STRAY LIGHT INTERFERENCE IN THE SPECTROPHOTOMETRIC MEASUREMENT OF ENZYME ACTIVITY AND DETERMINATION OF KINETIC PARAMETERS

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SUMMARY: Adenylosuccinate synthetase (E.C. 6.3.4.4) (GTP + IMP + L-aspartate $\xrightarrow{Mg^{++}}$ adenylosuccinate + GDP + Pi) was assayed spectrophotometrically at 280 nm using two double-beam instruments with different stray light specifications, and by a radioactive assay. Using the spectrophotometric assay, inhibition by high concentration of GTP and by GMP is observed; a Hill plot of the GMP inhibition data obtained with the instrument of higher stray light seems to show cooperativity in GMP binding. The radioactive assay shows no inhibition by GTP at the concentrations studied and no cooperativity in the binding of GMP. This example illustrates the care that must be exercised in the interpretation of results obtained spectrophotometrically in solution of high absorbance.

Spectrophotometric enzyme assays have been facilitated in the last few years by the introduction of medium-priced instruments with recorders and other time-saving devices. These instruments are convenient and useful in a considerable range of applications, but their monochromators may not be adequate for the measurement of enzyme activities in solution of high optical absorbance. This limitation is due to stray light which causes increasing non-linearity of absorbance as a function of concentration (1).

In this paper, some kinetic properties of the enzyme adenylosuccinate synthetase, as observed with a spectrophotometric assay using two double-beam spectrophotometers with monochromators of different quality and a radioactive assay, are compared. The results obtained with the lower-priced spectrophotometer would lead to seriously erroneous interpretation as to the properties of the enzyme. Cavalieri and Sable (2,3), using a single-beam instrument, have recently shown an apparent inhibition of glucose-6-phosphate dehydrogenase by DPNH caused by stray light.

MATERIALS AND METHODS

The spectrophotometers used were a Techtron Model 635 (stray light at

220 nm, 0.1%, according to the manufacturer's specifications) and a Cary Model 16 (stray light at 220 nm, 0.001%, according to specifications), both produced by Varian Instruments, Palo Alto, California.

Chemicals and enzyme used were obtained from commercial suppliers. Fast running F-254 silica gel-Kieselguhr precoated thin layer chromatography sheets (on aluminum) were obtained from E. Merck Co..

Adenylosuccinate synthetase was partially purified from Escherichia coli B (procedure to be published elsewhere). The spectrophotometric assay used is a modification of the method of Lieberman (4) and Rudolph and Fromm (5), measuring the increase in absorbance at 280 nm produced by the formation of adenylosuccinate. All assays were performed at 30° using silica cuvettes of 1 cm light path. A chart recorder with a full-scale range of 0.1 absorbance unit was used, and the activity is expressed in spectrophotometric units, where one unit corresponds to a change in absorbance of 0.001 per minute. The initial absorbance of a normal assay (containing 0.15 mM IMP and 0.15 mM GTP) was 1.45. The reactions were linear with time for periods of at least 1 to 2 minutes. Details for the individual assays are given in the figure legends.

The radioactive assay used was based on the incorporation of [^{14}C] aspartate into adenylosuccinate. The reaction mixture (50 µl) contained 50 mM HEPES, pH 7.7, 0.25 mM IMP, 2.5 mM $MgSO_4$, 3.3 mM [^{14}C] L-aspartate (0.1 µCi/assay), GTP at variable concentrations, 0.2 mg/ml phosphoenolpyruvate and 0.03 mg/ml pyruvate kinase. The reaction was started by addition of 10 µl of enzyme, incubated for 1 minute at 30°, and stopped with 10 µl 50 mM EDTA. 30 µl of each reaction mixture was plated on thin layer chromatography plates, with 5 µl of 10 mM carrier adenylosuccinate. The chromatograms were developed for 1.5 hours in ethanol-NH_4OH (70:30 v/v); the adenylosuccinate spots (R_F = 0.04) were located with UV light and the aspartate spots (R_F = 0.32) with ninhydrin. Each assay was performed in duplicate and with a zero-time control. The adenylosuccinate spots were cut out and counted in 0.4% Omnifluor in toluene in a scintillation counter. Enzyme activity is expressed in counts

per minute of [^{14}C] adenylosuccinate formed per minute after subtracting the zero-time values. The reaction was linear with time under the conditions used.

