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Assay of Glycogen Phosphorylase by Differential Spectropolarimetry[1]

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Glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) catalyzes the reversible phosphorolytic cleavage of monomeric subunits from the nonreducing end-groups of glycogen according to the equation:

$$\text{inorganic phosphate} + (C_6H_{10}O_5)_n \rightleftharpoons \text{glucose 1-phosphate} + (C_6H_{10}O_5)_{n-1}$$

Measurements of the velocity of this reaction are widely used for estimating the concentrations of both phosphorylase *a* and phosphorylase *b* and for examining their catalytic properties. They have also been used to study the properties of related enzymes (phosphatases and kinases) that inactive or activate the phosphorylases and to assay for pyridoxal phosphate (1), which activates apophosphorylases. 3′,5′-Cyclic AMP concentrations have also been measured by the degree of activation of dephosphophosphorylase in the presence of activating kinases.

Two methods are ordinarily used for measuring the above reaction. The formation of inorganic phosphate[2] (P_i) under incubation conditions described by Illingworth and Cori (3) is usually measured by the Fisk-

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[2] Abbreviations: AMP, adenosine 5′-monophosphate; P_i, inorganic phosphate; G-1-P, glucose 1-phosphate; NADP and $NADPH_2$, oxidized and reduced forms of triphosphopyridine nucleotide, respectively.

SubbaRow colorimetric method (4). The generation of glucose 1-phosphate (G-1-P) by the reaction has been measured spectrophotometrically by a coupled enzyme assay which generates $NADPH_2$ from NADP in the presence of large excesses of phosphoglucomutase and glucose-6-phosphate dehydrogenase (5). Although both methods are adequate for many purposes, each has limitations that restrict its use. The P_i method does not yield a continuous record of enzyme action, so many samples must be taken with precise timing and appropriate colorimetric controls in order to accurately describe the course of the reaction. The coupled enzyme assay does yield a continuous record but this requires the consumption of a reaction product and the introduction of additional enzymes, substrates, and cofactors to the reaction mixture. It is not adaptable to the measurement of the reverse reaction.

The present polarimetric method provides a continuous record of enzyme action and can measure the reaction in either direction. It does not require the introduction of aditional enzymes, substrates, and cofactors and does not disturb the reaction equilibrium. In addition to its usefulness as an alternative to the methods described above, the method illustrates a type of measurement that can be more widely exploited for kinetic studies.

METHODS

Principle of Measurement

Differential spectropolarimetry can be used to measure small differences in the rotation of polarized light by two samples of optically active solutions. The recording spectropolarimeter, in effect, subtracts the rotation of one sample from that of the other and records only the difference. As in differential spectrophotometry, this process can be accomplished with a double-beam optical system. However, the Cary 60 recording spectropolarimeter, which was used in this study, has a single-beam system in which the polarized light that has passed through one sample is reflected through the second. The second solution rotates the plane of the reflected light in a direction opposite to that resulting from the action of the first. If the solutions and cell characteristics are identical, this process reverses the plane of rotation essentially to that produced by the polarizer. Differences in rotation may arise in two initially identical solutions as the result of a chemical reaction started in one of them by the addition of a catalyst. If the difference in rotation is proportional to the extent of chemical change, the recorded difference may be used to describe the course of the reaction.

Conditions of Measurement

Phosphorylase *b* was prepared from frozen rabbit muscle by the method of Fischer and Krebs (6), as modified by DeLange *et al.* (7). It was recrystallized at least twice and was stored as the crystalline suspension at 3° to 5°C, usually in concentrations of 10 to 20 mg/ml. For the kinetic measurements, aliquots of the suspension were diluted with several volumes of freshly prepared 0.03 *M* cysteine solution. The composition of the substrate solutions is described in the legends of the figures. To allow variation in composition, the complete mixtures (see legends of figures) were prepared as needed by mixing solutions (pH 6.8) of 0.04 *M* sodium β-glycerophosphate, 0.064 *M* sodium glucose 1-phosphate, 4% shellfish glycogen, 14 *M* β-mercaptoethanol, and 0.1 *M* sodium AMP. These component solutions, excepting the mercaptoethanol, were stored in the freezer.

Any particulate material present in the substrate solutions was removed by filtration at room temperature with a Millipore filter just before addition to the polarimetry cells.

Equal aliquots (2.80 ml) of substrate solution were placed in two matched cylindrical quartz cells of 3.0 ml capacity and 1 cm light path. The stoppered cells were placed on the differential platform, lowered into the sample compartment, and retained there for at least 15 min for temperature equilibration. Solutions that were more than 5° cooler than the operating temperature of the polarimeter (usualy 30°, in this study) were warmed in a 30° water bath to assist equilibration. A baseline (rotation vs time) was recorded, using the instrument settings required for the subsequent kinetic measurement.

