A Method for Estimating Glycine in the Presence of Excess Glutamate with *o*-Phthaldialdehyde

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Dedicated to the memory of Dr. Torben K. With

Glycine, Glutamate, *o*-Phthaldialdehyde

We present a method to determine concentrations of glycine between 1 and 3 mM in the simultaneous presence of 25–100 mM glutamate, which is a modification of the procedure originally described by Klein and Linser, Z. Physiol. Chem. **205,** 251 (1932).

Glycine and glutamate react with 150 mM *o*-phthaldialdehyde, glycine is quantitatively converted into a colored *o*-phthaldialdehyde-glycine-glutamate complex. By recording the absorbance of the complex at its maximum peak (around 540 nm), at 600 nm and 480 nm and applying a correction formula is possible to obtain a very good linear correlation when plotting the values of corrected absorbance versus the concentration of glycine. From these plots an extinction coefficient of 7.0×10^2 cm^{-1} M^{-1} was calculated for the complex. The method is highly reproducible and sensitive and solved the problem of glutamate interference when estimating low concentrations of glycine in mixture containing 10 to 50 times concentrations of the former aminoacid.

Introduction

So far, the activity of 4,5-dioxovaleric acid-transaminase could not be separated from that of glyoxylate-transaminase in different sources [1, 2]. Both enzymes can use either alanine (Ala) or glutamate (Glu) as the amino-donor [1–3]. Independently of which amino-donor is used, the reaction course could be estimated by measuring either 5-aminolevulinic acid or glycine (Gly), which are transamination products of 4,5-dioxovaleric acid and glyoxylate respectively. No difficulties exist to quantitate the amount of 5-aminolevulinic acid, however the measuring of glycine is neither specific nor simple. In the latter case, it should be taken into account that low quantities of the product Gly are to be determined in the simultaneous presence of relatively much higher concentrations of the amino-donor Ala or Glu (10- to 100-fold). Then, to be able to estimate the activity of glyoxylate: glutamate aminotransferase, we had to develop first a method for quantitating Gly in the presence of excess Glu.

Nearly 60 years ago, Klein and Linser [4] published a method for determining Gly by measuring spectrophotometrically the color of a complex formed between the aminoacid and *o*-phthaldialdehyde (OPT). In 1982 Foley and Beale [2] used the Klein and Linser [4] procedure to quantitate 1–3 mM Gly, but with low reproducibility and limitations. Consequently we have re-investigated the Klein and Linser's method and accordingly modified the experimental design so as to be able to measure low levels of Gly when the simultaneous concentrations of Glu are 10 to 100 times higher.

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Materials and Methods

Glycine and *o*-phthaldialdehyde were from Sigma Chem. Co., London, U.K. Sodium glutamate was from BDH Chemicals Ltd., Poole, England. All other chemicals were of the highest purity commercially available.

Standard solutions of Gly and Glu were prepared by dissolving the solid reagent in 5% (w/v) TCA or water and then adding an equal volume of 10% TCA respectives so to make the final TCA concentration to 5% and be in the same conditions attained when the enzymic reaction is stopped by addition of 10% TCA volume to volume.

A 150 mM OPT solution was freshly prepared by dissolving the chemical in a mixture of ethanol: H_2O (2:1, v/v).

Assay conditions

1 ml of the aminoacid solution, 0.4 ml of 1 M sodium phosphate buffer pH 8.0, 0.1 ml of 3 N

OHNa (to adjust final pH to around 7.0) and 0.35 ml of 150 mM OPT are mixed in a test tube. Reaction is allowed to proceed for 2 min at room temperature, then 2.5 ml of ethanol: H_2SO_4 (30:5, v/v) are added. The colored derivative formed is extracted with 2 ml of $CHCl_3$, 1 ml of the lower organic layer is taken, to this aliquot 0.2 ml of ethanol is added to eliminate any possible turbidity; 20 min later the complex formed can be spectrophotometrically estimated (Shimadzu UV 210 A) by measuring the absorbance at the wavelengths indicated below.

Results and Discussion

Interference of glutamate in the estimation of glycine

Preliminary experiments lead us to adapt the method of Klein and Linser [4] to the conditions described in Materials and Methods.

When the procedure was applied to estimate Gly alone, a very good linear correlation (regression coefficient r = 0.98–1.00) for concentrations of the aminoacid between 1 and 3 mM and absorption maxima recorded at 560 nm was obtained, with an absorption coefficient for the OPT–Gly derivative of 2.2×10^2 cm^{-1} M^{-1} (data not shown). However reproducibility from experiment to experiment was poor, meaning that in every assay it was necessary to run the calibration curve.

Klein and Linser [4] established that Glu did not interfere in the colorimetric determination of Gly, at least at low and nearly equal concentrations of both aminoacids. As our experimental conditions when measuring the activity of glyoxylate: glutamate aminotransferase, are far from those described by Klein and Linser [4], we investigated what sort of effect, if any produced Glu on the quantitation of Gly by reacting with OPT. First step was to record the absorption spectra of the colored complexes obtained when using Gly, Glu and both aminoacids together (Fig. 1). The quantitative formation of the complex between the reagent and each aminoacid (OPT–Gly and OPT–Glu) occurred at a concentration of 1 mM and 20 mM for Gly and Glu respectively. The former complex has a blue-violaceous color, with a maximum at 560 nm as already indicated, while the maximum absorption peak of the latter is at 500 nm and is pinky.