RESULTS

Figure 1 presents the observed relationship between absorbance at 280 nm

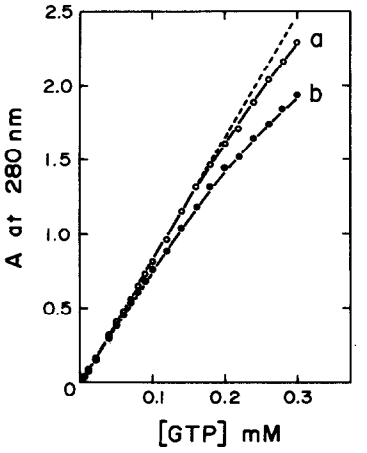

Figure 1. Relationship between GTP concentration and apparent absorbance at 280 nm using the Cary 16 (curve a) and the Techtron 635 (curve b) spectophotometers. Readings were performed in cuvettes of 1 cm light path against a water blank.

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and GTP concentration (the highest absorbing component of the enzyme reaction mixture at this wavelength). A larger deviation from linearity is observed with the Techtron 635 spectophotometer.

Curves showing apparent reaction rate as a function of GTP concentration, obtained with the two instruments and with the radioassay, are presented in Figure 2. The curves obtained with the spectophotometric assay, especially with the Techtron 635, would suggest the existence of a significant degree of substrate inhibition. No substrate inhibition is apparent with the radioassay.

GMP is a known inhibitor of adenylosuccinate synthetase (6). Hill plots of the effect of GMP on the enzyme are shown in Figure 3. The results obtained with the Techtron 635 do not give a linear plot, and the Hill coefficient

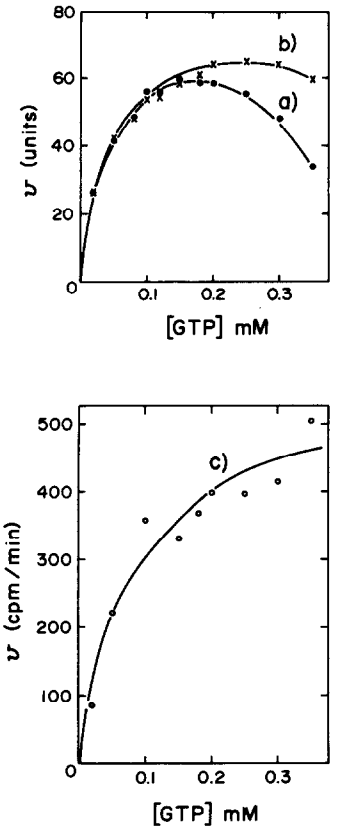

Figure 2. Velocity of the reaction catalyzed by adenylosuccinate synthetase as a function of the concentration of GTP. Curves a) and b) were obtained with the spectophotometric assay using a) the Techtron 635 and b) the Cary 16 spectophotometers. The assay mixtures contained 0.06 M K-HEPES buffer pH 7.7, 1 mM $MgSO_4$, 0.15 mM IMP, 5 mM L-aspartate, 1 mg Na-phosphoenolpyruvate, 120 µg of adenylosuccinate synthetase and variable amounts of GTP in a total volume of 3 ml. The reactions were started by addition of aspartate. Curve c) was obtained with the radioassay.

calculated at v/(Vo-v) = 1 is n = 2.14. This value suggests binding cooperativity for GMP. A value of n = 1.05, however, is obtained using the Cary 16, and the lack of cooperativity is confirmed with the radioactive assay (not shown), which gave a value of n = 1.27.

