REPRINTED FROM
Methods in Enzymology

Volume LVII

Bioluminescence and Chemiluminescence

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ACADEMIC PRESS New York San Francisco London 1978
A Subsidiary of Harcourt Brace Jovanovich, Publishers

[9] Determination of GTP, GDP, and GMP in Cell and Tissue Extracts

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Over the past two decades, an enormous amount of research effort has been directed toward determining the concentrations, and elucidating the physiological roles, of adenine nucleotides (ATP, ADP, and AMP) within cells and tissues; however, relatively little effort has been invested in obtaining comparable information on the additional intracellular purine and pyrimidine nucleotides. In recent years, guanosine triphosphate (GTP) has been shown to be an essential factor for the initiation, the aminoacyl tRNA binding, and the translocation processes of protein biosynthesis.[1] Routine quantitative analyses of guanine nucleotides (GTP, GDP, and GMP) in cell and tissue extracts have generally been neglected owing to the lack of a rapid, sensitive, and specific assay procedure comparable to the firefly luciferase bioluminescent assay for the determination of ATP. Several methods have been described for isolating GTP from cell and tissue nucleotide mixtures, including various separation procedures such as paper[2] thin-layer ion-exchange,[3,4] and high-pressure liquid[5] chromatography.

[1] F. Lucas-Lenard and F. Lipmann, *Annu. Rev. Biochem.* **40**, 409 (1971).
[2] G. Zweig and J. R. Whitaker, "Paper Chromatography and Electrophoresis," Vol. 1. Academic Press, New York, 1967.
[3] K. Randerath, "Thin-Layer Chromatography." Academic Press, New York, 1966.
[4] M. S. P. Manandhar and K. Van Dyke, *Anal. Biochem.* **60**, 122 (1974).
[5] P. R. Brown, *J. Chromatogr.* **52**, 257 (1970).

METHODS IN ENZYMOLOGY, VOL. LVII

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ISBN 0-12-181957-4

This chapter describes a simple, sensitive, and reproducible method for determining the concentrations of GTP, GDP, and GMP in extracts of biological materials, without prior chromatographic separations. This procedure is based upon the selective enzymic degradation of specific nucleotide triphosphates (NTP's) within cell extracts, and the fact that crude firefly luciferase preparations contain transphosphorylase enzymes and ADP, which together result in the quantitative production of ATP (and therefore light) in response to the addition of a wide variety of ribose and deoxyribose nucleotide triphosphates.[6,7] In addition to a complete description of the experimental procedures and equipment necessary for the determination of GTP, GDP, and GMP, a brief discussion of the pertinent analytical principles, assay limitations, and possible sources of interference is presented.

Assay Principles

$$\text{D-glucose} + \text{ATP} \overset{\text{HK}}{\rightleftharpoons} \text{D-glucose-6-P} + \text{ADP} + \text{H}^+ \quad (1)$$

$$\text{D-glucose-6-P} + \text{NADP}^+ \overset{\text{G6P-DH}}{\longrightarrow} \text{gluconate-6-P} + \text{NADPH} + \text{H}^+$$

$$\alpha\text{-D-glucose-1-P} + \text{UTP} \overset{\text{UDPG-PP}}{\longrightarrow} \text{UDP-glucose} + \text{PP}_i \quad (2)$$

$$\text{GMP} + \text{ATP} \overset{\text{GK}}{\rightleftharpoons} + \text{GDP} + \text{ADP} \quad (3)$$

$$\text{GDP} + \text{phosphoenolpyruvate} \overset{\text{PK}}{\longrightarrow} \text{pyruvate} + \text{GTP} \quad (4)$$

$$\text{ADP} + \text{GTP} \overset{\text{NDPK}}{\rightleftharpoons} \text{GDP} + \text{ATP} \quad (5)$$

$$\text{ATP} + \text{luciferin} \overset{\text{L}}{\rightarrow} \text{product} + \text{PP}_i + \textit{light} \quad (6)$$

where HK = hexokinase (EC 2.7.1.1), G6P-DH = glucose-6-phosphate dehydrogenase (EC 1.1.1.49), UDPG-PP = UDP-glucose pyrophosphorylase (EC 2.7.7.9), GK = guanosine-5'-monophosphate kinase (EC 2.7.4.8), PK = pyruvate kinase (EC 2.7.1.40), NDPK = nucleoside-5'-diphosphate kinase (EC 2.7.4.6), and L = firefly luciferase (EC 2.7. .)

