each beam can be focused separately in a different cell (double wavelength-two cells mode). By means of the chopper modulation, the beams strike sequentially and alternatively on their focusing point, about 17 miliseconds apart from each other. After the beams traverse the cell or cells their light intensity is detected by a photomultiplier placed very close to the cell or cells. The photomultiplier and electric amplification system separately recognize the intensity of both beams by means of a time sharing device which is synchronized with the modulating chopper. Each beam is converted to an electric siganl and the signal corresponding to the beam λ_1 is subtracter from the signal corresponding to beam λ_2, and the difference is recorded. Thus, in the double wavelength-one cell mode, the apparatus records the absorbancy of the sample at λ_2 mμ minus the absorbancy of the sample at λ_1 mμ. In the double-wavelength-two cells mode, the apparatus records the absorbancy of the sample placed in the cell passed through λ_2 minus the absorbancy of the sample placed in the cell passed through λ_1. The wavelength pair, λ_2-λ_1 is usually selected in a way so that λ_2 corresponds to the peak of absorption of the substante to be measured and λ_1 corresponds to a wavelength in which the nonspecfic absorption of the turbid sample can be substrated. In the double beam

mode λ_1 and λ_2 are made equal and therefore, two cells are always used, one cell with the sample plus the reference material and the other cell only with the reference.

The calibration of the apparatus is set to record quantitatively in optical density units, by means of the use of the optical attenuators. When the sample or samples are placed in the cell or cells, the absorbancy at both wavelengths are made equal by adjusting the optical attenuators. For example, if the absorbancy at λ_2 is higher than the absorbancy at λ_1, the optical attenuator of λ_2 is closed so that the beam λ_2 becomes thinner. Thus, the difference between the absorbancy at both wavelengths in made zero and the signal in the recorder corresponds to zero optical density. Any increment in absorbing material in the sample or any decrease of absorbing material in the sample would be represented by increasing (positive) or decresing (negative) optical density variations respectively which can be recorded in the electrically precalibrated scales of the apparatus. Any variation in optical density is always the result of the difference of absorbancy af both wavelengths.

Results

The principle of operation of the dual beam spectrophotometer permits the determination of small variations of optical density in turbid samples. Using the double wavelength mode, very small variations of optical density in very turbid samples can be recorded. Thus, this spectrophotometer can be applied to measure the activity of an enzyme present in a turbid suspension as long as the enzyme activity could be coupled to the appearance or disappearance of a chromophore with a specific peak of absorption and as long as the turbid material has a stable absorbancy.

As several enzymes can be measured spectrophotometrically, coupled to the disappearance or appearance of NADH, we selected NADH as the chromophore. On the other hand, as isolated mitochondria form suspensions of high turbidity, and the mitochondrial membrane presents a number of highly absorbing pigments, isolated mitochondria were selected as a source of turbidity.

Figure 1 offers a description of the performance of the dual wavelength spectrophotometer, detecting varying concentrations of NADH in the presence of high turbidity caused by the addition of mitochondria. The spectrophotometer was used in the dual wavelength-two cells mode. The sample cell was passed through a beam at 340 mμ and the reference cell was passed through a beam at 374 mμ. The experiment had the same results when using the double beam mode, using 340 as the wavelength.

The upper left graph of figure 1 represents the optical density changes recorded, increasing the concentration of NADH in the sample cell in the presence of various amounts of mitochandria present in the sample and reference cells. Rotenone was present in both cells to inhibit mitochondrial respiration and therefore keep endogenous mitochondrial pyridine nucleotides completely reduced. The reference cell canceled out all turbidity because, prior to the NADH addition to the sample cell, the difference of the absorption of mitochondria between both cells was made zero through optical attenuation. The graph shows that the change in optical density is linear with increasing concentrations of NADH in spite of the presence of increasing amounts of mitochondria. However, this linearity is only conserved between 0.0 and 0.1 units of optical density. As shown in the upper right graph of figure 1, the linearity does not occur for higher NADH concentrations in the presence of different amounts of mitochondria. This lack of linearity is due to the different optical attenuation for each beam at each mitochondrial