DISCUSSION

The results presented here indicate that considerable care must be exercised when studying enzyme kinetics with reaction mixtures of high absorbance. As pointed out by Cook and Jankow (1), a reaction measured spectro-

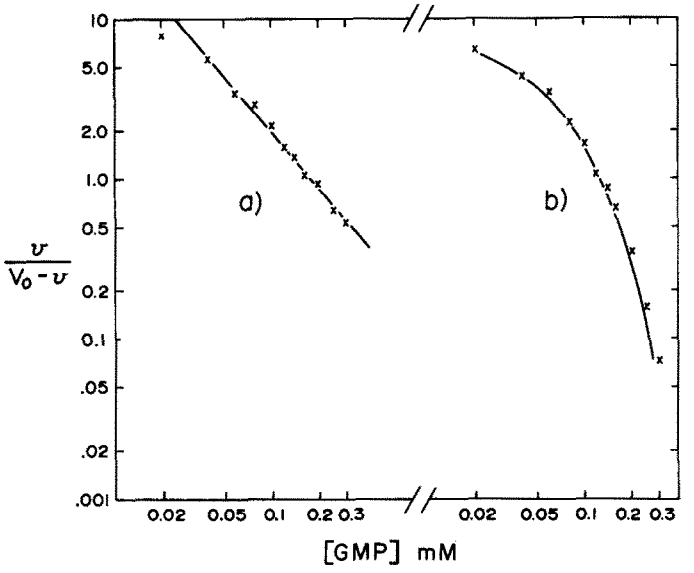

Figure 3. Hill plot of the effect of GMP on the activity of adenylosuccinate synthetase, as measured in a) the Cary 16 and b) the Techtron 635 spectophotometers. The assay mixtures contained 0.06 M K-HEPES buffer pH 7.7, 1 mM $MgSO_4$, 0.15 mM IMP, 0.15 mM GTP, 5 mM L-aspartate, 1 mg Na-phosphoenolpyruvate, 20 µg of pyruvate kinase, 120 µg of adenylosuccinate synthetase and variable amounts of GMP in a total volume of 3 ml. The reactions were started by addition of aspartate.

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photometrically may appear linear with time when examined in a narrow absorbance range (as is the case here), but a lower rate than the true rate will be observed if there is no linear relationship between measured absorbance and concentration of the absorbing compound. This explains the apparent substrate inhibition observed in Figure 2; thus inhibition is larger with the Techtron 635 because of the greater error introduced by stray light in this instrument, as shown in Figure 1.

An apparently small error due to stray light may have a large effect, however, in results like those shown in Figure 3. An increase in slope of the Hill plot with increase in GMP concentration is observed when the Techtron 635 is used. The molar absorbance of GMP at 280 nm is similar to that of GTP, so its addition increases the absorbance of the reaction mixture proportionately. The concomitant increase in stray light interference produces lower

apparent initial velocities, and therefore an increase in the slope of the curve. Large errors in Hill coefficient can thus result. Errors caused by stray light are not only quantitative in nature; the degree of non-linearity seen in Figure 1 can lead to qualitatively erroneous conclusions, such as the existence of substrate inhibition or markedly cooperative inhibition.

Stray light is essentially an optical problem and therefore cannot be compensated electronically in the instrument. A double-beam spectrophotometer will not show any advantage over a single-beam instrument with monochromator of similar stray light characteristics, since the stray light will always be present in the sample beam.

As shown in Figure 1, stray light interference can occur at relatively low absorbance levels (> 0.8 absorbance units in the Techtron 635). Therefore possible interference should be considered in any spectophotometric assay where one or more assay components absorb at the wavelength used. The best and simplest way to establish such interference is to determine the degree of deviation from linearity between the apparent absorbance and the concentration of the absorbing components. When interference is found, and a better spectophotometer is not available, it can sometimes be eliminated by using cuvettes of shorter light path, as suggested by Cavalieri and Sable (2). These authors have also proposed a method of calculation to correct for stray-light error (3).

In conclusion, the evidence presented here and by Cavalieri and Sable (2,3) indicates that the possibility of stray light interference must be eliminated before the apparent inhibitory effect of a compound on an enzyme, observed in a spectophotometric assay, can be truly accepted.

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