Equipment and Reagents

Equipment

Glassware: All glassware used for nucleotide analyses should be thoroughly cleaned in order to eliminate the possibility of contamination; if possible, disposable glassware should be used. (a) Pyrex

[6] B. L. Strehler, *Methods Biochem. Anal.* **16**, 99 (1968).
[7] M. S. P. Manandhar and K. Van Dyke, *Microchem. J.* **19**, 42 (1974).

glass flasks, 50 and 250 ml, (b) glass culture tubes, 12 × 75 mm (Kimble), (c) glass vials, 18 × 45 mm (Scientific Products)

Pipettes: (a) 5- and 10-ml glass or disposable plastic; (b) automatic pipettes of 50-μl and 200-μl volume, with disposable tips; (c) automatic pipette 0.1–1.0 ml, with disposable tips

Incubation equipment: a water bath to maintain a uniform temperature (30°) during the period of the enzymic reactions

Boiling water bath

Instrument for quantitative light detection: Any one of a number of commercially available fluorometers, spectrophotometers, liquid scintillation counters, or ATP photometers is suitable for this purpose.[6,8]

Strip-chart recorder or oscilloscope

Automatic pipette: This is essential for peak-height measurements in order to ensure reproducible sample injection velocities.

Reagents

Potassium phosphate buffer, 225 m*M*, pH 7.4

$MgCl_2$ solution, 45 m*M*

D-Glucose/α-D-glucose-1-P solution, 1.5 m*M* each (Sigma Chemical Co.)

Phosphoenolpyruvate solution, 1.5 m*M* (Sigma Chemical Co.)

$NADP^+$ solution, 1.5 m*M* (Sigma Chemical Co.)

Tris(hydroxymethyl)aminomethane-HCl buffer (Tris buffer), 20 m*M*, pH 7.75 (Sigma Chemical Co.)

Sodium salts of GTP, GDP, GMP, ATP, ADP, and UTP (Sigma Chemical Co.)

$MgSO_4$ solution, 40 m*M*

Sodium arsenate buffer, 100 m*M*, pH 7.4

D-Luciferin, synthetic (Sigma Chemical Co.); optional

Firefly lantern extract (Sigma Chemical Co., catalog No. FLE-50)

HK/G6P-DH, crystalline, from yeast, 2:1 activity ratio (Sigma Chemical Co.)

PK, crystalline, from rabbit muscle, 350–500 units per milligram of protein (Sigma Chemical Co.)

GK, from hog brain, 10 units per milligram of protein (Boehringer Mannheim)

UDPG-PP, from beef liver, 100 units per milligram of protein (Boehringer Mannheim)

NDPK, from yeast (Sigma Chemical Co.); optional

[8] Picciolo *et al.*, this volume [43].

Methods

Sample Collection and Nucleotide Extraction

Extraction of guanine nucleotides from the appropriate sample material is the first step in the quantitative assay procedure. A number of different extraction media and methodologies are currently available for nucleotide determinations; these include boiling buffers,[9-12] sulfuric acid,[13] perchloric acid,[14] trichloroacetic acid,[15] *n*-butanol,[16] chloroform,[17] and *n*-bromosuccinimide.[18] The procedure finally selected for routine nucleotide extractions will depend upon the nature of the biological material to be analyzed (i.e., bacterial cells, mammalian tissue, etc.). Several different extraction techniques should be tested, using the biological material in question, to evaluate such analytical properties as the rate of nucleotide release, ionic interference of the extraction fluid(s), residual enzyme activity, chemical stability of the nucleotides, ease of operation, reproducibility, and the absolute levels of GTP resulting in the final cell extracts.