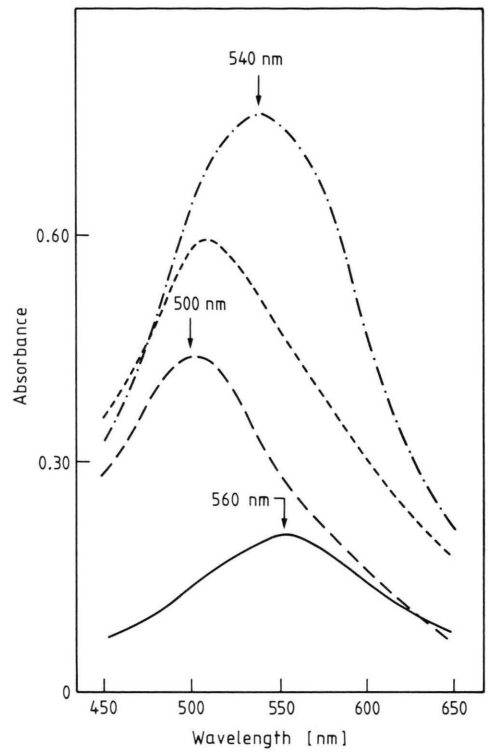

Fig. 1. Absorption spectra of the coloured compounds formed when glycine (—), glutamate (– –) or both (–·–) reacted with *o*-phthaldialdehyde. (----) represent the arithmetic sum of the absorbance of the absorption spectra of glycine (—) and glutamate (– –) separately. (Glycine) = 1.75 mM; (glutamate) = 150 mM.

When a mixture of Gly and Glu reacts with OPT, the color of the complex is that of the OPT–Gly derivative and the maximum is at 540 nm; however the resulting absorption curve is not the sum of the individual curves for each aminoacid, but higher. Therefore the system can not be analyzed as if it were a mixture of the separate complexes but more as if it were the product of another complex between the reagent and both aminoacids, that is OPT–Gly–Glu.

Results illustrated in Fig. 2 are in agreement with this assumption and show further the influence of different and increasing concentrations of Glu on the quantitation of Gly. When the absorbance at 560 nm due to the OPT-Gly complex is deducted from that produced by the OPT–Gly–Glu derivative, values 3 to 5 times higher than those corresponding to the OPT–Gly complex are ob-

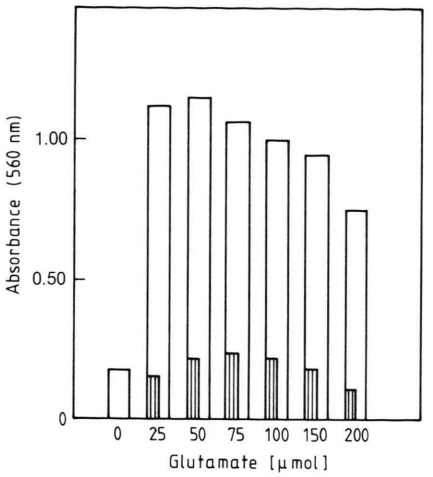

Fig. 2. Effect of varying concentration of glutamate on the simultaneous quantitation of glycine. Different bars correspond to absorbance at 560 nm of the coloured complexe formed: (□) 1.75 µmol of glycine plus variable concentration of glutamate as shown and (▥) glutamate at concentration indicated.

tained at certain concentrations of Glu. This potentiating effect of Glu appears to be inversily correlated with its concentration and could be attributed in part, to certain competition of the aminoacids for the reagent. At the concentrations of the aminoacids tested, Glu 15 to 50 times higher than Gly, the type of derivatives more likely to be produced will be OPT−Gly−Glu and OPT−Glu under these conditions OPT concentration limits the formation of OPT-Glu but not that of the complexes containing Gly (data not shown). Using 150 mM OPT, in the presence of 1 to 3 mM Gly and up to 75 mM Glu, the latter does not compete with Gly for OPT; its competitive effect becomes only evident at concentrations of Glu 100 mM and above. We have therefore assumed that maximum absorption at 540 nm corresponds to the OPT−Gly−Glu complex.

Reproducibility of the method

Using concentrations of 1−3 mM Gly, up to 75 mM Glu and 150 mM OPT (to convert all Gly in the corresponding OPT−Gly−Glu complex), when the value of the absorption at 540 nm was taken as a parameter proportional to the concentration of Gly, reproducibility of the method was poor. This was attributed to some turbidity in the chloroformic solution of the colored complex not completely prevented by addition of ethanol, which in fact changed not only the absolute value but also the maximum absorption peak towards lower or higher wavelengths.

