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ULTRASENSITIVE ASSAY FOR RIBONUCLEOSIDE TRIPHOSPHATES IN 50–1000 CELLS

APPLICATION TO STUDIES WITH PYRAZOFURIN AND MYCOPHENOLIC ACID

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Abstract—New methods have been developed for the measurement of ATP, GTP, and UTP pools of microscopic cell colonies of 50–1000 cells. These methods are based on stochiometric generation of ATP from ADP with GTP and UTP serving as phosphate donors, followed by measurement of ATP with firefly luciferase. This technique has been used to generate dose–response curves describing the effects of mycophenolic acid and pyrazofurin on individual colonies of Chinese hamster ovary cells.

Although high performance liquid chromatography has made the measurement of ribonucleoside triphosphate pools a routine procedure, the sensitivity of current methods requires about 10^4 cells for accurate determinations. For some applications such sensitivity is a limitation. For example, many types of cells including stem cells of B and T lymphocytes and human solid tumors are available only as colonies of 50–1000 cells growing in semi-solid medium [1–4]. To perform studies of nucleotide metabolism with such colonies and other systems where only small numbers of cells are available, more sensitive techniques are required.

The reaction catalyzed by firefly luciferase is the basis for measurements of ATP in the femtomole range [5, 6]. The present report describes procedures exploiting this reaction for measurement of not only ATP, but also GTP and UTP pools in the subpicomole range. These procedures are used here to describe the effects of pyrazofurin on UTP concentrations and mycophenolic acid on GTP concentrations of individual cell colonies.

MATERIALS AND METHODS

Materials. Nucleoside mono-, di-, and triphosphates, succinic acid, and glucose-1-phosphate were purchased from the Sigma Chemical Co. (St. Louis, MO). Yeast nucleoside 5′-diphosphate kinase (1000 units/mg protein), yeast uridine 5′-diphosphoglucose pyrophosphorylase (200 units/mg), and crystallized yeast hexokinase (340 units/mg protein) were also obtained from Sigma. Glucose and Tris base were from the Fisher Chemical Co. (Fair Lawn, NJ). Porcine succinyl-CoA-synthetase, purified by a modification of the procedure of Cha [7], (35 units/mg) was a gift from Dr. W. Bridger of the University of Alberta.

Luciferase-luciferin was a product of the Analytical Luminescence Laboratory (San Diego, CA) and was reconstituted in 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) buffer as suggested by the supplier and then diluted to half strength with sterile distilled water. A similar product sold by Lumac B.V. (Shaesberg, Netherlands) was also found to be suitable for this procedure.

Coenzyme A was purchased from Sigma or P-L Biochemicals (Milwaukee, WI). Both coenzyme A and ADP as obtained contained ATP at a concentration of >0.1%. Coenzyme A was purified by reverse phase chromatography on a μBondapack C18 column (Waters Co., Milford, MA) eluted with 2% methanol in 4 mM potassium phosphate, pH 6.5. The phosphate buffer was not removed but did not interfere with the assay. ADP was purified enzymatically [8] or by reverse phase chromatography.

Measurement of ATP. Samples or standard solutions of ATP were prepared in sterile 50 mM Tris acetate, pH 8.0, with 2 mM magnesium chloride. The sample (100 μl) was placed in a plastic cuvette, and 100 μl luciferin-luciferase reagent was injected into the cuvette with a plastic pipette. The light resulting from the reaction at ambient temperature was measured by a Turner model 20 photometer and averaged over the interval 1 sec to 18 sec after addition of luciferase. The light signal was nearly constant over this period (<2% change) and directly proportional to the concentration of ATP in the sample over the range of 10^{-10} M to 10^{-7} M. The light emission from the luciferase-luciferin and buffer (background) was equivalent to an ATP concentration of 2×10^{-11} M. For the measurement of ATP in colonies, standards were prepared from a 1 mM stock solution and used within 2 hr. Very dilute ATP solutions ($<10^{-7}$ M) were observed to show erratic decomposition on standing.

