Biochem. J. (1973) **135**, 893–896
Printed in Great Britain

Assay for Glutamine Synthetase Activity

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(*Received* 10 *September* 1973)

The use of phosphoenolpyruvate plus pyruvate kinase as an ATP-generating system in the assay for glutamine synthetase activity via the formation of γ-glutamylhydroxamate from glutamate and hydroxylamine with crude tissue preparations is shown to give values far in excess of the true glutamine synthetase activity of the tissue. This is due to the generation of pyruvate, which reacts with hydroxylamine to give a compound that is chromogenic with the ferric chloride reagent used for measuring γ-glutamylhydroxamate.

Of the several reactions catalysed by glutamine synthetase [L-glutamate–ammonia ligase (ADP), EC 6.3.1.2] (Tate & Meister, 1973), the formation of γ-glutamylhydroxamate is commonly used for determining enzyme activity:

$$\text{Glutamate} + NH_2OH + \text{ATP} \xrightarrow{Mg^{2+}} \gamma\text{-glutamylhydroxamate} + \text{ADP} + P_i \quad (1)$$

The product γ-glutamylhydroxamate is readily measured colorimetrically by its reaction with ferric chloride (Lipmann & Tuttle, 1945). High ADP/ATP ratios inhibit glutamine synthetase (Elliott, 1951) and it is customary to include an ATP-generating system in the reaction mixture to reconvert ADP into ATP (Berl, 1966). As we show here, when the ATP-generating system phosphoenolpyruvate plus pyruvate kinase is used, ATPase (adenosine triphosphatase) activity in crude tissue preparations causes the formation of high concentrations of pyruvate, which result in an artifact due to the chromogenicity of the product of pyruvate, hydroxylamine and the ferric chloride reagent.

Liver homogenates (10%, w/v), prepared in 0.25M-sucrose, subjected to sonic disruption and frozen overnight, were thawed and diluted with an equal volume of 50mM-Tris–acetate, pH7.4, and 0.1ml of the solution was used as the enzyme source. The species used were the laboratory rat (Sprague–Dawley), domestic chicken (female White Leghorn), catfish (*Ictalurus catus*) and snail (*Otalia lactea*). The basic reaction mixture contained 80mM-Tris–acetate, pH7.4, 40mM-hydroxylamine (the hydrochloride neutralized with KOH to pH7–7.4) (colorimetric assay) or 40mM-NH_4Cl (radiometric assay), 20mM-$MgSO_4$, 20mM-2-mercaptoethanol, 60mM-potassium L-glutamate, pH7.4, and 10mM-ATP (sodium salt, pH7). Either 10mM-potassium phosphoenolpyruvate plus 0.5–0.8 EC unit of pyruvate kinase (Sigma Chemical Co., St. Louis, Mo., U.S.A.) or 10mM-creatine phosphate plus 1.2–1.3 EC units of creatine kinase (Sigma Chemical Co.) were added as ATP-generating systems. The former is subsequently designated as 'plus phosphoenolpyruvate' and the latter as 'plus creatine phosphate'. Ouabain (0.5mM) and oligomycin (1.6μg/ml) were added as ATPase inhibitors. This is indicated as 'plus inhibitors'. For the colorimetric assay, the total volume was 0.5 or 1ml and for the radiometric assay 0.5ml. Incubation times and temperatures were: chicken, 15min at 37°C; rat, 25min at 37°C; catfish and snail, 60min at 30°C. In the colorimetric assay, the reaction was stopped by adding 1.5ml of ferric chloride reagent (Pamiljans *et al.*, 1962)/ml. The solution was centrifuged, left at room temperature and the E_{500} measured exactly 45min after the reaction was stopped. The apparent amount of the hydroxamate formed was estimated from the ε of the product of the hydroxamate and the ferric chloride reagent. The average ε observed was 1004 litre·mol^{-1}·cm^{-1}.

In the radiometric assay, potassium L-[U-^{14}C]-glutamate (60mM; sp. radioactivity 0.03μCi/μmol; made by dilution with unlabelled glutamate of [U-^{14}C]-glutamic acid from New England Nuclear Corp., Boston, Mass., U.S.A.) was used and the reaction was stopped by adding 0.1ml of 12% (w/v) $HClO_4$. The solution was neutralized with 50μl of 2.5M-KOH and the $KClO_4$ removed by centrifuging. Samples (20–50μl) of the supernatant fluid were spotted on 2.5cm×30cm strips of Whatman 3MM paper and picric acid was added as a tracking dye. The strips were electrophoresed in 50mM-sodium barbital buffer, pH8.6, at 17V/cm until the picrate had moved 8–9cm. Glutamate and glutamine separate by a minimum of 2cm under these conditions. The strips were dried and cut into 1cm sections, which were placed in 10ml of scintillation fluid [composed of 5.5g of Permablend (Packard Instrument Co.)/l of toluene] for radioactivity counting. The amount of [^{14}C]glutamine formed was calculated from the specific radioactivity of the glutamate substrate.