To initiate the reaction, 5 to 30 μl of the diluted enzyme solution was added to the sample cell immediately following addition of an equal volume of 0.03 *M* cysteine solution to the reference cell. The cell contents were mixed carefully, avoiding the formation of air bubbles, and the cells were replaced in the same positions in the polarimeter. Chart progression was started with the master switch and the pen, set initially at 0.5 on the chart, was lowered to start the record. The time period from the addition of enzyme to the lowering of the pen was standardized at 25 sec. The recorded curves were extrapolated to this starting time by an extension of the initial approximately linear region. Some saving substrate solution and temperature equilibration time was effected by using the sample cells in which reaction equilibrium had occurred as reference cells for the subsequent reactions. In this case, reversal of the direction of pen movement was prevented by interchanging the position of the two cells;

the base line was adjusted by the zero suppression control. Unless stated otherwise in the legends to the figures, the measurements were recorded at 300 mμ with a period setting of 3 and a range setting of 0.04° full scale. The slit was set manually at maximal width. The scan rate (50 seconds per scale division) was controlled by the synchronous drive, using gears 7 and 8. Enzyme levels were usually adjusted to give reaction equilibria in 30 min or less.

RESULTS

One can calculate from the recorded specific rotations of glycogen (8) and glucose 1-phosphate (9) that the former compound has a slightly greater average positive rotation per glucosyl residue than the latter. Synthesis of glycogen from glucose 1-phosphate was expected therefore to result in increased positive rotation and the reverse reaction in a decrease. It was not valid to assume, however, that these shifts in rotation would accurately reflect the course of the reaction, especially when it was recognized that the newly synthesized glucosyl chains are unbranched and that they have a more peripheral position in the polymer, and therefore have a somewhat different environment than those of the natural glycogen primer. Thus it seemed essential to test whether the catalytic properties reflected in the rotational changes agreed satisfactorily with those determined by the conventional methods. Figure 1 shows records obtained in both synthesis of glycogen and its phosphorolysis. The changes in rotation were in the direction expected from the above-mentioned comparison of rotations per glucosyl residue. Doubling the substrate concentration (P_i or G-1-P) approximately doubled the rotations connected with the enzyme-catalysed attainment of reaction equilibrium.

Since the phosphorylase reaction does not result in a net change in the number of nonreducing end-groups of glycogen (10), glycogen concentration is cancelled out of the equation for the equilibrium constant:

$$K_{eq} = \frac{[P_i][\text{glycogen}]}{[\text{G-1-P}][\text{glycogen}]} = \frac{[P_i]}{[\text{G-1-P}]}$$

The reaction course, as expected from this formulation, is first order (10). The equilibrium occurring under reaction conditions similar to those used in our study favors glycogen synthesis strongly (3). Although we made no attempt to determine an accurate equilibrium constant, the data recorded in Fig. 1 indicate a $[P_i]/[\text{G-1-P}]$ ratio of 4, a figure in reasonable agreement with the recorded values.

Figure 2 is derived plot in which the approach to equilibrium as measured by the change in rotation is compared with that observed by measur-

