GUANINE NUCLEOTIDE DETERMINATION IN EXTRACTS OF WHEAT EMBRYO

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1. Introduction

A number of recent studies have been concerned with the relationship between the rates of RNA and protein synthesis and the cellular levels of nucleotide triphosphates [1–4]. Prerequisite for such studies are methods for accurate nucleotide analysis. While in the case of the adenine nucleotides a number of satisfactory procedures have been described [5–7], a direct method for the determination of the guanine nucleotides is as yet unavailable. In the present communication we present such a method for guanine nucleotide analysis with a sensitivity in the picomole range. We also describe its application to the analysis of extracts of wheat embryos.

2. Materials and methods

2.1. *Preparation of [^{32}P]phosphoenolpyruvate*

[^{32}P]PeP was prepared according to Lauppe et al. [8] with all reagents purified by distillation and stored under anhydrous conditions. A mixture containing 1 mCi of carrier-free ^{32}P, 0.2 μmol NaH_2PO_4, 7 mg β-chlorolactate, and 2 ml of acetonitrile was evaporated to dryness at room temperature in a flash evaporator. The residue was evaporated twice more to dryness, each time from 2 ml of acetonitrile. A freshly prepared solution of 42 μl (300 μmol) of triethylamine and 4 μl (40 μmol) of trichloroacetonitrile in 0.5 ml of dimethyl sulfoxide was added and the mixture was incubated for 2 h at 37°C. The reaction was stopped by the addition of 5 ml of distilled water and the product was purified by chromatography on a 3.0 × 0.7 cm Dowex-1 Cl^- column after two treatments with 50 mg of acid-washed charcoal. P_i was removed by washing the column with 25 ml of 0.006 N HCl and 10 ml of 0.01 N HCl, and the ^{32}PEP was eluted with 10 ml of 0.01 N HCl, 0.2 M KCl, the solution being neutralized with 1 M $KHCO_3$ immediately upon elution. The specific activity of the [^{32}P]PEP was determined as previously described [7].

Pyruvate kinase (rabbit muscle) and guanosine-5′-monophosphate kinase were obtained from Boehringer; hexokinase (Baker's yeast, 150 U/mg in 50% glycerol) from Miles Laboratories; nucleotide pyrophosphatase (Type III from Crotalus atrox venom), and guanine nucleotides from Sigma Chemical Co.

Pyruvate kinase was diluted for use with a solution containing 10 mM Tris–HCl, pH 7.6, 1 mM EDTA and 0.1% bovine serum albumin. Thirty two units of nucleotide pyrophosphatase (4.92 U/mg protein) were dissolved in 2 ml of 20% glycerol buffer (20 mM KCl, 2 mM Tris–HCl, pH 7.6, and 1 mM $MgCl_2$) and stored at 1°C.

2.2. *Assays*

2.2.1. GDP

A reaction mixture in a volume of 60 μl containing 4 mM $MgCl_2$, 70 mM KCl, 50 mM Tris–HCl, pH 7.6, 2 μg pyruvate kinase, 500 pmol [^{32}P]PEP (250 000 cpm with a biological activity of 50% [7]), 1 nmol ATP, and guanine nucleotides at a level between 25 and 100 pmol is incubated for 10 min at 30°C. After incubation, the mixture is heated for 2 min at 90°C, then cooled in ice for 2 min. Five microliters of 10 mM GTP (50 nmol) is added followed by 10 μl containing 2 μg hexokinase, 20 mM glucose, Tris–HCl, pH 7.6, and salts to maintain the initial concentrations. After careful mixing, a second incubation is carried out for 10 min at 30°C and the reaction is then

stopped by heating for 2 min at 90°C. A 30 μl aliquot is spotted in 10 μl increments at a distance of 3 cm from the bottom of a 20 cm PEI-cellulose thin-layer-chromatographic sheet (Brinkmann) that has been pre-washed with water. Salts are removed after application of the samples by soaking in methanol for 5 min. The chromatogram is developed in 0.75 M KH_2PO_4, pH 3.4 [9], until the solvent reaches the top; the GTP area is located with an ultraviolet lamp, cut out from the sheet and counted in a toluene based scintillator.

2.2.2. GMP

The assay is that described for GDP with the addition of 4 μg of GMP kinase to the initial reaction mixture. The level of GMP is calculated by subtracting the GDP content as determined from the assay in the absence of GMP kinase.