CAUTION: Since the rate of nucleotide triphosphate turnover in growing cells is extremely fast (i.e., for bacteria, 1–2 sec), it is essential that the biological material be extracted immediately after collection. Centrifugation[19] and vacuum filtration[20] may result in a decrease in the level of NTPs within the cell extracts.

Luciferase Enzyme Preparations

Each vial of lyophilized firefly lantern extract (50 mg/vial) is reconstituted by adding 5 ml of distilled water, 10 ml of 100 m*M* sodium arsenate buffer (pH 7.4), 10 ml of $MgSO_4$ (40 m*M*), and 2.5 μg of ADP. The enzyme mixture is allowed to stand for 3–4 hr at room temperature (23°–25°) in order to reduce the level of background light emission. After aging, the insoluble residue is removed by vacuum filtration or centrifugation. The

[9] E. Beutler and M. C. Baluda, *Blood* 23, 688 (1964).
[10] O. Holm-Hansen and C. R. Booth, *Limnol. Oceanogr.* 11, 510 (1966).
[11] S. Lin and H. P. Cohen, *Anal. Biochem.* 24, 531 (1968).
[12] K. Bancroft, E. A. Paul, and W. J. Wiebe, *Limnol. Oceanogr.* 21, 473 (1976).
[13] C. C. Lee, R. F. Harris, J. D. Williams, D. E. Armstrong, and J. K. Syers, *Soil Sci. Soc. Am., Proc.* 35, 82 (1971).
[14] R. E. Strange, H. E. Wade, and F. A. Dark, *Nature (London)* 199, 55 (1963).
[15] E. Beutler and K. Mathai, *Blood* 30, 311 (1967).
[16] E. W. Chappelle and G. V. Levin, *Biochem. Med.* 2, 41 (1968).
[17] A. M. Dhople and J. H. Hanks, *Appl. Microbiol.* 26, 399 (1973).
[18] N. H. MacLoed, E. W. Chappelle, and A. M. Crawford, *Nature (London)* 223, 267 (1969).
[19] H. A. Cole, J. W. T. Wimpenny, and D. E. Hughes, *Biochim. Biophys. Acta* 143, 445 (1967).
[20] O. Holm-Hansen and D. M. Karl, this volume [8].

clear filtrate or supernatant is transferred to a clean flask, and the enzyme preparation is now ready for use. Since considerable variation occurs between individual vials of firefly lantern extract, the contents of several vials should be pooled whenever large volumes of enzyme are required.

Standard Nucleotide Solutions

For each firefly luciferase preparation (1 or more vials), a set of nucleotide standards must be analyzed. Stock GTP, GDP, and GMP solutions (2 μM) are prepared in Tris buffer and stored frozen (−20°) in 5-ml aliquots. When required, a single vial is thawed and working solutions (1 nM to 100 nM) are made up by dilution of the stock with Tris buffer. A separate set of standard solutions is prepared for each assay and discarded after a single day's use. This procedure will eliminate any problems due to GTP hydrolysis.

GTP Determinations

Two-hundred microliters are pipetted into a disposable glass culture tube (12 × 75 mm), labeled and placed into a test tube rack. A series of GTP standard solutions covering the full range of expected experimental values, as well as reagent blanks, are also processed simultaneously. Fifty microliters of a solution containing 75 mM potassium phosphate buffer (pH 7.4), 0.5 mM $NADP^+$, 15 mM $MgCl_2$, 0.5 mM D-glucose, 0.5 mM α-D-glucose-1-P,HK/G6P-DH (2 units ml^{-1}) and UDPG-PP (5 units ml^{-1}) are pipetted into each tube. The samples are incubated for 15–30 min at 30°, followed by a 2-min enzyme deactivation period in a boiling water bath (100°). The actual time required for the complete conversion of ATP and UTP to ADP and UDP-glucose, respectively, should be determined by conducting a time-course experiment using ATP and UTP concentrations similar to those present in the nucleotide extracts. After heat deactivation of the enzymes, the samples are brought to room temperature prior to the actual GTP assay procedure.