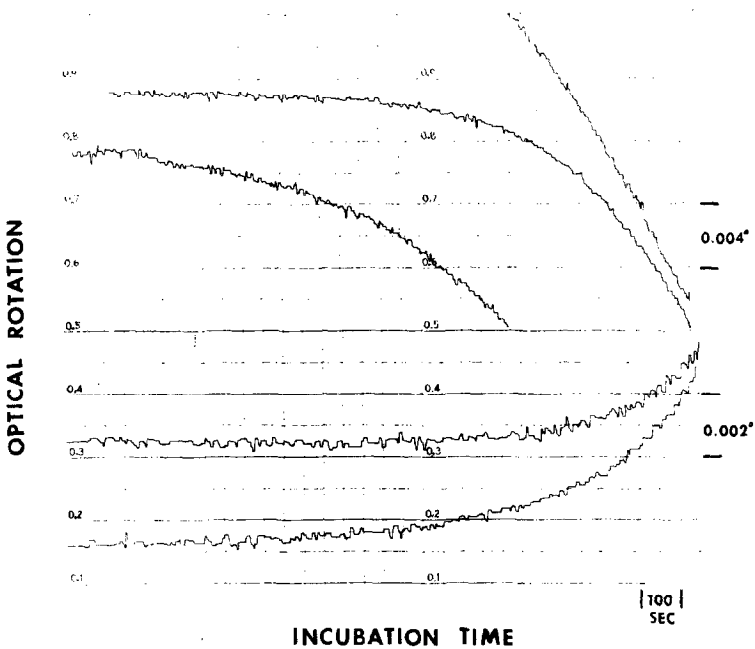

FIG. 1. Spectropolarimetric measurement of the glycogen phosphorylase reaction. Instrument settings were as described in the text except that a range of 0.02° full-scale was used for the lower curves. Pen center was switched from 0.5 to 0.0 during the run represented by (1) below, in order to expand the record. All four substrate solutions contained 1% glycogen, 0.001 *M* AMP, and 0.014 *M* β-mercaptoethanol. The concentrations of glucose 1-phosphate (or inorganic phosphate) and glycerol phosphate in these solutions, reading from top to bottom, were (1) 0.032 *M* glucose 1-phosphate, 0.01 *M* glycerol phosphate; (2) 0.016 *M* glucose 1-phosphate, 0.02 *M* glycerol phosphate; (3) 0.016 *M* inorganic phosphate, 0.02 *M* glycerol phosphate; (4) 0.032 *M* inorganic phosphate, 0.01 *M* glycerol phosphate.

ing P_i liberation in a reaction mixture incubated under the same conditions in an external bath. The close agreement of the two sets of data indicates that the rotational changes accurately reflect the course of the reaction under the reaction condition studied.

Figure 2 also presents a first-order plot of the reaction course as measured by the rotational change. As expected from their agreement with the results obtained with the phosphate method, the data describe a straight line and are consistent with the first-order course generally observed with the phosphorylase reaction under similar reaction conditions. Similar plots obtained at various concentrations of enzyme (Figs. 3 and 4) demonstrate that rate constants derived in this way provide a satisfactory measurement of phosphorylase activity.

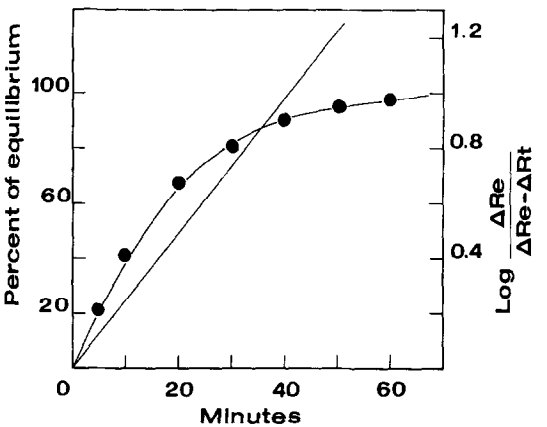

FIG. 2. Course of the glycogen phosphorylase reaction as determined by differential spectropolarimetry (solid curved line) and by measurement of inorganic phosphate liberation (points). Instrument settings were as described in the text except that the range was 0.1° full-scale and the temperature was 28°. Substrate composition was: 0.032 *M* glucose 1-phosphate, 1% glycogen, 0.02 *M* glycerol phosphate, 0.001 *M* AMP, 0.014 *M* mercaptoethanol. Phosphate analyses were done on 0.20 ml aliquots of reaction mixture by the method of Illingworth and Cori (2). R_e represents change in rotation occurring from the extrapolated zero time to equilibrium, and R_t is that occurring in a specified reaction period, t.

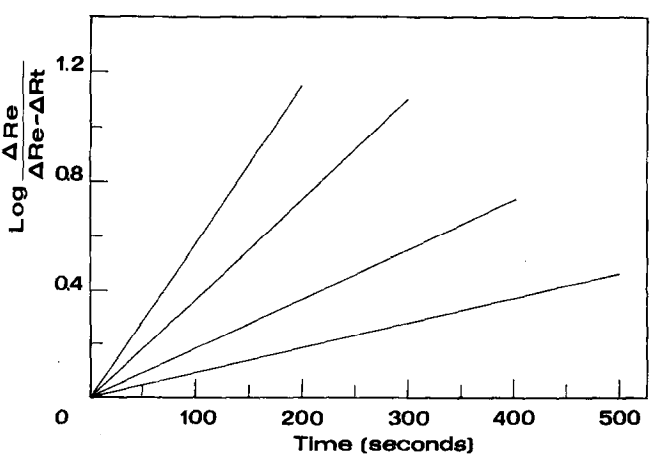

FIG. 3. First-order plots of the glycogen phosphorylase reaction as measured by differential spectropolarimetry. Instrument settings are as described in the text. Substrate composition was: 0.015 *M* glucose 1-phosphate, 1% glycogen, 0.02 *M* glycerol phosphate, 0.000 *M* AMP, 0.014 *M* β-mercaptothanol. Enzyme volumes for the different curves, reading from top to bottom, were 15, 10, 5, and 2.5 μl, respectively.

