Adenosine triphosphate: enzymatic spectrophotometric determination

Keith Tornheim and Vera Schultz

Division of Diabetes and Metabolism, Department of Biochemistry, Boston University School of Medicine, Boston, MA, USA

Overview

ATP is the major energy currency of the cell, and the ATP level and the ATP to ADP ratio are measures of the cellular energy state. In this enzymatic assay, ATP is used in the hexokinase reaction to phosphorylate glucose. The resulting glucose-6-P is oxidized by glucose-6-P dehydrogenase in the presence of NADP. The stoichiometric production of NADPH is followed spectrophotometrically at 340 nm. The ATP must first be extracted from tissue/cells by treatment with perchloric or trichloroacetic acid and centrifugation to remove the precipitated protein. The deproteinized supernatant must then be neutralized before the enzymatic analysis.

Reagents

Use distilled/deionized water for all solutions.

0.1 M Triethanolamine buffer, pH 7.6. Add 1.49 g triethanolamine (certified grade, Fisher Scientific, Medford, MA, USA, catalog no. T407) to 50 ml water. Adjust pH to 7.6 with 1 N HCl. Make up volume to 100 ml. Store refrigerated. The solution is stable for at least 1 month.

1 M $MgCl_2$. Add 5.08 g $MgCl_2$ (hexahydrate, Fisher) to 25 ml total volume with water. Store refrigerated. The solution is stable for at least 1 month.

Hexokinase buffer (0.1 M triethanolamine, pH 7.6, 10 mM $MgCl_2$). Add 50 ml triethanolamine buffer, pH 7.6, to 0.5 ml 1 M $MgCl_2$. Store refrigerated. The solution is stable for at least 1 month.

NADP, 10 mg/ml. Add 50 mg NADP (Boehringer Mannheim, Indianapolis, IN, USA) to 5 ml water. Adjust pH to 6 with 1 N NaOH (approximately 0.07 ml). If too much NaOH is added, titrate back with HCl, since NADP is not stable under alkaline conditions. Store frozen. The material is stable for at least 1 year.

Modified from Lamprecht, W., and Trautschold, I. (1974). Adenosine-5'-triphosphate determination with hexokinase and glucose-6-phosphate dehydrogenase. In *Methods of Enzymatic Analysis*, 2nd ed. (H.U. Bergmeyer, ed.), pp. 2101–2110, Academic Press, New York.

Address reprint requests to Dr. Keith Tornheim, Boston University School of Medicine, 80 E. Concord St., E211, Boston, MA 02118, USA.

10 mM glucose. Add 90 mg glucose (Fisher) to 50 ml water. Make fresh or store frozen. This is an excellent bacterial growth milieu and should be discarded if not clear.

Glucose 6-phosphate dehydrogenase, 0.2 mg/ml. Add 50 μl of 1 mg/ml suspension (from yeast, grade I, Boehringer Mannheim) to 0.2 ml water. (Note that enzyme solutions should be mixed gently. Vigorous shaking may denature and inactivate them.) This material may be kept refrigerated for several weeks.

Hexokinase, 1 mg/ml. Add 50 μl of 10 mg/ml suspension (Boehringer Mannheim) to 0.45 ml water. This may be kept refrigerated for several weeks.

10 mM ATP. Add 30 mg ATP (disodium salt, Boehringer Mannheim) to 5 ml water. Neutralize (pH 6.5 to 7.5) with about 70 μl 1 N NaOH. Store frozen. The substance is stable for at least several months. To calibrate the solution, measure the absorbance at 259 nm of a 500-fold dilution (e.g., 0.2 ml diluted to 100 ml); a quartz cuvette must be used. The concentration (in mM) is then $A_{259} \times 500/15.4$. Although ADP (and AMP) have the same absorbance as ATP, the level of ADP contamination in the commercial ATP is normally 1% or less and can be ignored. The 1-mM ATP solution recommended to be used to check the enzymatic assay can be prepared by suitable dilution of this stock solution.