Either the initial rise of the light-emission curve, the peak height of luminescence, or some integrated portion of the subsequent reaction kinetics can be used to relate unknown sample extracts to the light emitted from standard GTP solutions. It is recommended that the photometer (or similar light-detection instrument) be interfaced with an analog recorder, in order to follow the reaction kinetics.

CAUTION: All GTP assays should be conducted in reduced illumination, and fluorescence lighting should be avoided because of the chemiluminescent properties of certain glassware and plasticware.

Initial Rise and Peak-Height Measurements. Pipette 1 ml of the firefly

luciferase preparation into a small glass vial (18 × 45 mm), and record the background light emission. Using an automatic pipette, withdraw 200 μl of the appropriate sample extract (or GTP standard solution) and position the pipette assembly onto the photometer. Inject the sample into the enzyme preparation and record the photometer digital display; or alternatively, measure the peak height of light emission directly from the strip-chart recorder trace.

NOTE: Reliable initial rise or peak height measurements require specially designed equipment for rapid and reproducible injection velocities to ensure complete mixing of the reagents. To the author's knowledge, only three instruments are commercially available at the present time that have this extended capability. The analytical characteristics and properties of these instruments have recently been evaluated and compared.[8]

Integrated Light-Flux Determinations. Pipette 1 ml of the firefly luciferase preparation into a small glass vial (18 × 45 mm), and record the background light emission. Remove the vial from the photometer, inject 200 μl of the appropriate sample extract (or GTP standard solution) into the enzyme preparation, mix the contents thoroughly for 5 sec, and replace the vial into the photometer. The light flux is integrated, either electronically or manually, over a predetermined portion of the light-emission curve. It is important that integrated light-flux determinations be restricted to the early portion of the reaction (<30 sec) in order to reduce various sources of analytical interference (see Fig. 1 and the section on analytical interferences and troubleshooting, this chapter, pp. 93–94).

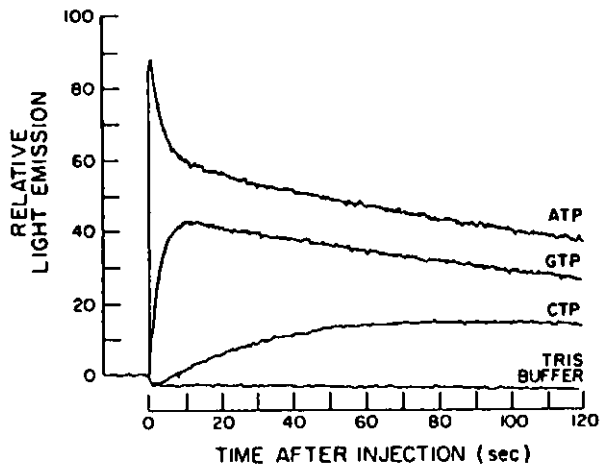


FIG. 1. Light emission kinetics and relative rates of ATP, GTP, and CTP reactivity upon injection of the samples into crude luciferase enzyme preparations. The zero light-emission level represents the enzyme background activity, and values < 0 indicate dilution of this endogenous reactivity. The concentration of all three nucleotides was 20 n*M*.

