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A Rapid Sensitive Method for the Measurement of Guanine Ribonucleotides in Bacterial and Environmental Extracts

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A method was devised for the quantitative determination of guanine ribonucleotides (GTP, GDP, and GMP) in extracts of biological materials. The technique is based upon selective enzymatic hydrolysis of UTP and ATP contained within the cell extracts, followed by a quantitative determination of GTP. GTP is measured using a nucleoside diphosphate kinase–firefly luciferase coupled bioluminescent reaction, during which the GTP is enzymatically coupled to ATP production, resulting in ATP-dependent light emission. The methods are simple and reproducible and extremely sensitive ($\leq 10^{-9}$ M GTP), and require no preparatory chromatographic separation procedures. Methods are also presented for the enzymatic conversions of GDP and GMP to GTP in addition to the determination of GTP.

Although the majority of energy-requiring and energy-producing reactions require direct coupling with the adenine nucleotides (ATP, ADP, and AMP), many important biosynthetic reactions proceed only as an indirect result of the adenylate system. For example, the replication of DNA and the transcription of RNA require the presence of intracellular pools of a variety of nucleotide triphosphate (NTP)[2] precursors, including ATP, dATP, CTP, dCTP, GTP, dGTP, TTP, and UTP. Moreover, GTP has been shown to be an essential factor for the initiation, the aminoacyl tRNA binding, and the translocation processes of protein biosynthesis (1).

Several techniques are available for determining GTP levels in extracts of biological materials (2–4); however, since these methods require tedious

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[2] Abbreviations used: dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate; NTP, nucleoside triphosphate; NDPK, Nucleoside-5′-diphosphate kinase (EC 2.7.4.6); PPi, pyrophosphate; 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); L, firefly luciferase (EC 2.7.-.-); and pyr, pyruvate.

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and time-consuming chromatographic separation procedures, they have not been utilized for routine GTP analyses. Although crystalline firefly luciferase is specific for ATP (5,6), commercially available enzyme preparations contain ADP and the enzyme nucleoside diphosphate kinase (NDPK), which together result in the production of ATP, and therefore light, upon the addition of a variety of purine and pyrimidine nucleotide triphosphate precursors (7–10). This nonspecific reactivity of the crude luciferase reagents has been previously discussed as a potential source of analytical interference on the quantitative determination of ATP (11,12), but has also been used as the basis for a quantitative assay procedure for ribose and deoxyribose nucleotide triphosphates (4,13).

This report describes a new method for the determination of GTP in mixtures of nucleotides, without the need of prior chromatographic purification procedures. This technique is based upon selective enzymatic hydrolysis of specific nucleotides (UTP and ATP) present within the cell extracts, followed by a quantitative assay for GTP using a modified firefly luciferin–luciferase preparation. In addition, methods are also presented for the quantitative determinations of GDP and GMP as well, thereby enabling the calculation of the relative intracellular concentrations of all three of the guanine ribonucleotides.

ASSAY PRINCIPLES

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

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

$$\alpha\text{-D-glucose 1-P} + \text{UTP} \xrightarrow{\text{UDPG-PP}} \text{UDP-glucose} + \text{PP}_\text{i} \quad (2)$$

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

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

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

$$\text{ATP} + \text{luciferin} \xrightarrow{\text{L}} \text{product} + \text{PP}_\text{i} + \text{light} \quad (6)$$

Reactions (1) and (2) are designed to eliminate all of the ATP and UTP present within the sample extracts, since both of these nucleotides interfere with the quantitative assay of GTP. GMP and GDP can be enzymatically converted to equivalent concentrations of GTP [Reactions (3) and (4)] prior to GTP analyses. The quantitative assay for GTP is based upon the linear luminescent response of crude firefly lantern extracts to the addition of GTP [Reactions (5) and (6)] when ADP is present in excess. Light emission can be measured using any one of a number of light detection instruments (8), and the actual GTP concentration can be determined

by relating this emitted light to the reactivity resulting from standard GTP solutions.