1 N perchloric acid (10% wt/vol). Add 6 ml 70% perchloric acid (PCA) (Fisher) to water, for a final volume of 70 ml.

2 N KOH/0.5 M triethanolamine. To 14.9 g triethanolamine, add 150 ml water. While stirring with a glass rod, add 26 g KOH (approximately 85% pure) (Fisher). (Note that the KOH pellets will stick to the glass beaker unless they are well-stirred. Also, the dissolving of the KOH generates much heat; it is therefore desirable to have the beaker partially immersed in a container of water.) Make up to 200 ml with water. This solution may develop a yellowish tinge after 1 month and should then be discarded.

0.6 M Trichloroacetic acid. Add 9.8 g trichloroacetic acid (TCA; Fisher) to 100 ml water for a final volume of 100 ml.

Phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). Add 8 g NaCl, 0.2 g KCl, 2.1 g Na_2HPO_4 (heptahydrate), and 0.20 g KH_2PO_4 (Fisher), to a total volume of 1,000 ml. Adjust pH to 7.4 with NaOH or HCl as needed. Make fresh or store refrigerated for no more than several days.

Procedure

The enzymes, substrates (glucose and NADP), and samples should be kept on ice. Having the buffer at room temperature should prevent fogging of the cuvette. See Discussion for spectrophotometer settings and sample preparation.

1. In a 1-ml cuvette, add:
   0.5 ml HK buffer;
   0.05 ml NADP, 10 mg/ml (use a 1 mg/ml solution for amounts of ATP < 5 nmol);
   0.05 ml glucose, 10 mM;
   0.4 ml sample plus water (for example, 0.2 and 0.2 ml) (note that TCA-extracted samples may need to be weighed), see Discussion; and
   5 μl glucose-6-P dehydrogenase, 0.2 mg/ml.
2. Mix by covering the top of the cuvette with Parafilm (Fisher) and inverting several times.
3. Place cuvette in the spectrophotometer, and take baseline measurements over 5 minutes to allow consumption of endogenous glucose-6-P.
4. Add 5 μl hexokinase (1 mg/ml) and mix. Avoid altering the position of the cuvette. This is most easily done by pipetting the enzyme solution onto a glass or, preferably, plastic stirring rod and mixing this into the

cuvette. We use a plastic "plumper" (Hellma Cells, Jamaica, NY, USA) which has a shelf at the end that can be trimmed down as needed to fit the cuvette.

5. Follow the absorbance change to completion of the reaction (5 to 10 minutes). Extrapolate any drift back to the time of hexokinase addition. The ATP concentration (in mM) in the sample is

$$\frac{(absorbance\ change)}{(extinction\ coefficient)} \times \frac{(total\ volume\ =\ 1.01\ ml)}{(sample\ volume)}$$

This value must be multiplied by any dilution during sample processing. The millimolar extinction coefficient is 6.2 or 5.9, depending on instrument configuration (see Discussion). If the absorbance change is greater than about 0.6, the assay should be repeated with less of the sample. The assay can be checked by adding 50 μl of 1 mM ATP in place of the sample; this should give an absorbance change of about 0.3. It is also advisable to run a blank reaction with no sample (or with neutralized PCA or TCA dummy samples) to check for any contamination of the reagents.

Discussion

ATP is the major energy currency of the cell, and the ATP level and the ATP/ADP ratio are measurements of the cellular energy state. (The assay of ADP will be described in a subsequent Methods of Nutritional Biochemistry.) Since ATP may be rapidly depleted in excised tissues at room temperature, or even in broken cells due to ATPases and phosphatases, samples must be quickly frozen and/or deproteinized. Cells on culture dishes or in suspension may be washed several times with cold phosphate-buffered saline, then extracted directly with cold 0.5 to 1 N PCA or 0.6 M TCA.[1] Small tissues (for example, small isolated muscles) may be quickly frozen in liquid nitrogen (or freon cooled with liquid nitrogen) to stop metabolism, and then homogenized with 1 N PCA. Larger samples or perfused organs should be quickly frozen using aluminum tongs cooled in liquid nitrogen, then powdered in a stainless steel or porcelain mortar and pestle cooled with liquid nitrogen. Aliquots of the powder can then be extracted with PCA. In some cases, for rapid acidification of the tissue in an ethanol/dry ice bath, it is recommended to mix the powder initially with 0.1 N HCl in 99% methanol before the addition of PCA.[2,3] Furthermore, since calcium carbonate or calcium phosphate precipitates can bind ATP, inclusion of EDTA or EGTA is recommended in cases in which there may be a substantial amount of calcium.[2,3]