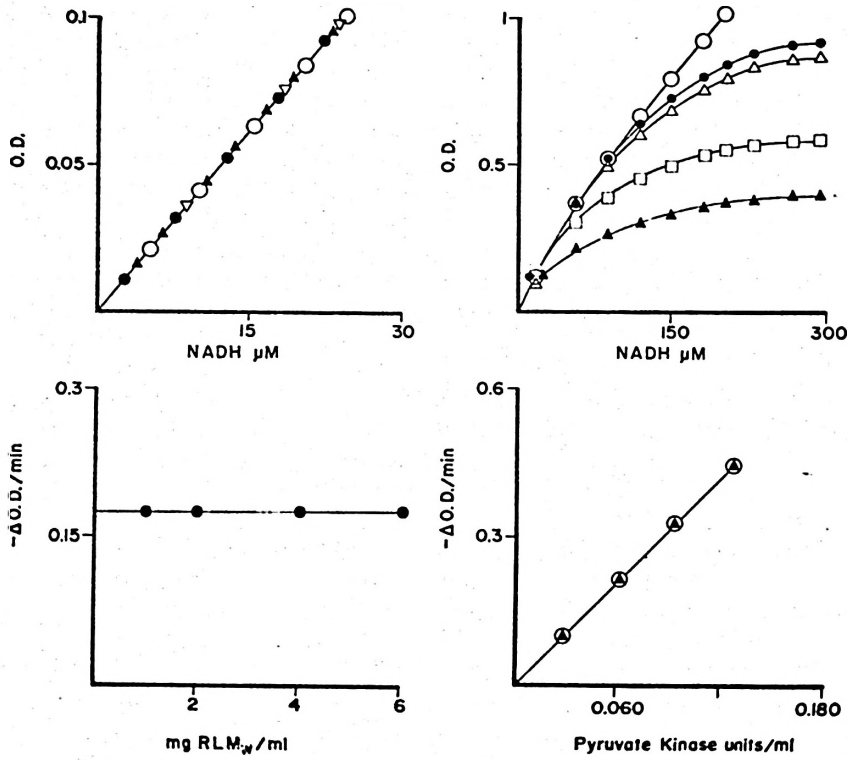

Fig. 1. *Double-wavelength-two cells mode operation.*
Upper graph: Increase of optical density with increasing NADH concentration in the presence of different amounts of mitochondria inhibited by 10 μM Rotenone. The NADH was added to the sample cell; the final volume was 3 ml. Left: ○: no mitochondria; ●: 1 mg protein/ml; △: 2 mg protein/ml; □: 3 mg protein/ml; ▲: 6 mg protein/ml. Lower graph: Decrease in optical density per minute with addition of, left: 10 milliunits of pyruvate kinase in the presence of different amounts of mitochondria and a coupling system composed of 30 μM NADH, 5 mM phosphoenol pyruvate, 1 mM ADP, 0.5 units/ml lactic dehydrogenase and 10 μM Rotenone. Right: different amounts of pyruvate kinase in the absence (○) or presence (▲) of 6 mg mitochondrial protein/ml. For more details, see text.

concentration. Thus, the linearity, in the absence of mitochondria (clear circles, upper right graph) reaches almost 1.0 optical density. The linearity with 1 and 3 mg mitochnodrial protein per ml (black circles and clear triangles) reaches 0.5 optical density and the linearity in the presence of 4 and 6 mg of mitochondrial protein per ml (black triangles and crosses) only reaches 0.1 optical density. This means that for high turbidity the double beam or double wavelength two cells method can only be used to measure very small concentrations of NADH or for measuring initial rates of NADH disappearance.