MATERIALS AND METHODS

Chemicals

D-Glucose, Tris(hydroxymethyl) aminomethane–HCl (pH 7.7), and the sodium salts of ATP, ADP, CTP, GTP, GDP, GMP, UTP, NADP, phosphoenolpyruvate and α-D-glucose 1-phosphate were all obtained from Sigma Chemical Co. (St. Louis, Missouri). Firefly luciferase (crude, catalog FLE-50), hexokinase/glucose-6-phosphate dehydrogenase (crystalline, from yeast; 2:1 activity ratio), and pyruvate kinase (crystalline, from rabbit muscle; 350–500 U/mg of protein) were also obtained from Sigma Chemical Co. Guanylate kinase (from hog brain; 10 U/mg of protein) and UDP-glucose pyrophosphorylase (from beef liver; 100 U/mg of protein) were purchased from Boehringer/Mannheim (Indianapolis, Indiana). All other chemicals used in this report were analytical grade reagents.

Standard Nucleotide Solutions

ATP, ADP, CTP, GTP, GDP, GMP, and UTP solutions (2×10^{-6} M) were prepared in 0.02 M Tris buffer and stored frozen in 5-ml aliquots. When required, a single vial was thawed and working solutions (1×10^{-9} to 1×10^{-7} M) were prepared by dilution with Tris buffer. A separate set of guanine nucleotide standards was prepared for each assay and discarded after a single day of use. This procedure eliminated problems of GTP hydrolysis.

Luciferase Enzyme Preparations

Prior to use, lyophilized firefly lantern extracts were stored desiccated at −20°C. When required, each vial was reconstituted by adding 5 ml of distilled water, 10 ml of sodium arsenate buffer (100 mM; pH 7.4), 10 ml of $MgSO_4$ (40 mM), and 2.5 μg of ADP. The enzyme mixture was allowed to stand for 3 to 4 h at room temperature (23–25°C) to reduce the level of background light emission. After aging, the insoluble residue was removed by vacuum filtration or centrifugation. The clear filtrate or supernatant was transferred to a clean flask, and the enzyme preparation was then ready for use. Since considerable variation occurs between individual vials of firefly lantern extract, the contents of several vials should be pooled when large volumes of enzyme are required.

For GTP determinations only

Two hundred microliters of each sample extract are pipetted into a series of disposable glass culture tubes (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, is also processed simultaneously. Fifty microliters of a solution containing 75 mM potassium phosphate buffer (pH 7.4), 15 mM $MgCl_2$, 0.5 mM NADP, 0.5 mM D-glucose, 0.5 mM α-D-glucose 1-P, HK/G6P-DH (2 U/ml), and UDPG-PP (5 U/ml) is pipetted into each tube. The samples are incubated for 30 min at 30°C, followed by a 2-min enzyme deactivation period in a boiling water bath (100°C). After heat deactivation of the enzymes, the samples are allowed to adjust to room temperature prior to the actual GTP assay procedure (see GTP assay procedure).

For GTP, GDP, and GMP Determinations

For each set of nucleotide determinations, 200 μl of each sample extract is pipetted into a series of four 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 mM potassium phosphate buffer (pH 7.4) and 15 mM $MgCl_2$ is added to each tube. For GTP plus GDP determinations (tube B), 50 μl of a solution containing 75 mM potassium phosphate buffer (pH 7.4), 15 mM $MgCl_2$, 0.5 mM PEP, and 20 μg of PK is added to each tube. For GTP plus GDP plus GMP determinations (tube C), 50 μl of a solution containing 75 mM potassium phosphate buffer (pH 7.4), 15 mM $MgCl_2$, 0.5 mM PEP, 10 ng of ATP, 20 μg of PK, and 5 μg of GK is added to each tube. To determine the efficiency of coupled Reactions (3) and (4), 50 μl of a solution containing 75 mM potassium phosphate buffer (pH 7.4), 15 mM $MgCl_2$, 0.5 mM PEP, 10 ng of ATP, 20 μg of PK, 5 μg of GK, and an internal GMP standard is added to each D tube. The concentration of GMP 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 sets of reagents (A–D).