Phosphorylation of ADP with UTP or GTP as phosphate donor. A reagent consisting of 10 units/ml nucleoside diphosphate kinase and 25 μM ADP

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(purified as described) was freshly prepared in 50 mM Tris–acetate, pH 8.0. A 10-μl aliquot of this reagent was added to 100 μl of sample, and the solution was incubated at 37° for 10 min.

Selective removal of ATP, GTP, or UTP. A reagent consisting of 25 mM succinic acid, 2 mM magnesium chloride, 5 mM glucose-1-phosphate, 10 mM glucose, and 0.2 mM coenzyme A (purified as above) was prepared in sterile, 50 mM Tris–acetate buffer, pH 8.0. To remove all three triphosphates (ATP, GTP and UTP), the following enzymes were added: hexokinase, 2 units/ml; UDP-glucose pyrophosphorylase, 25 units/ml; and succinyl thiokinase, 0.5 units/ml. If only two nucleotides were to be removed, the appropriate enzyme was omitted. An aliquot of 10 μl of this reagent was added to 100 μl of sample and incubation was at 37° for 5 min. The enzymes were then inactivated by placing the tube in a boiling water bath for 2 min.

Growth of CHO cell colonies. Chinese hamster ovary (CHO) cells grown in α-Minimum essential medium (α-MEM) without nucleosides (Gibco, Grand Island, NY) supplemented with 10% dialyzed fetal calf serum were diluted to 10–100 cells/ml, and the suspension was mixed with an equal volume of medium containing 1.8% methyl cellulose. Aliquots of 1 ml were then spread over 0.5-ml underlayers of medium with 1.8% methyl cellulose in 24-well plates (Flow Laboratories, Hamden, CT). The plates were incubated at 37° in a humidified incubator containing 5% CO_2 in air. Cloning efficiency was >90%.

The cell number per colony was determined on days 2 and 3 by examination with an inverted stage microscope and on day 7 by disaggregation in saline and enumeration with a Coulter Counter. The results indicated an exponential increase in cell number with a doubling time of 12–14 hr over the first 7 days after plating.

Extraction of nucleotides from colonies. Individual colonies were removed from semi-solid culture with a pasteur pipette or by use of a 100-μl capillary tube directed by a micro-manipulator. The colony, with a small amount of associated gel (1–2 μl), was placed in a 1.5-ml tube, 200 μl of boiling 50 mM Tris-acetate, 2 mM magnesium chloride was added, and the tube was placed in a boiling water bath for 2 min. A similar extraction procedure has been reported previously for other cells [9, 10]. This method was compared to extraction with 0.3 M ice-cold perchloric acid; when day 8 CHO colonies were extracted, the two methods gave ATP/colony values which agreed within 5%.

RESULTS

The methods described here are based on the various reactions listed below and on the luciferin-luciferase reaction for the measurement of ATP.

1. ATP + glucose $\xrightarrow{\text{hexokinase}}$ ADP + glucose-6-phosphate
2. GTP + succinate + coenzyme A $\xrightarrow{\text{succinyl thiokinase}}$ GDP + succinyl-CoA
3. UTP + glucose-1-phosphate $\xrightarrow{\text{UDPG-pyrophosphorlyase}}$ UDPG + pyrophosphate
4. NTP + ADP $\xrightarrow{\text{nucleoside 5'-diphosphate kinase}}$ NDP + ATP

Although the reactions listed are reversible, under the conditions described all reactions strongly favor the direction indicated. For the measurement of ATP in colonies, the luciferase reaction can be used directly. For measurement of UTP or GTP the sample is split and one-half is treated to remove ATP, UTP, and GTP as indicated in reactions 1, 2, and 3. The other portion is treated with a reagent lacking the enzyme for reaction 2 (for GTP), or reaction 3 (for UTP). Both portions are then incubated with a reagent containing ADP and nucleoside 5'-diphosphate kinase to generate ATP (reaction 4). The excess amount of ATP generated in the absence of succinyl thiokinase (for GTP) or UDPG-pyrophosphorlyase (for UTP) is a measure of the concentration of GTP or UTP.