GTP, GDP, and GMP Determinations

For each set of guanine nucleotide determinations, 200 μl of the sample are pipetted into a series of 4 disposable culture tubes (12 × 75 mm), labeled (A–D) and placed into a test tube rack. For GTP determinations (tube A), 50 μl of a solution containing 75 m*M* potassium phosphate buffer (pH 7.4), and 15 m*M* $MgCl_2$ are added to each tube. For GTP plus GDP determinations (tube B), 50 μl of a solution containing 75 m*M* potassium phosphate buffer (pH 7.4), 15 m*M* $MgCl_2$, 0.5 m*M* PEP, and 20 μg of PK are added to each tube. For GTP plus GDP plus GMP determinations (tube C), 50 μl of a solution containing 75 m*M* potassium phosphate buffer (pH 7.4), 15 m*M* $MgCl_2$, 0.5 m*M* PEP, 10 ng of ATP, 20 μg of PK, and 5 μg of GK are added to each tube. In order to determine the efficiency of coupled reactions (3) and (4), 50 μl of a solution containing 75 m*M* potassium phosphate buffer (pH 7.4), 15 m*M* $MgCl_2$, 0.5 m*M* PEP, 10 ng of ATP, 20 μg of PK, 5 μg of GK, and an internal GMP standard are added to each D tube. The actual concentration of GMP added will depend upon the experimental conditions; however, the internal standard concentration should be kept well within the linear range of the GTP assay and, ideally, should be approximately equal to the concentration of GMP already contained within the sample extract. Standard GTP solutions should also be processed, simultaneously, with each of the four reagents.

In addition to the GTP standard solutions, it is advisable to prepare, and allow to react, several GDP and GMP standards, as well as standard solutions containing known molar ratios of GTP, GDP, and GMP together, in order to monitor the efficiency of reactions (3) and (4). Enzyme blanks (Tris buffer plus the appropriate enzyme solutions) should also be prepared, since certain commercial enzyme preparations contain contaminating nucleotides. All samples are incubated at 30° for 60 min and then place into a boiling water bath (100°, 2 min) in order to denature the enzymes.

NOTE: It is essential that the PK be deactivated since the addition of PEP and active PK to crude firefly luciferase preparations will cause rapid production of ATP from ADP contained within the enzyme mixture. Once the samples have adjusted to room temperature, they are treated as GTP samples, and assayed as described in the preceding section (GTP determinations).

Data Reduction

GTP Data. Determine the net light emission by subtracting the appropriate enzyme blank value from the total light emitted in each of the sample or GTP standard reactions. Construct a standard curve relating net light emission (initial rise, peak or integrated) to GTP concentrations. From this

curve, the GTP concentration in each sample extract can be calculated, and, knowing the proportion of the total sample volume actually assayed and the volume (or weight) of material originally extracted, the [GTP] ml^{-1} (or [GTP] $cell^{-1}$, [GTP] g^{-1} of tissue, etc.) of the biological material can be determined.

GTP, GDP, and GMP Data. Determine the net light emission by subtracting the appropriate enzyme blank value from the total light emitted in each of the sample or GTP standard reactions. Standard curves are constructed by plotting net light emission (initial rise, peak, or integrated) versus GTP concentrations. The GTP concentration in each of the A tubes is calculated from the GTP standard curve prepared with A reagents; likewise, the GTP concentration in tubes B, C, and D are calculated from the GTP standard curves prepared with B, C, and D reagents, respectively. GDP concentrations are determined by subtracting tube A determinations from tube B determinations. GMP concentrations are determined by subtracting tube B determinations from tube C determinations, and by correcting for the efficiency of the GK reaction (if necessary), using the internal standard data as follows:

$$\% \text{ recovery} = \frac{[\text{tube D}] - [\text{tube C}]}{[\text{internal standard}]} \times 100$$

$$[\text{GMP}] = \frac{[\text{tube C}] - [\text{tube B}]}{\text{decimal equivalent of \% recovery}}$$

Capabilities

The sensitivity of the GTP assay procedure is greatly influenced by the instrument used to detect the light emission. With a commercial ATP photometer (SAI Technology Co., San Diego, California), the procedure as described in this report can detect 1 ng of GTP ml^{-1} of solution (~2 n*M*). If additional sensitivity is required, synthetic D-luciferin may be added to saturate the crude enzyme preparation,[21] thereby extending the limit of detection of 1 pg of GTP ml^{-1} (~2 p*M*). If measurements are made of the initial rate of light emission, the addition of exogenous NDPK to the crude enzyme preparation will also significantly increase the sensitivity of the GTP assay. The luminescent response is linear to a maximum GTP concentration of 50 ng ml^{-1} of solution (100 n*M*). For a given operator, variation between replicate standard sample analyses is generally ±3%, using the commercial ATP photometer.