In addition to the GTP standard solutions, it is advisable to prepare and to react several mixtures containing known molar ratios of GTP, GDP, and GMP, to monitor the efficiency of coupled Reactions (3) and (4). Enzyme blanks (Tris buffer plus the appropriate enzyme solution) should also be prepared since certain commercial enzyme preparations contain substantial levels of contaminating nucleotides. All samples are incubated for 60 min at 30°C and then placed into a boiling water bath (100°C) for 2 min, to deactivate the enzymes. This heat deactivation procedure is essential since the addition of PEP and active PK to crude firefly luciferase preparations will cause an immediate (<1 s) production of ATP, and therefore light, from ADP contained with the firefly enzyme mixture. Once the samples have adjusted to room temperature, they are treated as GTP samples and reacted and assayed as described in the GTP sections of this report.

GTP Assay Procedure

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 (see Results and Discussion). It is recommended that the photometer (or similar light detection instrument) is interfaced to an analog recorder in order to monitor the reaction kinetics. 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 commercially available instruments have this extended capability. These are the Chem-Glow Photometer (American Instrument Co., Silver Springs, Maryland), the Luminescence Biometer (DuPont Instrument Co., Wilmington, Delaware), and the ATP Photometer (SAI Technology Co., LaJolla, California). The analytical characteristics and properties of these instruments have recently been evaluated and compared (14). When integrated determinations are being used, the light flux is measured, either electronically or manually, over a predetermined portion of the light emission curve. It is important that the integrated light flux determinations be restricted to the early portion of the reaction, in order to reduce the various sources of analytical interference on quantitative GTP determinations (see Results and Discussion).

Data reduction. Net light emission is determined by subtracting the appropriate blank value from each of the initial rise, peak height, or integrated light flux measurements. Standard curves are prepared by plotting net light emission versus GTP concentration. The GTP concentration in each of the A tubes is calculated from the GTP standard curve prepared with A reagents; likewise, the GTP concentrations in tubes B, C, and D are calculated from the GTP standard curves prepared with reagents B, C, and D, respectively. The GDP concentration in each sample is determined by subtracting the GTP concentration in tube A from that measured in tube B. GMP concentrations are determined by subtracting the GTP concentration in tube B from that measured in tube C, and by correcting for the efficiency of the coupled GK/PK reactions (if necessary) using the internal GMP standard data as follows:

$$\text{Percentage recovery} = \frac{[\text{GTP}] \text{ in tube D} - [\text{GTP}] \text{ in tube C}}{[\text{Internal GMP standard}]} \times 100,$$

$$[\text{GMP}] = \frac{[\text{GTP}] \text{ in tube C} - [\text{GTP}] \text{ in tube B}}{\text{Decimal equivalent of percentage recovery}},$$

By knowing the proportion of the sample actually assayed and the volume (or weight) of material originally extracted, one can determine

the [GTP] (or [GDP], [GMP]) ml^{-1} (or $cell^{-1}$, g^{-1} of tissue, etc.) of the biological material.

RESULTS AND DISCUSSION

Nucleotide Extractions

Extraction of the guanine ribonucleotides is the first step in the quantitative assay procedure. A number of different extraction media and methodologies are available for nucleotide determinations, including boiling buffers (7,15,16), sulfuric acid (17), perchloric acid (18), trichloroacetic acid (19), butanol (20), chloroform (21), and *n*-bromosuccinimide (22), to name a few. In the past few years, a number of reports have been published comparing the efficiencies of various techniques for extracting nucleotides from living cells and tissue samples (12,16,17,21,23–28). Prior to the selection of a preferred technique, several different procedures should be tested using the specific biological material in question, to evaluate analytical properties such as the rate of cell death and nucleotide release, ionic interference of the extraction fluid(s), residual enzyme activity, chemical stability of the nucleotides, ease of operation, reproducibility of the methods, and the absolute GTP concentrations resulting in the final cell extracts. It should be mentioned that SO_4^{2-} ions can reduce the activity of the UDPG-PP; therefore the rate of UTP hydrolysis in cell extracts containing high concentrations of SO_4^{2-} (e.g. H_2SO_4) should be monitored through the use of UTP internal standards, and, if required, the cell extracts should be diluted with Tris buffer (0.02 M, pH 7.7) to restore maximal enzyme activity.