The lower left graph of figure 1 represents an experiment in which the activity of 40 miliunits of pyruvate kinase per ml was measured in the presence of increasing mitochondrial concentrations. In this experiment, both cells had the indicated mitochondria and the appropriate amounts of phosphoenol pyruvate, ADP

and lactic dehydrogenase to couple the activity of pyruvate kinase to NADH disappearance. The sample cell was calibrated by adding 30 μMolar NADH and the reaction was started by adding the aliquote of pyruvate kinase to the sample cell. The enzyme activity was recorded in optical density and in less than one minute, all NADH disappeared. The graph shows that the activity of the enzyme was constant in spite of increasing the concentration of mitochondria. The lower right graph of figure 1 depicts a similar experiment in which increasing concentrations of the enzyme were measured in the absence (open circles) or presence (dark triangles) of mitochondria. There is a linear increase of optical density variation per minute with increasing enzyme concentrationns. Again, this experiment had the same result when using the double beam method with 340 nm as the wavelength.

These experiments of figure 1 demonstrate taht the double beam method or the two wavelength-two cells method permits enzymatic measurements coupled to the disappearance of NADH in the presence of turbid suspensions, as long as only small quatities of NADH are used. This method has been applied to measure the competition between pyruvate kinase and respiring coupled mitochondria (9). In such a case, the double-wavelength method was used because the reference wavelength, 374 mμ, represents an isosbestic point for mitochondria, a wavelength at which there is not an absorption change when the mitochondrial pigments are oxidized or reduced and therefore is ideal to cancel turbidity. The endogenous mitochondrial pyridine nucleotides suffer an oxidation with the addition of ADP, needed for the coupling system of the pyruvate kinase assay, when using respiring, coupled mitochondria. This represents a very small, initial decrease in optical density when measuring pyruvate kinase but does not interfere with the assay because after such initial oxidation, the level of the mitochondrial, reduced pyridine nucleotides remains in a steady state as long as there is oxygen present (4). Although, for the specific purpose of the competition between mitochondria and glycolytic enzymes, the method used was the two wavelength-two cells mode, for most purposes, in which the reference does not change at the sample's specific wavelength, the advisable modality would be to use a double beam apparatus set at 340 mμ. Such a modality is available in most laboratories.

The above described method is only useful for measuring small enzyme activities if the suspension is very turbid because the NADH concentration and its linearity are limiting factors. Figure 2 illustrates another modality of using the double wavelength spectrophotometer for measuring enzyme activities in turbid suspensions in excess of NADH. In this case, the spectrophotometer is used in the double wavelength-one cell mode and again, the wavelength pair is 340-374 mμ. It should be noted that NADH absorbs at 374 mμ, about one third of its absorption at 340 mμ.

The upper graph of figure 2 shows the variation of optical density recorded with increasing additions of NADH to the cell in the presence of different concentrations of isolated mitochondria. The variation of optical density with increasing NADH is bell-shaped at any mitochondrial concentration and only shows linearity in the descendent branch of the bell. The bell shape occurs because the absorption of NADH at 340 mμ becomes saturated very soon while the absorption at 374 mμ is not saturated with even large concentrations of NADH. This is explained in the upper graph of figure 2 in which the absorption changes, which occur at the two wavelengths with NADH additions in the presence of the indicated mitochondria, are measured separately. There is a linear increase in absorption at 374