ADP, phosphoenolpyruvate and pyruvate were determined enzymically essentially as described by Adam (1963), Czok & Eckert (1963) and Bücher *et al.* (1963) respectively. For this, the reaction mixture for

Table 1. *Glutamine synthetase assay with rat liver homogenate*

The basic reaction mixture and incubation conditions are described in the text. ΔE_{500} refers to the difference from the zero-time control. Corrected activity (row f) is the difference between values in rows (d) and (e).

		Products (μmol/ml of reaction mixture)					
Assay modifications	ΔE_{500}	'Glutamyl-hydroxamate'	P_i	Pyruvate	Phosphoenol-pyruvate	ADP	Glutamine
Colorimetric assay							
(a) None	0.092	0.23	2.64	—	—	1.70	—
(b) Plus phosphoenol-pyruvate	0.452	1.13	4.63	—	4.24	0	—
(c) Plus phosphoenol-pyruvate, minus glutamate	0.226	0.56	4.65	6.80*	3.82	0	—
(d) Plus phosphoenol-pyruvate, plus inhibitors	0.245	0.61	1.96	—	6.71	0	—
(e) Plus phosphoenol-pyruvate, plus inhibitors, minus glutamate	0.072	0.18	1.95	2.17*	6.95	0	—
(f) Corrected activity	—	0.43	—	—	—	—	—
(g) Plus creatine phosphate	0.134	0.33	—	—	—	0.12	—
Radiometric assay							
(a) Plus phosphoenolpyruvate	—	—	3.62	2.80	—	—	0.10
(b) Plus creatine phosphate	—	—	—	—	—	0.11	0.25

* Based on molar extinction coefficient of product of the reaction between pyruvate–hydroxylamine and the ferric chloride reagent.

the colorimetric assay was divided into two 0.5ml portions. To one portion, ferric chloride reagent was added for measuring the amount of hydroxamate formed. To the other, 0.1ml of 12% $HClO_4$ was added and ADP, phosphoenolpyruvate and pyruvate were determined after the removal of the perchlorate. P_i was also measured in these latter solutions as described by Fiske & SubbaRow (1925).

As shown in Table 1, when the basic reaction mixture was used with rat liver homogenates, 0.23μmol of γ-glutamylhydroxamate could be measured colorimetrically. When the ATP-generating system containing phosphoenolpyruvate was added to the basic reaction mixture, there was an apparent 5-fold increase in the amount of hydroxamate formed. However, the omission of L-glutamate from the reaction mixture indicated that at least 0.56μmol of the 'γ-glutamylhydroxamate' measured in the presence of the phosphoenolpyruvate system was an artifact. Dialysis of the enzyme preparation showed that the high 'minus-glutamate' value was not due to endogenous glutamate. Our conclusion that this artifact is due to the chromogenicity with the ferric chloride reagent of the product formed between hydroxylamine and pyruvate generated by the phosphoenolpyruvate system during the conversion of ADP into ATP is based on the following observations. Even though the ϵ of the product is low (determined by us to be 83 litre$\cdot$mol$^{-1}\cdot$cm^{-1} under the same conditions used for measuring γ-glutamylhydroxamate), the amount of pyruvate generated is sufficient to account for most if not all of the E_{500} measured in the absence of glutamate. As shown in Table 1, 3.82μmol of the original 10μmol of phosphoenolpyruvate added remained at the end of the reaction, so theoretically 6.18μmol of pyruvate was formed. This correlates well with the value of 6.80μmol estimated from the ϵ of the pyruvate–hydroxylamine compound. In reaction mixtures containing hydroxylamine, no pyruvate could be determined enzymically with lactate dehydrogenase, which was shown not to be significantly inhibited by any of the reaction-mixture components; it is therefore assumed that the pyruvate–hydroxylamine product does not serve as a substrate for this enzyme. The value of 2.8μmol of pyruvate measured enzymically in reaction mixtures containing NH_4Cl for rat liver homogenate (Table 1) may possibly be in error, since for chicken liver homogenate a value of 7.5μmol was found under the same conditions. This latter value correlated with a value of

Table 2. *Glutamine synthetase assay with homogenates of chicken and catfish liver and of snail hepatopancreas*

The basic reaction mixture and incubation conditions are described in the text. Corrected activity (row f) is the difference between values in rows (d) and (e).