The specificity and sensitivity of this method of measuring GTP and UTP depend on the specificity, completeness, and background contribution of the four reactions. The reactions were individually characterized as described below.

Reaction 1: *Removal of ATP by the hexokinase reaction.* Under the conditions described, ATP (10^{-7} M) was quantitatively (97 ± 3%) converted to ADP, while CTP, UTP, and GTP were essentially unaffected (<2.5% loss). Kinetic studies showed that this reaction was complete by 1 min. Neither the glucose nor the hexokinase increased the background ATP level as measured by the luciferase reaction. Higher levels of hexokinase (25 units/ml) produced some loss of other triphosphates, possibly due to a contaminating activity. This procedure is a modification of a method reported earlier [11].

Reaction 2: *Removal of GTP by succinyl thiokinase.* Under the conditions described, GTP (10^{-7} M) was quantitatively removed (99 ± 0.7%), while, CTP, ATP, and UTP were essentially unaffected (<4% loss). This reaction was shown to be complete at 1 min, although routine incubations are for 5 min. If purified coenzyme A is used, this reagent does not increase the background of the ATP assay.

Reaction 3: *Removal of UTP by UDPG-pyrophosphorylase.* Removal of UTP (10^{-7} M) was 99.3 ± 0.3%, while CTP, ATP, and GTP were virtually unaffected (<2% loss). Neither UDPG-pyrophosphorylase nor glucose-1-phosphate increased the background of the ATP determination.

Reaction 4: *Stoichiometric generation of ATP from ADP by nucleoside diphosphate kinase.* This reaction quantitatively transfers a phosphate group from UTP, CTP, or GTP (10^{-7} to 10^{-9} M) to form ATP within 2 min and the ATP formed is stable for at least 25 min. If a freshly prepared reagent is used together with chromatographically purified ADP, the background contributed by 10 μl of this reagent is equivalent to 0.01 pmole of ATP or a concentration of 10^{-10} M ATP in the sample (100 μl). On standing on ice for several hours the ATP content of this reagent increases, so it should be freshly prepared for greatest sensitivity. The standard deviation for the measurement of CTP, UTP, or GTP by this method was ≤3% for 1 pmole samples (N = 6).

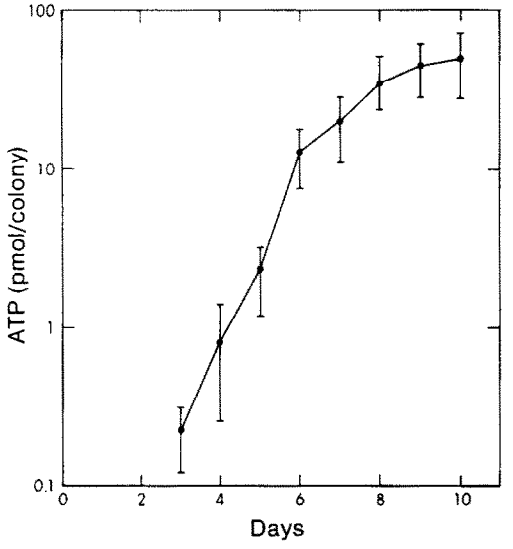

Fig. 1. ATP content of CHO cell colonies. Individual colonies were plucked on days 3–10 after plating and assayed for ATP as given in Materials and Methods. The mean and S.D. are indicated for 8 or 10 colonies.