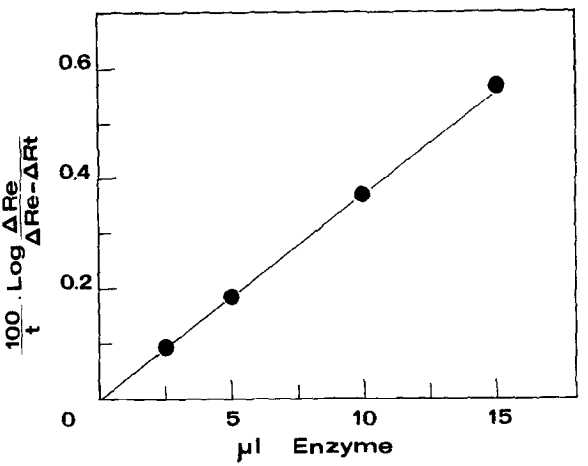

FIG. 4. Plot of first-order rate constants as a function of enzyme concentration. The points are derived from the slopes of the lines in Fig. 3.

DISCUSSION

In the phosphorylase assay method of Illingworth and Cori (3), one unit of activity is defined as one-thousandth of the first-order rate constant. Specific activities based on this assay have been reported for freshly prepared, thrice-crystallized phosphorylase *a* (3) and *b* (6). Specific activities calculated in a similar way from the first-order rate constants determined by spectropolarimetry may be compared with these recorded values, provided the incubation conditions are the same.

Illingworth and Cori specified the assay of samples removed from the incubation mixture at 5, 10, and 15 min as a check on the conformity of the reaction during that time interval to first-order kinetics. Variation of specific activity with time as a valuable signal of inappropriate assay conditions. The spectropolarimetric method described here provides this check throughout the course of the reaction.

It is apparent in Fig. 3 that the measurements required for each point of a standard curve (Fig. 4) are relatively rapid (5 to 10 min). Determination of the equilibrium rotation (R_e) can be accomplished within that time range and is therefore not tedious. If desired, however, one can apply the Guggenheim equation (11) to obtain first-order constants for reactions that do not reach equilibrium.

Under the conditions studied, glucose 1-phosphate concentrations varied from 0 to 0.032 M and glycogen concentrations from approximately 0.9 to 1.4% without deviation from first-order kinetics. Although we made no attempt to validate the application of the method for other conditions of assay, there is no reason to believe that the method could not be applied

generally. Additional tests of its validity would of course be desirable in any studies that use markedly different pH, temperature, or substrate composition.

Although the method was developed with the Cary 60 spectropolarimeter, it should be adaptable to less expensive and less versatile spectropolarimeters provided that they allow differential measurements. We have used the Cary 60 instrument with a fixed slit width with satisfactory results. The choice of 300 mμ as the wavelength was somewhat arbitrary; a relatively small diminution of the differential rotation as the wavelength was varied between 300 and 350 mμ indicated that higher wavelength settings would not seriously reduce the precision of the method. The method might therefore be adapted to instruments with fixed or manually controlled slits and with more restricted wavelength range.

Except for the restriction to optically active compounds, the limitations of spectropolarimetric methods for kinetic measurements are similar to those of spectrophotometric methods. Problems encountered in regulating the temperature and in recording the early stag of reaction are similar; both types of measurement require transparent solutions. In differential applications, however, polarimetry is not as severely limited by strong absorption of the incident light; wavelengths can usually be selected at which the optically active solutes do not absorb light significantly. In the studies reported here, for example, the rotation differential was generally less than 1% of the total rotation of the reaction mixture. An analogous relationship in differential spectrophotometry would create an intolerable noise-to-signal ratio due to the very weak signal provided by light that would escape absorption.

In applying differential spectropolarimetry for kinetic measurements, it is important to remember that such a large difference between the total and differential rotations may exist. Small differences in the substrate concentration in the two cells, which may be introduced by pipetting errors, may lead to substantial shifts in the baseline. Such shifts would occur immediately and would not seriously affect the interpretation of the kinetic curves. Care must also be exercised by adequate equilibration to obtain equal temperatures in the substrate solutions of the two cells. Significant differences in solute concentration and in the temperature dependence of the specific rotation of the substrate components may arise from relatively small temperature differences. These differences may result in slowly changing differentials of rotation as the two solutions approach the same temperature.

The application of differential spectropolarimetric measurements described here have led us to believe that the general method of analysis may be applicable to other reactions in which relatively slight changes in

optical rotation occur. Its use for the measurement of other reactions in which an optically active polymer (carbohydrate, polynucleotide, or protein, for example) is one of the reactants may be of special interest because such reactions are in many cases difficult or tedious to measure by present methods.

SUMMARY

A differential spectropolarimetric method has been developed for the measurement of glycogen phosphorylase activity. The reaction course measured in this way was first order, in agreement with results obtained by measuring inorganic phosphate liberation. The rate constants derived from the reaction curves were proportional to enzyme concentration.

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