Comparison of Methods for Extraction of Bacterial Adenine Nucleotides Determined by Firefly Assay

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Adenine nucleotides in Escherichia coli, Bacillus cereus, Klebsiella pneumoniae, Staphylococcus aureus, and Pseudomonas aeruginosa were extracted using 10 different methods. Extracts were assayed for adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), and adenosine 5'-monophosphate (AMP) by the firefly method using an improved procedure. Analytical interference by bacterial enzymes not inactivated during the extraction was found to be a major problem. However, these enzymes were inactivated to a considerable extent by the inclusion of ethylenediaminetetraacetate in the extraction reagent. The 10 extraction methods were compared with respect to yield of adenine nucleotides, interference with the enzymic assay, reproducibility of the method, and stability of the extracts. Results indicated that extraction with trichloroacetic acid was the method most closely reflecting actual levels of ATP, ADP, and AMP in intact bacterial cells. However, for the extraction of ATP in some bacterial strains several other methods may be used and may be advantageous from a practical point of view.

The assay of adenine nucleotides is of continuous interest in the study of intermediary metabolism and many methods for the assay of adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), and adenosine 5'-triphosphate (ATP) have been devised. One of these methods, gaining widespread use, is the firefly luciferase assay of ATP (17, 23), which may be modified to include the assay of ADP and AMP (20). Due to its high sensitivity, firefly luciferase assay of ATP has also been used to estimate minute quantities of biomass (10, 14, 19), mainly in connection with bacteriological work (5, 22, 24). The latter application has prompted the development of several methods for rapid extraction of ATP from microorganisms, since the classical acid extraction procedures have practical disadvantages for routine use. Among the suggested methods are: boiling buffer (5, 10, 19), boiling ethanol (21), butanol (22), chloroform (7), sulfuric acid (8, 14), and formic acid (13).

So far none of these newer extraction procedures has been generally accepted for use in metabolic studies, partly due to insufficient characterization of their performance. With some of the methods comparative studies regarding the extraction of ATP have been performed in plants (9), lake sediments (14), blood (3), mycobacteria (7), and Escherichia coli (2, 7). However, in many biochemical studies there is an interest in being able to assay ADP and AMP as well.

The present study was undertaken to compare and, if possible, improve the performance of the various proposed extraction procedures, with respect to efficiency of extraction of the adenine nucleotides in bacteria, compatibility of the extracts with the firefly luciferase assay including the enzymic conversion of ADP and AMP to ATP, and the stability of the nucleotides when extracted. The 10 methods selected for study and tested in five bacterial strains included extraction with acids, organic solvents, and boiling aqueous solutions.

MATERIALS AND METHODS

Preparation of reagents. All metabolites and enzymes were obtained from Sigma. Metabolites were dissolved in "Tris-EDTA," which was a tris(hydroxymethyl)aminomethane (Tris)-H2SO4 buffer, pH 7.75, containing 20 mM Tris and 2 mM ethylenediaminetetraacetate (EDTA). Stock solutions of sodium salts of ATP, ADP, and AMP (2 mM) and of the tricyclohexylamine salt of phosphoenolpyruvate (100 mM) were stored at -25 °C and were stable for several months. Dilutions were made in Tris-EDTA in which they were stable for several days when refrigerated.

The firefly luciferin-luciferase reagent, Sigma FLE-50, was prepared and stored as previously described (17).

Pyruvate kinase and adenylate kinase from rabbit muscle were obtained from Sigma as suspensions in (NH4)2SO4. The pyruvate kinase suspension was diluted 10-fold in Tris-H2SO4 buffer, pH 7.75, containing 20 mM Tris and 0.1% bovine serum albumin. The adenylate kinase suspension was centrifuged,

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and the pellet was dissolved to the original volume in the same buffer as pyruvate kinase. The resulting reduction of the \((\text{NH}_4)\text{SO}_4\) concentration was necessary to decrease enzyme inhibition. After preparation the enzymes were stored on ice.

Assay of adenine nucleotides. The firefly assay of ATP was performed by injecting 0.4 ml of firefly reagent into a 1.0-ml sample measuring maximal light intensity of the resulting bioluminescence. Assay procedures and equipment have been described previously (17).

As described by Pradet (20), all three adenine nucleotides may be assayed by the firefly method using pyruvate kinase and adenylate kinase for the stepwise conversion of ADP and AMP to ATP. In the present work, 10 \(\mu\)l of the enzyme preparations in Tris-\(\text{H}_2\text{SO}_4\) buffer was used, and the assay was done at room temperature using 1.0-ml samples to which 5 \(\mu\)mol of \(\text{MgSO}_4\) 12.5 \(\mu\)mol of \(\text{K}_2\text{SO}_4\), and 0.1 \(\mu\)mol of phosphoenolpyruvate were added in a small volume (40 \(\mu\)l). At each step in the assay procedure 2 aliquots (0.1 ml) were diluted in Tris-EDTA buffer (0.9 ml) and assayed by the firefly method.

Finally, the inhibition of the bioluminescence was determined by the constant addition technique assaying a 0.1-ml aliquot added to 0.9 ml of ATP solution of known concentration (17). The 10-fold dilution of the extracts inherent in the described procedure decreased the inhibition to less than 20% in all extracts, allowing the use of the constant addition technique (17). As shown in Table 1, the inhibition in undiluted extracts was considerably higher and also strongly variable. There were no systematic differences between bacterial strains, and therefore we felt justified in calculating averages for each extraction method. The inhibition in extracts prepared with boiling Tris-EDTA buffer is caused by components from the bacterial culture, whereas the greater inhibition obtained with other extraction methods is predominantly caused by remaining inhibitory components from the extraction reagents.