Since the rate of nucleotide turnover in growing cells is extremely rapid [i.e., ~1–2 s for bacteria (29)], the biological material in question should be extracted as soon as possible after sample collection. Centrifugation (30) and vacuum filtration (31,32) may cause a decrease in the level of NTPs in the final cell extracts, presumably as a result of metabolic stress imposed by these cell concentration techniques.

Specificity of Crude Luciferase Preparations

Crystalline firefly luciferase is specific for ATP (5,6); however, a number of additional phosphorylated compounds will result in light emission if injected into impure luciferase preparations (7–10). Figure 1 presents composite curves obtained for the time course and relative intensity of light emission resulting from the injection of each of the four most predominant intracellular NTPs into a preparation of crude firefly luciferase. Although all four nucleotides stimulate light emission, the reaction kinetics and the extent of reactivity are quite variable. If quantitative determinations of GTP are to be made within nucleotide extracts of biological materials, it is apparent that ATP, UTP, and CTP will all interfere with

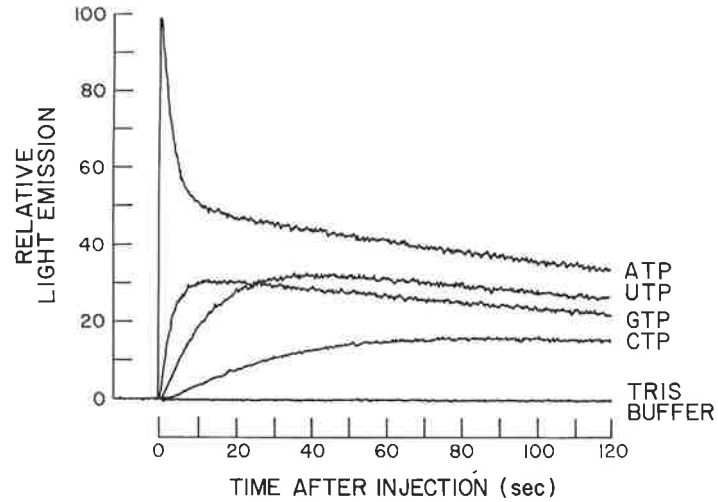


FIG. 1. Kinetics of ATP-, GTP-, UTP-, and CTP-dependent light emission using crude luciferase preparations. The concentration of each nucleotide was 4×10^{-8} M.

the measurement of GTP. The extent of this analytical interference, however, will vary, depending upon the relative concentration of each NTP, and also upon the method used to quantitate light emission (i.e., initial rise, peak height, or integrated light flux). Investigations were undertaken to develop methods for selective enzymatic NTP hydrolysis, to eliminate these potential sources of interference.

Selective ATP Hydrolysis

Figure 2 presents the results of an experimental procedure designed to eliminate ATP reactivity. It is apparent from these data that the hexokinase/glucose 6-phosphate dehydrogenase (HK-G6PDH) reaction, as described in this report, is specific for ATP throughout the entire range of nucleotide concentrations tested ($\sim 10^{-9}$ to 10^{-7} M). Within the first 30 min of incubation at 30°C, the concentration of ATP decreased from 100 ng/ml to limits below detection (<0.1 ng/ml), whereas the reactivity resulting from an equimolar concentration of GTP remained constant relative to the control sample (Fig. 2). Similar experiments conducted with UTP and CTP indicate that these nucleotides are likewise unaffected by incubation with the HK-G6PDH reaction mixture (data not shown). These results are consistent with the kinetic substrate specificity experiments reported previously by Purich *et al.* (33); nevertheless, it should be mentioned that yeast hexokinase is not absolutely specific for ATP, and therefore it is essential to denature the enzyme (100°C, 2 min) following ATP hydrolysis, to eliminate enzymatic hydrolysis of GTP catalyzed by extended incubation periods (i.e., >90 min).