Extraction with either PCA or TCA not only gives a low pH, at which enzymes are inactive, thus preventing further metabolism, but also causes the precipitation of proteins (including enzymes). Therefore, the enzymes can be physically removed by centrifugation (10 minutes in a clinical centrifuge). The deproteinized supernatant, which contains the soluble metabolites, is then neutralized so that the analytic enzymes used in the enzymatic assays will not be inactivated. Perchloric acid extracts are neutralized (pH 6.6 to 7.5) with 2 N KOH/0.5 M triethanolamine. KOH is used, rather than NaOH, because the resulting potassium perchlorate salt is largely insoluble and can be removed by centrifugation (15 minutes at 30,000 × *g*) after standing on ice for 30 minutes. Trichloroacetic acid extracts are neutralized by ether extraction: add 1 volume ethyl ether, vortex, centrifuge, remove the ether (upper) layer totally by aspiration, repeat four more times—this should raise the pH to at least 4. Removal of the last of the ether layer is facilitated by rapidly revolving the aspirating Pasteur pipette (connected to a vacuum line) around the inside of the tube, just above the solution, so that the stream of air evaporates the ether; the resulting cooling of the tube gives a brief misting. If the ether layer is not totally removed, a substantially greater number of extraction cycles will be needed. For small samples, processing in a microfuge is convenient. The neutralized, deproteinized sample should be quickly frozen (dry ice/ethanol

bath) if not analyzed that day. With small amounts of material, such as from cultured cells, we routinely assay ATP and ADP after no more than 1 day of storage, since the ATP to ADP ratio sometimes appeared to decline thereafter. More concentrated samples are stable for longer periods.

Use of PCA (rather than TCA) extraction has the advantage that the neutralization is more straightforward and complete. However, even though much of the potassium perchlorate is removed, the remainder can still inhibit some analytic enzymes. Thus, in the spectrophotometric assay for ADP with pyruvate kinase, no more than 25% of the reaction mixture volume can be PCA-extracted sample. Trichloroacetic acid extraction is also preferable if one wishes to concentrate the sample. Although these considerations do not apply specifically to the ATP assay, they may be important if one wishes to assay certain other metabolites on the same processed sample. It should also be noted that with the TCA/ether extraction, some ether remains dissolved in the water layer. This residual ether can cause a 5% to 20% error in sample pipetting with automatic pipets, which tend to drip because of lowered surface tension and volatilization of the ether. We therefore routinely measure TCA sample volumes by weighing what is added to the assay cuvette.

A spectrophotometer set to measure at 340 nm can be used for NADPH- or NADH-linked assays. Since the NAD(P)H absorption peak is very broad, a wide band pass (e.g., 10 nm) increases the stability and decreases signal noise without decreasing the extinction coefficient significantly. For greater stability and sensitivity, we use a Hewlett-Packard (Palo Alto, CA) diode array spectrophotometer (model 8450A) set to measure $A_{335\text{-}345} - A_{390\text{-}400}$. Averaging over the 11-nm range, together with a longer integration time (6 seconds), substantially reduces the signal noise, to about ± 0.0001 A. Subtraction of absorbance near 400 nm, where there should be no change due to NAD(P)H, eliminates much of the interference by any light scatter, dust in the solutions, precipitation, etc., which can be a significant problem at high sensitivity. The UV lamp is used since it has greater stability than the visible lamp, even though its light output is less. In this optical configuration, the millimolar extinction coefficient is reduced slightly to 5.9, from the value of 6.2 at 340 nm.