2.2.3. GTP

The reaction mixture in a volume of 30 μl contains 2 mM $MgCl_2$, 35 mM KCl, 25 mM Tris–HCl, pH 7.6, guanine nucleotides (25–100 pmol) and 0.032 units nucleotide pyrophosphatase. After incubation for 10 min at 30°C, the mixture is heated at 90°C for 2 min, then cooled in ice for 2 min. A 30 μl mixture containing 2 μg pyruvate kinase, 4 μg GMP kinase, 500 pmol ^{32}PEP (approximately 250 000 cpm), 1 nmol ATP, Tris–buffer and salts to maintain 4 mM $MgCl_2$, 70 mM KCl, 50 mM Tris–HCl, pH 7.6, is added and a second incubation is carried out for 10 min at 30°C. The remainder of the procedure is that described in the GDP assay. The radioactivity on the PEI-cellulose sheet at the GTP spot includes that due to GTP, GDP and GMP. The level of GTP is therefore calculated by subtracting the amount of GDP and GMP as determined in the GMP assay.

3. Results

The principle of the assay is the conversion of GDP to [^{32}P]GTP utilizing [^{32}P]PEP in the pyruvate kinase reaction [10]. [^{32}P]ATP which is also formed as a major product is removed with glucose and hexokinase. The analysis of GMP is made possible by its phosphorylation of GDP with GMP kinase, while the analysis of

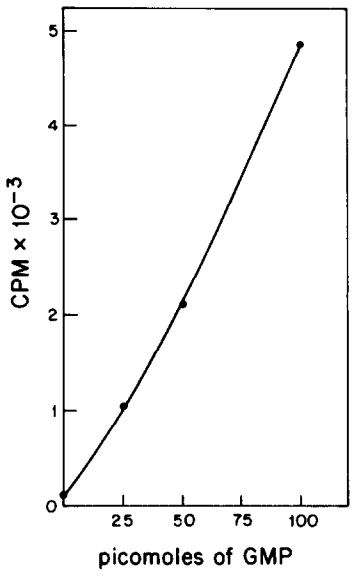

Fig.1. Analysis of GMP. The assay is that described in Materials and methods with the scale of the ordinate denoting the actual cpm obtained from the 30 μl aliquot chromatographed. The abscissa presents the pmoles of GMP present in the entire 75 μl incubation mixture.

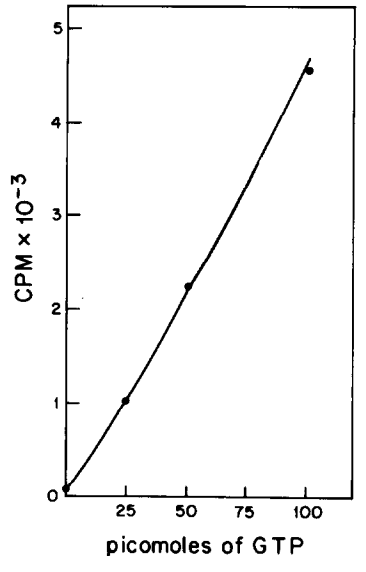

Fig.2. Analysis of GTP. The assay is that described in Materials and methods with details as presented in fig.1.

GTP is facilitated by its conversion to GMP with nucleotide pyrophosphatase. The reaction of [^{32}P]PEP with GDP does not go to completion at reasonable levels of PEP, so that a standard curve is required with each assay. The primary standard for the analyses of both GMP and GDP is GMP phosphorylated by ATP in the GMP kinase reaction. In the presence of PEP which serves to remove both ADP and GDP, the phosphorylation of GMP goes to completion [11]. GTP standards are also included in the GTP assays to ensure the completeness of the nucleotide pyrophosphatase reaction. Figures 1 and 2 show the application of the method. The assay is curvilinear probably because the reaction is carried out far below the K_m of GDP for the pyruvate kinase reaction. As a consequence of this curvilinearity, it is necessary to include standards throughout the range of assay.

Table 1 presents data examining the specificity of each of the assays. In these experiments charcoal adsorption is used to trap the ^{32}P-labeled nucleotides. The GDP assay is highly specific with essentially no reaction either with GMP or GTP. The efficiency of the hexokinase reaction can also be seen in that little ^{32}P-labeled nucleotide remains after the reaction with ADP. This is particularly crucial since adenine nucleotides are generally present in tissue extracts at 5–10 times the level of guanine nucleotides. The added step of determining the GTP radioactivity after thin layer chromatography also serves to eliminate any residual [^{32}P]ATP. In addition, it provides a substantial increase in the sensitivity of the assay (relative to charcoal adsorption) because of the low radioactivity blank in the GTP area of the chromatogram.