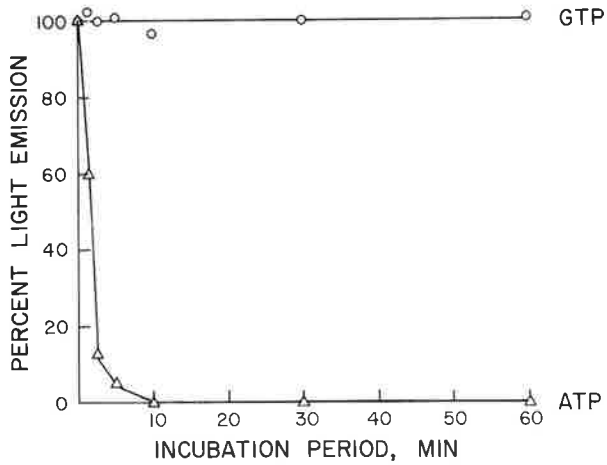


FIG. 2. Kinetics and extent of ATP and GTP reactivity for the hexokinase/glucose-6-phosphate dehydrogenase coupled reaction. The initial concentration of each nucleotide was 2×10^{-7} M. See Materials and Methods for experimental design.

Selective UTP Hydrolysis

Figure 3 presents the results of an experimental procedure designed to eliminate UTP reactivity. The UDP-glucose pyrophosphorylase (UDPG-PP) reaction, as described in this report, is highly specific for UTP at the nucleotide concentrations tested ($\sim 10^{-9}$ to 10^{-7} M). The reactivity of

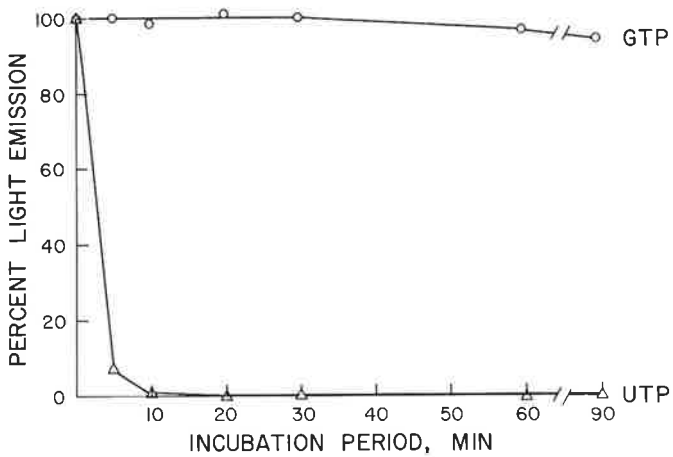


FIG. 3. Kinetics and extent of UTP and GTP reactivity for the UDP-glucose pyrophosphorylase reaction. The initial concentration of each nucleotide was 2×10^{-7} M. See Materials and Methods for experimental design.

GTP (see Fig. 3), CTP, and ATP (data not shown) remained constant throughout the first 30 min of incubation, whereas the reactivity of UTP dropped to below detectable limits ($<10^{-9}$ M). It should be mentioned, however that the concentrations of ATP and GTP decreased with extended incubation periods, although the apparent loss of GTP was not substantial (i.e., approximately 6% reduction of GTP reactivity after 90 min; see Fig. 3). The possibility of GTP hydrolysis can be eliminated by deactivating the UDPG-PP enzyme (100°C, 2 min) following the predetermined incubation period (usually 20–30 min). In practice, the hydrolysis of standard UTP solutions should be monitored in a time course experiment to determine the optimal incubation period.

When the HK-G6PDH reactions and the UDPG-PP reaction are combined and applied to nucleotide mixtures containing known amounts of GTP, ATP, and UTP, the results (i.e., reaction kinetics, reactivity, and specificity) are identical to those presented in Figs. 2 and 3 for pure nucleotide solutions.