An useful approach to overcome this interference is to obtain the graphic of the first derivative for each spectra (Fig. 3), so we can accurately determine the wavelength of the absorption maximum at the point where the first derivative is 0, at the same time the inflexion points will now be the maximum and minimum in the first derivative spectrum and thus we can mark out the interval corresponding to the spectral trace due to the complex.

In Fig. 3 we can clearly appreciate the spectral differences corresponding to the three complexes which nicely correlate with the direct absorption spectra previously recorded (Fig. 1). We are particularly interested in the formation of the OPT−Gly−Glu complex, which we emphasize is a measure of the concentration of Gly in a mixture containing both this aminoacid and Glu. In this particular case, the spectrum curve starts at 480 nm, its maximum is at 540 nm and it ends at 600 nm.

In 1950 Rimington and Sveinsson [5] and Allen [6] studying a similar problem related to the pres-

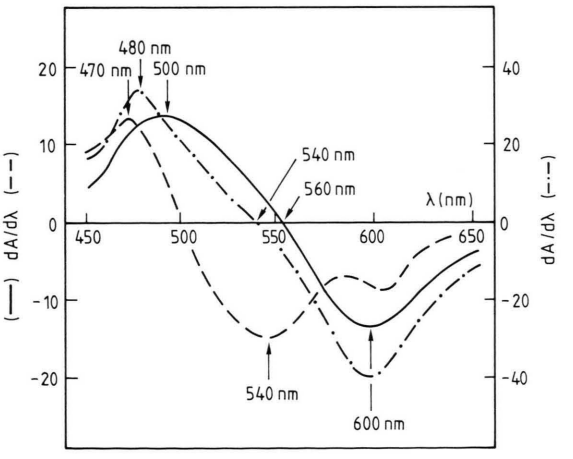

Fig. 3. First derivative spectroscopy (dA/dλ) of the absorption spectra corresponding to the coloured complexe formed from glycine (—), glutamate (– –) and both (–·–) with *o*-phthaldialdehyde. λ = wavelength (nm).

ence of interferences in the spectrophotometric determination of porphyrins and steroids respectively, came independently to the same conclusion and proposed a correction formula for the measuring of both porphyrins and steroids, which are still used today. By analogy with the Rimington and Sveinsson [5] and Allen [6] formula we have studied the relationship between the actual concentration of Gly and the absorption value obtained when applying the following equation:

$$A_c = 2\,A_{max} - (A_{600} + A_{480})$$

where A_c is the corrected absorption, A_{max} is the absorption at the maximum which is in the region of 540 nm and should be determined with accuracy recording the spectrum between 530 and 550 nm and obviously A_{600} and A_{480} are the absorbance values measured at 600 and 480 nm.

Using this correction formula we found a very good linear correlation ($r = 0.98-1.00$) between the corrected absorption and the concentration of Gly (1–3 mM) and reproducibility was excellent. Nearly superposed straight lines were obtained for concentrations of Glu between 25 and 75 mM (data not shown). Statistical treatment of the data gives a medium value for the slope of 0.178 ± 0.011 and absorbance of 0.123 ± 0.043 at concentration of Gly equal to 0. From these values the calculated absorption coefficient for the OPT–Gly–Glu was 7.0×10^2 cm^{-1} M^{-1}. The sensitivity of the assay was 3 times as that obtained when measuring absorbance only at 540 nm and results were now highly reproducible.

Conclusion

The original method of Klein and Linser [4] has been modified to allow to quantitate low concentrations of Gly (1–3 mM) in the simultaneous presence of much higher concentrations of Glu (25–75 mM). Following the procedure here described, recording the absorbance of the OPT–Gly–Glu complex at its maximum peak (between 530 and 550 nm), at 600 nm and 480 nm, and applying the correction formula $A_c = 2\,A_{max} - (A_{600} + A_{480})$ a very good linear correlation between the A_c and the concentrations of Gly is attained at concentrations of Glu ranging from 25 to 100 mM with an extinction coefficient of 7.0×10^2 cm^{-1} M^{-1}. The method is both very reproducible and sensitive and preliminary results would indicate that it can also be used to estimate Gly in the presence of aminoacids other than Glu. Employing the glycine quantification method here described, a parallel initial velocity pattern for the glyoxylate:glutamate aminotransferase, consistent with a ping-pong mechanism characteristic for transaminases, was obtained (in preparation). In contrast, no classic-kinetic behaviour could be established for this enzyme when Foley and Beale [2] measured aminotransferase activity using the original method of Klein and Linser [4].

Acknowledgements

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[1] T. Noguchi and R. Mori, J. Biol. Chem. **256,** 10335 (1981).
[2] T. Foley and S. I. Beale, Plant Physiol. **70,** 1495 (1982).
[3] B. L. W. Brock, D. A. Wilkinson, and J. King, Can. J. Biochem. **48,** 486 (1970).
[4] G. Klein and H. Linser, Z. Physiol. Chem. **205,** 251 (1932).
[5] C. Rimington and S. L. Sveinsson, Scand. J. Clin. Lab. Invest. **2,** 209 (1950).
[6] W. M. Allen, J. Clin. Endocrinol. **10,** 71 (1950).