It is possible to assay several metabolites along with ATP in the same cuvette, by sequential addition of appropriate enzymes. Thus, glucose-6-P can be measured by following the absorbance change on adding glucose-6-P dehydrogenase, rather than simply including that enzyme in the initial reaction mixture. Fructose-6-P can be assayed by subsequent addition of phosphoglucose isomerase. Creatine phosphate can be assayed after ATP, by the addition of creatine phosphokinase and supplementary ADP. More detailed discussions of these assays and general methods in enzymatic analysis are given in references 3 and 4.

Our assay could detect 0.2 nmol ATP. Somewhat greater sensitivity might be obtained by using a fluorometer to measure NADPH fluorescence at 460 nm; however, an ATP standard must be added subsequently to calibrate the fluorescence change. Amounts of ATP down to about 10^{-15} mol can be measured by bioluminescent assay with firefly luciferase[5]; this is the simplest, fastest, and most sensitive of the ATP assay procedures, but the reagents are relatively expensive, and a bioluminometer specifically designed for such measurements is a less common piece of laboratory equipment. A scintillation counter can be used for luciferase measurements, but it is more awkward. Adenosine triphosphate can also be separated from other nucleotides by anion exchange or reverse-phase high-pressure liquid chromatography (HPLC) and quantitated by its UV absorbance.[6,7] Sensitivity of the HPLC methods can be increased to the picomole range by prior chemical conversion of the adenine nucleotides to strongly fluorescent $1,N^6$-etheno derivatives.[8,9] The HPLC methods are more time-consuming, require careful calibration with standards, and the instrumentation is relatively expensive; however, they have the advantage that ATP, ADP, AMP, and other nucleotides can be quantitated at the same time.

References

1 Sussman, I., Carson, M.P., Schultz, V., Wu, X.P., McCall, A.L., Ruderman, N.B., and Tornheim, K. (1988). Chronic exposure to high glucose decreases myo-inositol in cultured cerebral microvascular pericytes but not in endothelium. *Diabetologia* **31,** 771–775

2 Schultz, V., and Lowenstein, J.M. (1978). The purine nucleotide cycle: Studies of ammonia production and interconversions of adenine and hypoxanthine nucleotides and nucleosides by rat brain in situ. *J. Biol. Chem.* **253,** 1938–1943

3 Lowry, O.H., and Passonneau, J.V. (1972). *A Flexible System of Enzymatic Analysis.* Academic Press, New York

4 Bergmeyer, H.U., ed. (1974). *Methods of Enzymatic Analysis,* 2nd ed., Academic Press, New York

5 McCall, A.L., Valente, J., Cordero, R., Ruderman, N.B., and Tornheim, K. (1988). Metabolic characterization of isolated cerebral microvessels: ATP and ADP concentrations. *Microvasc. Res.* **35,** 325–333

6 de Korte, D., Haverkort, W.A., van Gennip, A.H., and Roos, D. (1985). Nucleotide profiles of normal human blood cells determined by high-performance liquid chromatography. *Anal. Biochem.* **147,** 197–209

7 Rao, G.H.R., Peller, J.D., and White, J.G. (1981). Rapid separation of platelet nucleotides by reversed-phase, isocratic, high-performance liquid chromatography with a radially compressed column. *J. Chromatogr.* **226,** 466–470

8 Ramos-Salazar, A., and Baines, A.D. (1985). Fluorometric determination of adenine nucleotides and adenosine by ion-paired, reverse-phase, high-performance liquid chromatography. *Anal. Biochem.* **145,** 9–13

9 Yoshioka, M., Hishidate, K., Iizuka, H., Nakamura, A., El-Merzabani, M.M., Tamura, Z., and Miyazaki, T. (1984). Sensitive fluorimetry of adenine-containing compounds with high-performance liquid chromatography. *J. Chromatogr.* **309,** 63–71