Residual Analytical Interference

Even though the contribution of ATP and UTP to total light emission can be selectively eliminated, additional reactive phosphorylated compounds might be present in extracts of biological materials that could significantly affect quantitative GTP determinations. The only remaining intracellular nucleotides, however, that might be present at concentrations comparable to GTP are CTP and ADP. It is apparent from Fig. 1 that CTP reacts with crude firefly luciferase preparations at a much slower rate and to a lesser extent than does GTP, presumably because of the specificity of the enzyme NDPK. When initial rise measurements (see below) are being used, CTP results in between 0 and 4%, depending upon the concentration, of the light emission resulting from an equimolar concentration of GTP (see Fig. 1); furthermore, recent chromatographic investigations of nucleotide extracts from bacterial cells have revealed that the concentration of CTP is only about 20 to 30% of the cellular levels of GTP (34). Therefore, for most biological samples the contribution from CTP will probably be negligible, especially if initial rise measurements are conducted.

The addition of ADP to the crude firefly luciferase preparations stimulates light emission as a result of adenylate kinase (AK) contamination ($2\ ADP \rightleftharpoons AMP + ATP$). The amount of light emitted is less than 1% of the reactivity resulting from an equimolar concentration of GTP; if necessary, however, this source of analytical interference can be evaluated (and corrected for) by measuring the sum of the concentrations of ATP and ADP within each sample extract and by relating these values to the reactivity of standard ADP solutions.

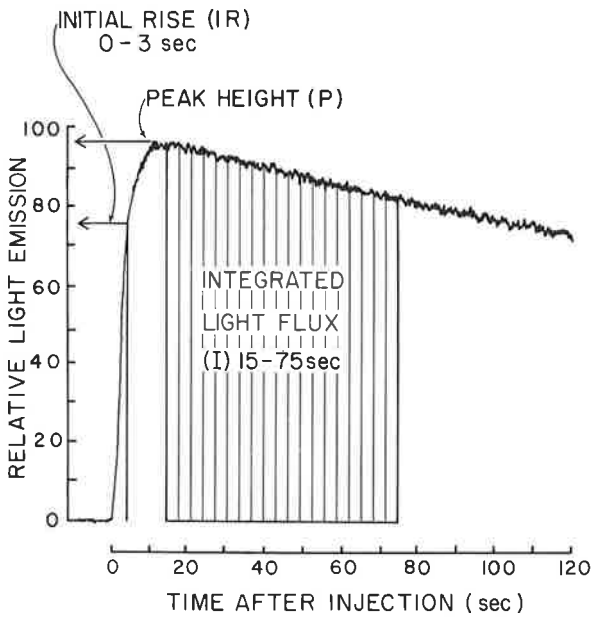


FIG. 4. Kinetics of GTP-dependent light emission from the nucleoside diphosphate/firefly luciferase coupled reaction. Light emission can be determined by measuring the initial rise, the peak height, or some integrated portion of the light emission curve.

GTP-Dependent Light Emission

An example of the characteristic kinetics of GTP-dependent light emission is reproduced in Fig. 4. When assaying sample extracts containing GTP, the initial rise of the luminescent curve, the peak height of luminescence, or an integrated portion of the light emission curve may be used to relate the GTP concentration in sample extracts to the reactivity of standard GTP solutions. Figure 5 displays the reaction kinetics for a series of GTP solutions and also presents the three representative standard curves resulting from these data. Although all three light detection methods yield linear standard curves, it should be emphasized that only initial rise measurements significantly reduce the interference resulting from CTP. The contribution of CTP (at an equimolar concentration, 2×10^{-8} M) to the initial rise, the peak height, and integrated GTP-dependent light emission is approximately <1%, 15%, and 50%, respectively (see Fig. 1).

As presented under Materials and Methods, ADP is added to crude luciferase preparations prior to the GTP assay. Figure 6 shows the effect of exogenous ADP addition on the resulting GTP-dependent light emission reaction. As ADP is added to crude luciferase preparations (from 0–200 ng of ADP/ml of extract) an increase is observed in both the initial rise and

net peak height of light emission. Since ADP is one of the two substrates required for the NDPK reaction, this stimulation in light emission can be interpreted in terms of saturation enzyme kinetics. With further additions of ADP (>200 ng of ADP/ml of extract), the kinetics of light emission are affected, resulting in a slower initial rise of luminescence without adversely affecting the relative peak height of luminescence (see Fig. 6). The amount of ADP added to the crude luciferase preparations will therefore depend upon the actual method used to quantitate light emission.