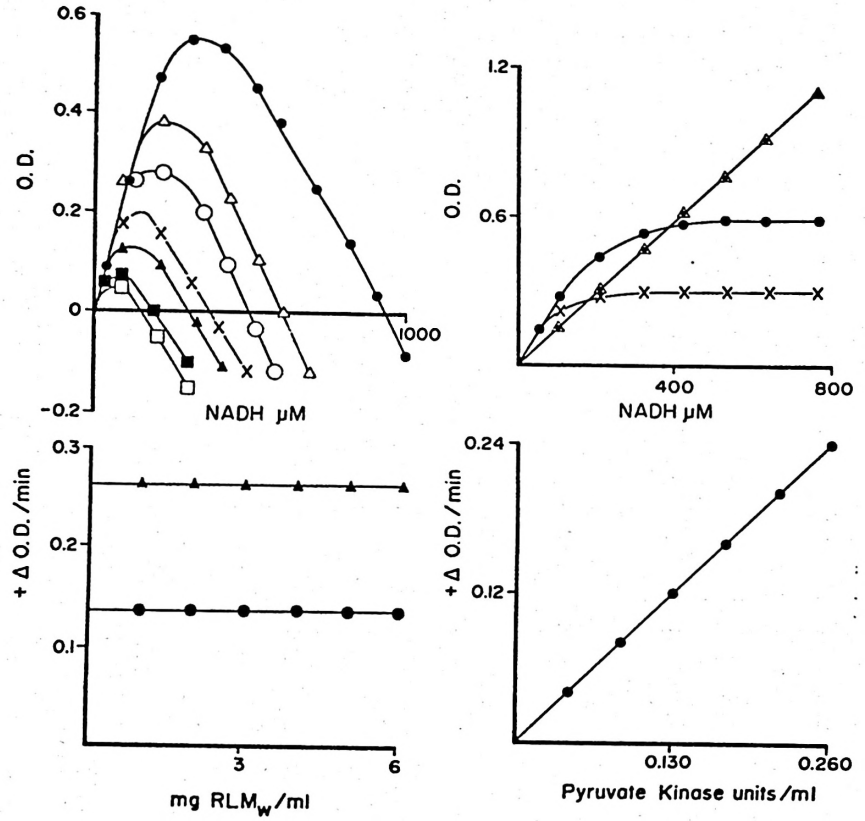

Fig. 2. *Two-wavelength-one cell mode operation.*
Upper graph: Changes in optical density with increasing NADH concentraions in the presence of different amounts of mitochondria plus 10 μM Rotenone. Left: at 340 and 374 mμ O: no mitochondria; Δ: 1 mg protein/ml; O: 2 mg protein/ml; ×: 3 mg protein/ml; ▲: 4 mg protein/ml; ■: 5 mg protein/ml; □: 6 mg protein/ml. Right: ●: at 340 mμ with 2 mg protein/ml; ×: at 340 mμ with 3 mg/ml; ●: at 374 mμ and no mitochondria; Δ: at 374 mμ with 1 mg/ml. NADH was first added to the sample cell. Lower graph: Increase in optical density per minute at 340 mμ and after at 374 mμ with addition of 130 milliunits (●) or 260 milliunits (▲) of pyruvate kinase, in the preesence of different amounts of rat liver mitochondria and a coupling system (left) or with addition of increasing concentrations of pyruvate kinase in the absence of mitochondria (right). For more details see text.

mμ with increasing NADH until 800 μm NADH either in the presence or the absence of mitochondria. However, at 340 mμ, the optical density increase which follows the addition of NADH, levels off at 0.2 or 0.6 optical density, respectively, depending on mitochondrial concentration. These experiments indicate that with the initial addition of NADH there is a positive optical density difference or absorbancy increase. A moment arrives, the sooner the higher mitochondrial concentrations, when the absorption at 340 mμ does not increase with increasing NADH concentrations because there is saturation at such a wavelength while the absorption at 374 mμ does not increase. As the instrument detects the difference

between both absorptions. There will be from this moment on, a linear decrease in optical density with increasing NADH. On this basis, the cell with the mitochondria can be loaded with NADH until, passing through the bell peak, the absorbance results again zero and the activity of an added enzyme coupled to NADH disappearance can be measured as optical density increase.

The left lower graph of figure 2 shows the activity of 130 and 260 miliunits of pyruvate kinase measured by this method in the presence of various amounts of mitochondria. The cell in each case was provided with the indicated amount of mitochondria, the amount of NADH required to obtain —0.1 units of optical density and the coupling system, composed of phosphoenol pyruvate, lactic dehydrogenase and ADP. The reaction was started by the addition of pyruvate kinase. Enzyme activity was recorded as an optical density increase which was followed linearly from one to five minutes, depending on the mitochondrial concentration. The lower right graph of figure 2 shows the variation of optical density per minute corresponding to various concentrations of the enzyme measured in the absence of mitochondria. In this experiment samples were taken from the cell at different times for determination of lactic acid, in order to calibrate the method. A change of 0.1 units of optical density corresponded to 100 nanomoles lactic acid per ml in the cell.