Assay modifications	'γ-Glutamylhydroxamate' (μmol/ml of reaction mixture)		
	Chicken	Catfish	Snail
(a) None	0.44	0.02	0.24
(b) Plus phosphoenolpyruvate	1.96	0.63	0.76
(c) Plus phosphoenolpyruvate, minus glutamate	0.59	0.45	0.33
(d) Plus phosphoenolpyruvate, plus inhibitors	1.73	0.37	0.56
(e) Plus phosphoenolpyruvate, plus inhibitors, minus glutamate	0.47	0.32	0.19
(f) Corrected activity	1.25 (0.95*)	0.05	0.37

* Value plus creatine phosphate.

7.1 μmol of pyruvate estimated from the ε of the pyruvate–hydroxylamine product in reaction mixtures containing hydroxylamine and chicken liver homogenate. The results obtained with the creatine phosphate system for rat liver also support the view of a pyruvate artifact. In the colorimetric assay, 0.33 μmol of γ-glutamylhydroxamate was obtained compared with a value of 1.13 μmol with the phosphoenolpyruvate system. Both were effective ATP-generating systems as judged by the low concentration of ADP remaining at the end of the reaction. Although not shown in Table 1, the ΔE_{500} observed in the absence of glutamate with the creatine phosphate system was zero; that is, with the creatine phosphate system the absorbance for the zero-time, minus-glutamate and heat-inactivated controls was the same. The decrease in the apparent amount of γ-glutamylhydroxamate formed in the presence of the ATPase inhibitors indicates that a large fraction of the pyruvate generated is the result of ADP formed by ATPase activity and not by glutamine synthetase.

We have consistently observed lower values for glutamine synthetase activity measured by the radiometric assay. The results for rat liver are shown in Table 1. With the phosphoenolpyruvate system, only 0.1 μmol of glutamine was determined; however, with the creatine phosphate system, 0.25 μmol was detected in the radiometric assay, which is in better agreement with the value of 0.33 μmol measured colorimetrically in the presence of the creatine phosphate system. When the phosphoenolpyruvate system is used in the colorimetric assay, even the 'best' control value (minus glutamate, plus inhibitors) gives a value at least 20% higher than that obtained with the creatine phosphate system because of the pyruvate artifact.

The marked species differences with respect to the magnitude of the error introduced by the pyruvate artifact can be illustrated by the data in Tables 1 and 2. Assuming that the glutamate-dependent formation of 'γ-glutamylhydroxamate' in the presence of the ATPase inhibitors (d minus e in the Tables) is a close approximation to the true glutamine synthetase activity of liver tissue from each species, then in the catfish this activity represents only 8% of the activity measured in the presence of the phosphoenolpyruvate system by using a zero-time control (*b* in the Tables). On the other hand, the effect with the chicken is much less, although the activity measured in the chicken with the creatine phosphate system is still only one-half that with the phosphoenolpyruvate system. It should be added that, using the various assay systems and controls described here as well as reaction mixtures not containing an ATP-generating system but in which the ADP/ATP ratio was controlled so as to not be inhibitory, we have obtained results on the mitochondrial localization of glutamine synthetase in chicken liver identical with those first reported (Vorhaben & Campbell, 1972) except that the total amount of activity is lower.

In summary, the use of phosphoenolpyruvate plus pyruvate kinase to generate ATP in the colorimetric assay of glutamine synthetase via reaction (1) by using crude tissue preparations introduces an artifact owing to the generation of pyruvate. Pyruvate, formed by both glutamine synthetase and ATPase activities, reacts with hydroxylamine, producing a product which is chromogenic with the ferric chloride reagent used to measure γ-glutamylhydroxamate. Neither a zero-time nor a heat-inactivated control will correct for this artifact. In view of this, studies in which these assay conditions have been used, for example on the distribution of glutamine synthetase in lower vertebrates (Lund & Goldstein, 1969) and on the non-identity of glutamine synthetase and glutamyltransferase activities in rat liver (Herzfeld, 1973), should

perhaps be reinvestigated by using other assay conditions.

This work was supported by U.S. National Science Foundation Grant GB-38138 and U.S. Public Health Service Grants AI 05006 and T01 DE 00118-10.

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