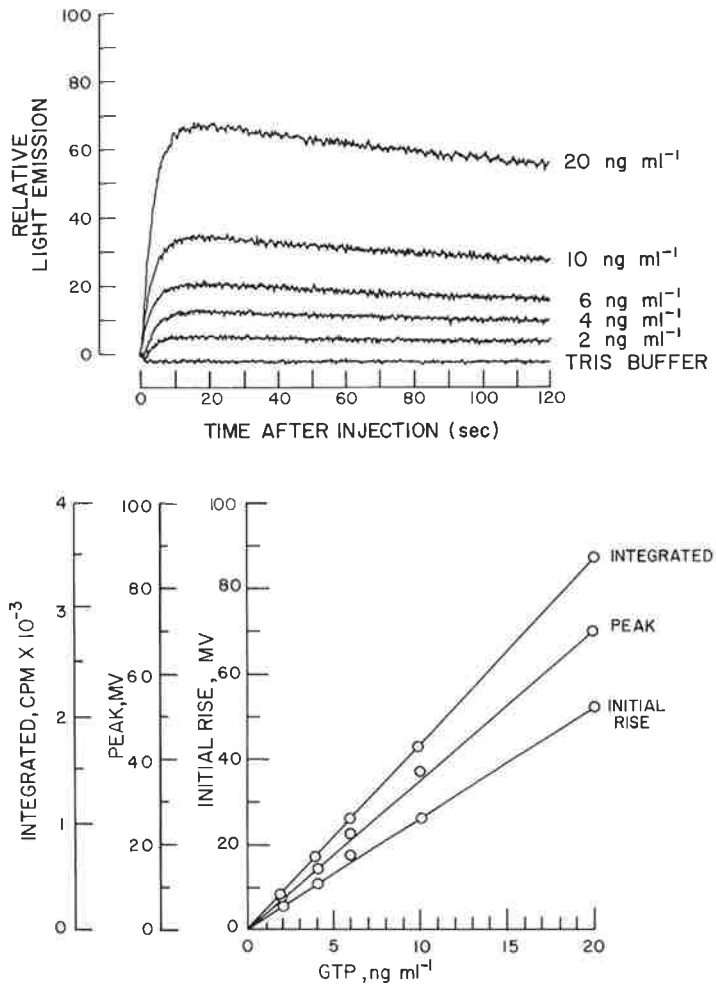


FIG. 5. Kinetics of GTP-dependent light emission and standardization of the GTP assay. The upper graph displays the reactivity of a range of GTP standard solutions; the lower graph presents these light emission data in the form of GTP standard curves.

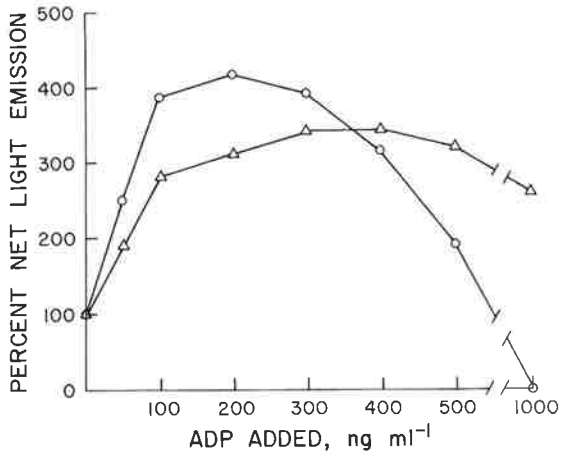


FIG. 6. The effect of addition of exogenous ADP on the net light emission resulting from a standard GTP solution. The concentration of GTP was 2×10^{-8} M. Symbols: circles, initial rise determinations; triangles, peak height determinations.