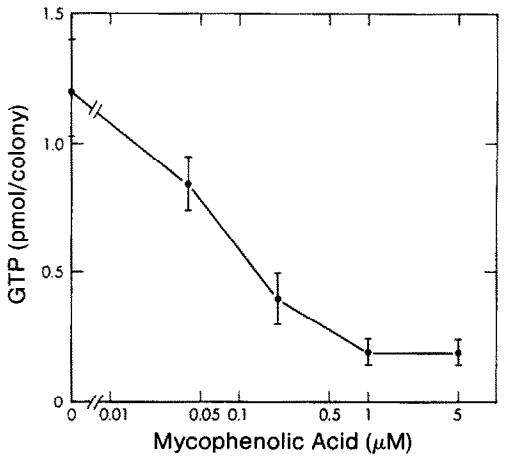

Fig. 3. Effect of mycophenolic acid on GTP pools of CHO cell colonies. On day 5 after plating, CHO colonies were exposed to the indicated concentration of mycophenolic acid by overlaying the gel with an appropriate concentration of sterile drug solution in 50 μl of saline. After 5 hr of exposure at 37°, individual colonies were plucked, extracted, and assayed for GTP as described in Materials and Methods. Data are given for the mean ± S.E., N = 6.

Measurement of ATP pools of cell colonies. CHO cell colonies were plucked on days 3–10 after plating and assayed for ATP as described in Materials and Methods. These colonies consist of approximately 60 cells on day 3 and 10,000 cells on day 10. The results (fig. 1) indicated that the average ATP content per colony increased exponentially until day 6 and reached a plateau from day 8 to 10. The content varied among colonies, reflecting a heterogeneity of size which could be clearly observed by phase-contrast microscopy. Portions of gel (2-5 μl) without colonies were also extracted as a control and had <0.01 pmole ATP/sample.

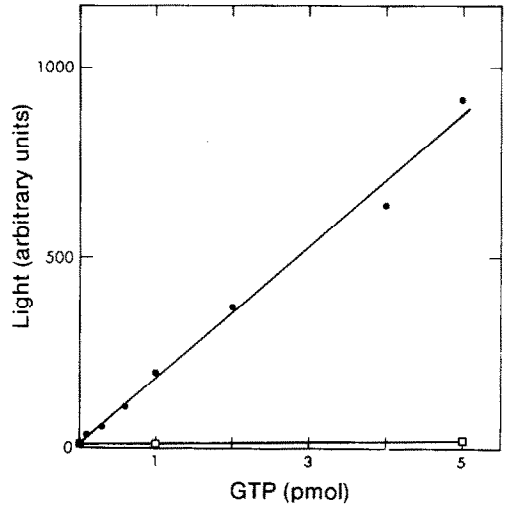

Fig. 2. GTP calibration curve. Standard solutions of GTP from 10^{-9} M to 5×10^{-8} M were assayed as described in Materials and Methods. Aliquots of 100 μl were treated with 10 μl of reagent either including succinyl thiokinase (□) or omitting this enzyme (●).

This procedure was also applied to human T-cell colonies grown by the method of Minden *et al.* [12]. The colonies on day 5 after plating had an ATP content of 2.4 ± 1.2 pmoles/colony (± S.D.).

Measurement of GTP and UTP content of colonies. The assays for GTP and UTP are less sensitive than that for ATP, and colonies of about 200 cells or more are required. The method for determining these nucleotides requires two measurements: in one the nucleotide of interest is removed, in the other this nucleotide remains and is converted to ATP. A typical calibration curve for GTP is shown in Fig. 2. The linearity of response in the absence of succinyl thiokinase and the quantitative removal of GTP by this enzyme are shown in the figure. Similar plots are obtained for the UTP assay. The assay is sensitive to 0.1 pmole of either UTP or GTP with a signal/background of 3–6 at this sensitivity. The precision when CHO cell extracts were assayed repeatedly was ± 11% S.D., a lower degree of precision than that seen with standards (± 3% S.D.).

To demonstrate the application of this assay we have examined the effect of inhibitors previously shown to deplete GTP or UTP pools in CHO cell cultures [13]. Mycophenolic acid, an inhibitor of GTP synthesis, and pyrazofurin, an inhibitor of UTP synthesis, were used in these experiments.