[21] D. M. Karl and O. Holm-Hansen, *Anal. Biochem.* 75, 100 (1976).

Reaction Specificities

As described in this report, the HK/G6P-DH coupled reaction is highly specific for ATP. No hydrolysis of GTP could be detected when a series of standard solutions were treated as experimental samples. Likewise, UDPG-PP showed no reactivity toward GTP at the concentrations and incubation period selected for this reaction. It is essential, however, that the HK/G6P-DH and the UDPG-PP enzyme activities be deactivated, after the appropriate incubation periods, in order to eliminate the possibility of subsequent GTP hydrolysis. GK is specific for GMP as the phosphoryl acceptor, and for ATP as the phosphoryl donor.[22]

PK catalyzes the phosphorylation of both ADP and GDP to ATP and GTP, respectively. This ATP regenerating system ensures maximum conversion of GMP to GTP in coupled reactions (3) and (4). NDPK will catalyze the production of ATP (from ADP) upon the addition of a wide variety of ribose and deoxyribose nucleotide triphosphates. Since ATP and UTP are hydrolyzed prior to the GTP assay, CTP is the only remaining intracellular NTP that may be present at concentrations comparable to GTP. When using initial rise measurements, however, CTP results in $<4\%$ of the light emission resulting from an equimolar concentration of GTP (see Fig. 1).

Analytical Interferences and Troubleshooting

Since the determination of GTP is dependent upon the production of ATP within the crude luciferase preparations [Reaction (5)], followed by an ATP-dependent light-emission reaction [Reaction (6)], the final light emitted from this GTP-dependent coupled light-emission reaction [Reactions (5) and (6)] will be subjected to the same sources of interference as have been described for the quantitative analysis of ATP.[6,23,24] The use of internal standards is strongly recommended in order to correct for all sources of analytical interference. It is important that $MgSO_4$ is not used as the source of Mg^{2+} for enzyme activation during the preliminary steps of this procedure [Reactions (1–4)], since UDPG-PP is strongly inhibited by SO_4^{2-}. Although complete conversion of GMP to GTP may be achieved through the addition of excess ATP, or by increasing the concentration of GK used per reaction tube [Reaction (3)], both of these modifications will result in a significant level of interference on the GTP assay due to the contamination of GK preparations by the enzyme myokinase (MK). Since MK is an

[22] R. P. Miech and R. E. Parks, *J. Biol. Chem.* **240**, 351 (1965).
[23] B. L. Strehler and W. D. McElroy, this series, Volume 3, p. 871.
[24] L. M. Aledort, R. I. Weed, and S. B. Troup, *Anal. Biochem.* **17**, 268 (1966).

extremely heat-stable protein, back production of ATP will occur (2 ADP $\overset{MK}{\rightleftharpoons}$ AMP + ATP) following the heat deactivation of coupled Reactions (3) and (4). By maintaining relatively low concentrations of ATP [i.e., ADP after Reaction (1)] and of GK, the effects of MK contamination are eliminated. If sufficiently high levels of ADP are present within the GTP extracts (>200 ng of ADP ml^{-1}), a small amount of light will be produced, after sample injection, owing to MK activity contained within the crude luciferase preparations. The amount of light emitted is less than 1% of the activity resulting from an equimolar concentration of GTP; however, if necessary, this source of interference can be evaluated (and corrected for) by measuring the sum of the concentrations of ATP and ADP within each sample extract[20] and relating these values to the reactivity of standard ADP solutions.

Acknowledgments

The author expresses his appreciation to Dr. O. Holm-Hansen for comments, criticism, and encouragement offered during the course of this research and the preparation of this chapter. Dr. F. Azam and Ms. L. Campbell critically reviewed the original manuscript and offered helpful suggestions for improvement. The methodology described in this report was developed under ERDA contract EY-76-C-03-0010 P.A. 20.