Conversion of GDP and GMP to GTP

To make quantitative determinations of GDP and GMP, these nucleotides are enzymatically converted to equivalent concentrations of GTP and measured as such. Pyruvate kinase (NDP + PEP $\rightleftharpoons$ NTP + pyr) displays a broad specificity for its nucleotide substrate, and will catalyze the production of NTPs from a wide variety of ribose and deoxyribose nucleotide triphosphates, in addition to its preferred substrate, ADP. The maximum velocity for the GDP-dependent reaction is comparable to the rate measured for ADP (35). When utilizing the procedure described under Materials and Methods, the conversion of GDP to GTP is 100% efficient throughout the range of concentrations tested (10^{-9} to 10^{-7} M).

Guanylate kinase (GMP + ATP $\rightleftharpoons$ GDP + ADP) is highly specific for GMP as the phosphoryl acceptor, and ATP as the phosphoryl donor (36). At the concentrations described in this report (10^{-9} to 10^{-7} M), the efficiency of the guanylate kinase/pyruvate kinase (GK/PK) coupled reactions (i.e., GMP $\rightarrow$ GTP) is usually less than 100% within the designated incubation period. The actual conversion efficiency is therefore determined for each sample extract, using a GMP internal standard. As described under Materials and Methods, the concentration of the internal GMP standard 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 nucleotide extract. The rate of conversion of GMP to GTP is dependent upon the concentration of ATP in solution as well as on the concentration of GK used. The greater the ATP concentration, the greater the amount of GTP formed from GMP

for a given incubation period. The use of PK results in the regeneration of ATP from ADP, thereby ensuring a maximum conversion efficiency of GMP to GTP. For a given ATP concentration, the production of GTP (from GMP) is a linear function of GMP in solution, even though the conversion is not 100% complete. Therefore, for data reduction, the concentration of GMP is determined by subtracting the GTP plus GDP value from the GTP plus GDP plus GMP value, and by correcting that concentration for the efficiency of the GK/PK coupled reaction (if necessary) using the internal standard data, as described under Materials and Methods.

Although complete conversion of GMP to GTP can be achieved through the addition of excess ATP (millimolar concentrations) or by increasing the concentration of GK used per reaction tube, both of these modifications will result in a significant level of interference on the GTP assay due to the contamination of commercial GK preparations with the enzyme adenylate kinase (AK). Since the AK is an extremely heat-stable protein, back production of ATP occurs (2 ADP $\rightleftharpoons$ ATP + AMP) following heat deactivation of GK and PK. When relatively low concentrations of adenine nucleotides and GK are maintained, the effects of AK contamination are eliminated.

Sensitivity and Reproducibility

The sensitivity of the GTP assay procedure is greatly influenced by the instrument used to detect light emission as well as by the actual mode of light detection (i.e., initial rise, peak height, or integrated light flux). When a commercial ATP photometer (SAI Technol. Co., Sorrento Valley, San Diego, California) and initial rise measurements are used, the procedure as described in this report can detect 1×10^{-9} M GTP. If measurements are made of the initial rise of luminescence, the addition of exogenous NDPK (from yeast, Sigma Chemical Co.) to the crude enzyme preparations will result in a substantial increase in activity, thereby extending the lower limit of GTP detection. If additional sensitivity is required, D-luciferin (Sigma Chemical Co.) may be added to saturate the crude enzyme preparation, as described by Karl and Holm-Hansen (37). For a given operator, variability between replicate standard sample analyses is ± 2 to 3%, using a commercial ATP photometer (SAI Technical Co.).

Since the determination of GTP is dependent upon the production of ATP within the crude luciferase preparations, followed by an ATP-dependent light emission reaction, the light emitted from this GTP-dependent coupled light emission reaction is subjected to the same sources of interference as having already been enumerated for the quantitative assay of ATP. The light emitted in the firefly bioluminescent reaction is adversely affected by the presence of ions (both cations and anions), turbidity, and color in the final cell extracts. The magnitude of this combined interference can be determined and corrected for using a GTP internal standard.

In conclusion, GTP, GDP, and GMP concentrations can now routinely be assayed in extracts of biological materials without using chromatographic purification procedures. The methods described in this report are sensitive, rapid, and reproducible, and require no special equipment or facilities beyond those already employed in many laboratories for ATP, ADP, and AMP analyses. Therefore, quantitative determinations of guanine ribonucleotides can easily be integrated into many ongoing research programs.

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