Discussion

The above described method represents, to our knowledge, the only present, existing method for measuring spectrophotometrically, enzyme activities in turbid suspensions. Although this method has been devised for measuring pyruvate kinase, in the presence of isolated mitochondria, it could easily be adapted to measure other enzymes in different turbid suspensions, as long as the enzyme activity could be coupled to the disappearance or appearance of a chromophore. The modality of election is the double beam mode in which the reference beam substracts the turbidity. This method is available to most laboratories. The two wavelength method is only applicable for special purposes in which a different reference wavelength must be selected, lying at an isosbestic point of the turbid sample which will not interfere with the absorption of the chromophore. The selection of the wavelength pair in these cases should be based on the spectra of both the chromophore and the turbid suspension.

The method can be adequately calibrated in its linear range with the addition of increasing quantities of the chromophore, however, for a precise quantitative calibration, a chemical determination of the product of the reaction in each condition is advisable.

Among the possible applications of this method stads the study of the competition between glycolytic enzymes and isolated mitochondria, the determination of the enzyme activities *in situ* in permeabilized cells (6, 8), the determination of enzymes bound to organalles and the determination of enzyme activities directly in undiluted tissue homogenates. In general, our method represents the possibility of measuring enzymes which are very scarce in biological materials without worrying about the turbidity introduced in the sample. The main drawback of the method is that, for very high turbidity, it can be used to measure low enzyme activities for a very short time. However, for most of the envisaged applications in which it is not necessary to reach extreme high turbidity, the range of measurable enzyme activities and the time of the measurement are quite similar to conventional spectrophotometric single beam method for the measurement of enzyme activity.

Resumen

Se describe un método para la determinación de actividades enzimáticas en suspensiones túrbidas. El método se basa en el uso de un espectrofotómetro de doble haz o de doble longitud de onda, midiendo a 340 mμ o con el par de longitud de onda 340 menos 374 mμ, la desaparición de NADH acoplada a la actividad del enzima. El método se ha aplicado para la determinación de piruvato kinasa en presencia de mitocondrias aisladas pero se discuten otras aplicaciones posibles.

References

1. BEINSENHERZ, G., BOLTZL, H. J., BUCHER, TH., CZOK, R.: *Z. Naturforsch*, **86**, 555-559, 1953.
2. BERGMEYER, H. V.: In «Methods of enzymatic analysis», Academic Press, New York, 1965.
3. CHANCE, B.: *Rev. Sci. Instruments*, **22**, 634-639, 1951.
4. CHANCE, B. and WILLIAMS, G. R.: *J. Biol. Chem.*, **217**, 409-415, 1955.
5. ESTABROOK, R. W.: In «Methods in Enzymology», Vol. 10 (R. W. Estabrook and Maynard, E. Pullman, eds.). Academic Press, New York, 1967, pp. 41-47.
6. FELIU, J. E., ARAGÓN, J. J. and SOLS, A.: 10th FEBS Meeting, Paris, July, 1975, Abstract 846.
7. GORNALL, A. G., BARDAWILL, G. J. and DAVIS, M. M.: *J. Biol. Chem.*, **177**, 751-766, 1949.
8. GOSÁLVEZ, M., GARCÍA-SUÁREZ, S. and LÓPEZ-ALARCÓN, L.: *Eur. J. Biochem.* In press, 1976.
9. GOSÁLVEZ, M., LÓPEZ-ALARCÓN, L., GARCÍA-SUÁREZ, S., MONTALVO, A. and WEINHOUSE, S.: *Eur. J. Biochem.*, **55**, 315-321, 1975.
10. HOHORST, H. J.: In «Methods in Enzymatic Analysis» (H. V. Bergmeyer, ed.). Academic Press, New York, 1965, pp. 266-270.
11. SCHNEIDER, W. C.: *J. Biol. Chem.*, **176**, 549-566, 1948.
12. SOLS, A.: In «Mechanisms of action and regulation of enzymes» (T. Keleti, ed.). *FEBS Symposium*, **32**, 211-221, North Holland Publishing Co., Amsterdam, 1975.