GTP is completely unreactive in the GMP assay i.e., in the absence of nucleotide pyrophosphatase, substantiating the validity of the assay for GTP. Superficially, this observation suggests that analysis of GTP could be carried out without thin layer separation since CTP and UTP would be hydrolyzed by the pyrophosphatase to UMP and CMP, both of which are unreactive with GMP kinase [11]. The analysis would involve determination by charcoal adsorption of ^{32}P transferred in the presence and absence of nucleotide pyrophosphatase. Careful consideration, however, shows that CDP and UDP would be included in the GMP assay, i.e., the determination in the absence of the pyrophosphatase, whereas both would be excluded from the GTP assay because of their prior conversion to CMP and UMP. We have made a direct test of this point by analyzing CDP and UDP in a GDP assay similar to that of table 1 and have found that a prior incubation with nucleotide pyrophosphatase completely removes their reactivity. The analysis for GTP therefore requires the thin-layer separation of the formed [^{32}P]GTP.

Application of the method to the analysis of trichloracetic acid extracts of wheat embryos is presented in table 2. The data show a substantial reserve of GMP and GDP in the dry embryo with only a low level of GTP. Upon germination, there is a marked decrease in the GMP and GDP pool concomittant with a substantial increase in the level of GTP. Particularly germane are the satisfactory recoveries of added GMP and GTP thus demonstrating that the sensitivity of the method is such that there is no interference from other components in the cell-free extract.

Table 1
Specificity of the guanine nucleotide assay

Nucleotide added (200 pmol)	^{32}P Adsorbed to charcoal (cpm)		
	GDP Assay	GMP Assay	GTP Assay
–	650	678	322
GDP	4154	4602	
ADP	942		
GMP	564	5730	4530
GTP	548	730	4014

The assays are those described in methods with 222 000 cpm in the experiments analyzing the GDP and GMP assays and 210 000 in the experiment analyzing the GTP assay. The incubation was terminated by the addition of 0.2 ml of 5% TCA containing 0.2% acid-washed charcoal and the remainder of the analysis was as previously described [7].

Table 2
Recovery of GMP and GTP from extracts of dry and germinated wheat embryos[a]

Extract	Nucleotide added	GMP Assay		GTP Assay		
		cpm[b]	pmol[c]	cpm[b]	pmol[d]	
–		52		72		
Dry embryos[e], 5 μl	–	675	37.5	912	42.5	(5.0)
	GMP (50 pmol)	2039	80.5			
	GTP (50 pmol)			2220	92	(54.5)
3 h-embryos, 5 μl	–	196	12	1182	54	(42)
	GMP (50 pmol)	1214	57			
	GTP (50 pmol)	225		2678	107	(95)

[a]The standard assay was used with the addition of 160 000 cpm [^{32}P]PEP. This level of [^{32}P]PEP is about 60% of that used in the experiments of figs.1 and 2, thus accounting for the lower levels of [^{32}P]GTP formed.
[b]Determined in the 30 μl aliquot analyzed by thin layer chromatography.
[c](GMP + GDP). The data are total nucleotides for the 75 μl reaction mixture.
[d]Total guanine nucleotides (GMP + GDP + GTP). The figure in parentheses is GTP, as determined by difference.
[e]125 mg of embryos extracted in 4.5 ml 5% TCA. After ether extraction and neutralization with 1 M Tris-base, the indicated aliquot was assayed.

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References

[1] Live, T. R. and Kaminskas, E. (1975) J. Biol. Chem. 250, 1786–1789.
[2] Swedes, J. S., Sedo, R. J. and Atkinson, D. E. (1975) J. Biol. Chem. 250, 6930–6938.
[3] Grummt, I. and Grummt, F. (1976) Cell 7, 447–453.
[4] Gross, K. J. and Pogo, A. O. (1976) Biochemistry 15, 2070–2081.
[5] Strehler, B. L. (1963) in: Methods of Enzymatic Analysis (Bergmeyer, H. U. ed) 559–568.
[6] Cooney, D. A., Jayaram, H. N., Homan, E. R. and Motley, C. F. (1974) Analyt. Biochem. 62, 157–165.
[7] Cheung, C. P. and Marcus, A. (1975) Analyt. Biochem. 69, 131–139.
[8] Lauppe, H. F., Rau, G. and Hengslenberg, W. (1972) FEBS Lett. 25, 357.
[9] Cashel, M., Lazzarini, R. A. and Kalbacker, B. (1969) J. Chromatog. 40, 103–109.
[10] Kayne, F. J. (1973) in: Enzymes (Boyer, P. D., ed) 3rd ed., Vol. 8, pp. 353–382, Academic Press, New York.
[11] Miech, R. P. and Parks, R. E. Jr. (1965) J. Biol. Chem. 240, 351–357.