Mycophenolic acid produced marked depletion of GTP pools in CHO cell colonies (Fig. 3). Pool depletion was dose dependent, with a concentration of 0.1 μM required to produce a 50% depletion at 5 hr. The maximum depletion seen, with 5 μM mycophenolic acid, was 84%, similar to that reported for CHO cells grown in suspension culture [13].

Pyrazofurin produced a marked decrease in UTP pools (Fig. 4). A clear dose–response relationship was also evident here with a concentration of 0.01 μM producing a 66% decrease after a 5-hr exposure. Pyrazofurin at 0.1 μM or greater produced a dep-

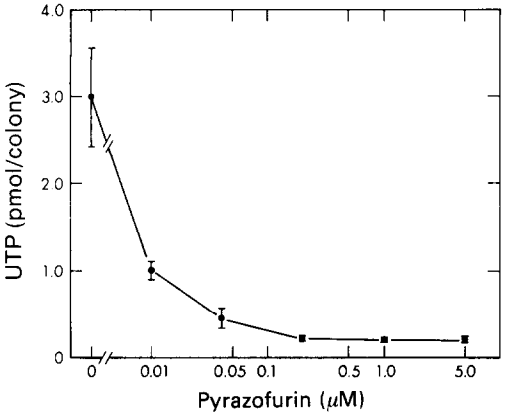

Fig. 4. Effect of pyrazofurin on UTP pools of CHO cell colonies. On day 5 after plating, CHO colonies were exposed to the indicated concentration of pyrazofurin by overlaying the gel with an appropriate concentration of sterile drug solution in 50 μl of saline. After 5 hr of exposure at 37°, individual colonies were plucked, extracted, and assayed for UTP as described in Materials and Methods. In some treated colonies, the UTP pool was below the level of accurate determination (0.2 pmole/colony); these were taken as 0.2 pmole/colony in calculating the average. Thus, the indicated pool size in colonies treated with 0.2 μM pyrazofurin or higher is an upper limit. Each value is the mean ± S.E., N = 5.

letion of >90% as was reported earlier for CHO cells growing in suspension cultures [13].

DISCUSSION

The procedure described here for measuring ATP, UTP, and GTP offers significant increases in sensitivity compared to other techniques such as liquid chromatography and previously published enzymatic assays. The reported lower limit of quantitation when nucleotides are separated on Whatman Partisil SAX columns is 30–200 pmoles [14–16], while enzymatic assays based on measurement of changes in u.v. absorbance are sensitive to 2 nmoles [17, 18]. An alternative enzymatic assay for UTP involves the formation of [^{3}H]uridine diphosphoglucose from uridine and radiolabeled glucose with a lower limit of sensitivity of 5 pmoles [19]. Karl [11] has reported an assay for GTP with subpicomole sensitivity which is based on generation of ATP by a contaminating nucleoside diphosphate kinase in crude luciferase. The assays described here can measure 0.01 pmoles of ATP and 0.1 pmoles of GTP or UTP. When such sensitivity is not required, however, the chromatographic method of course offers the advantage of the simultaneous determination of all four triphosphates.

This assay method cannot be used to measure CTP pools at present because an enzyme to specifically remove this nucleotide is not readily available. If enzymes such as ethanolamine phosphate cytidyltransferase (EC 2.7.7.14) or cholinephosphate cytidyltransferase (EC 2.7.7.15) were available, this assay could be extended to CTP. As indicated in the results, CTP is a satisfactory donor of phosphate for the quantitative synthesis of ATP from ADP in the presence of nucleoside diphosphate kinase.

This assay has been used to determine the sensitivity of cell colonies to drugs which influence nucleotide pool sizes (Figs. 3 and 4). The extension of this technique to human tumor stem cells [1] may be useful in examining the relation of biochemical sensitivity to therapeutic response, since several drugs currently under investigation affect nucleotide concentrations [20–22].

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