Standards and blanks were assayed at each step in the analytical procedure. Nucleotide concentrations corrected for standards, blanks, and inhibition were calculated on a DEC SYSTEM 10 computer (Digital Equipment Corporation) with a FOCAL program.

The described procedure may be carried out with 25 samples by one person in less than 2 h, including calculations. The sensitivity limit of the assay of ATP and ADP is determined by the firefly reagent, whereas the limit for AMP within reasonable incubation times is a few times \(10^{-9}\) M provided ATP or ADP of approximately the same concentration as that of AMP is present in the sample. This limit could not be significantly lowered by increasing the amount of adenylate kinase, since enzyme preparations are contaminated with nucleotides. Assuming a 10-fold dilution of the bacterial culture before the assay, the procedure in its present form allows the assay of ATP, ADP, and AMP in bacterial cultures containing \(\geq 10^9\) cells/ml.

From Table 2 it may be concluded that the assay is linear and that the assay of any one of the nucleotides is not influenced by the presence of the other two nucleotides. The method was also found to give results essentially identical to those obtained by the less sensitive adenyl acid deaminase method performed according to Munch-Petersen and Kalckar (18).

Cultivation of bacteria. \(E.\) \(coli\), \(Staphylococcus\) aureus, \(Bacillus\) cereus, \(Pseudomonas\) aeruginosa, and \(Klebsiella\) pneumoniae were grown at room temperature overnight in nutrient broth (Oxoid) on a rotary shaker in Erlenmeyer flasks. Bacterial dry weights in the cultures were 0.5 to 1.4 g/liter, depending on bacterial species.

Extraction procedures. After preliminary experiments 10 extraction methods were selected for the present study and modified to be comparable. The most important modifications were that EDTA (20 \(\mu\)mol/ml of sample) was included in all extraction reagents, and that aliquots were rapidly pipetted into the extraction reagent instead of vice versa. A thorough mixing was achieved with magnetic stirring during the whole extraction period (Tris-EDTA, Tris-EDTA plus KOH, Tris-EDTA plus arsenate and n-butanol [TAEB], and perchloric acid [PCA] methods, see Table 3) or with a Vortex mixer for 5 to 10 s (butanol, chloroform, HCOOH, \(\text{H}_2\text{SO}_4\), and trichloroacetic acid methods).

Adjustment of pH in extraction reagents and extracts was done with sulfuric acid or potassium hydroxide. All extracts prepared with acids or bases were brought to pH 7.75, which was found to be the pH optimum for the bioluminescence in the assay buffer used.

Finally, all extracts were diluted in 20 mM Tris-\(\text{H}_2\text{SO}_4\) buffer, pH 7.75, to contain 10% bacterial culture and 2 mM EDTA (the final concentration in TAEB extracts was 4 mM EDTA). Further details on the extraction methods are described in Table 3. Until assayed for nucleotides extracts were kept on ice or, if the assay could not be performed the same day, frozen at \(-20^\circ\) C.

**RESULTS**

In preliminary experiments the addition of \(\text{Mg}^{2+}\) to extracts of \(E.\) \(coli\) prepared with boiling
Tris-H$_2$SO$_4$ buffer, pH 7.75, was found to result in a rapid disappearance of ATP. This phenomenon was later observed with several other bacterial strains and in extracts prepared with other methods. Since Mg$^{2+}$ is a cofactor in the enzymatic assay of ADP and AMP, it was necessary to find a method to remove the activity.

Results on the ATP hydrolyzing activity in extracts of *E. coli* prepared with boiling Tris-H$_2$SO$_4$ buffer and with PCA are shown in Table 4. The activity was heat stable in the absence of EDTA. It could be removed by centrifugation but only in the presence of PCA. Additional experiments showed that the activity was sensitive to trypsin, could not be removed by dialysis, and could result in formation of ATP with high concentrations of ADP. Thus the extracts contained nucleotide converting enzymes, presumably including ATPase and adenylate kinase. A bacterial phosphatase stable to PCA extraction has been previously described (6).

The nucleotide converting enzymes could be inactivated by heating the extracts in the presence of EDTA or by including EDTA in the extraction reagent, as shown in Table 4. Experiments performed with *E. coli* and *Klebsiella aerogenes* grown in synthetic media containing various amounts of Mg$^{2+}$ indicated that the effect of EDTA was stoichiometrically related to the amount of divalent metal ions present in the bacterial culture. A constant amount of EDTA per milliliter of culture therefore was included in all extraction procedures in subsequent experiments.

In the experiment shown in Table 5, bacterial cultures were harvested by centrifugation and the pellet was extracted with trichloroacetic acid. The supernatant was also extracted, and nucleotide levels in the growth medium were subtracted from levels in the bacterial culture extracted without harvesting the culture. Harvesting resulted in a significant decrease of the total pool of adenine nucleotides and of the energy charge value defined as (ATP + $1/2$ ADP)/(ATP + ADP + AMP) (1). Furthermore, with the exception of extracellular AMP in cultures of *P. aeruginosa*, the growth medium contained only minor amounts of nucleotides and in the following experiments only the bacterial culture was extracted.

In bacterial cells that had not been harvested, the total pool was approximately twice as high in *S. aureus* as in the other bacterial strains. With the exception of *E. coli*, the energy charge was similar in different bacterial strains and also similar to values compiled from the literature by Chapman et al. (4). The low energy charge in *E. coli* was occasionally, but not always, found in other experiments.

A comparison of the various extraction methods with respect to yield of adenine nucleotides and analytical interference during the assay of ADP and AMP is shown in Table 6. Results are corrected for inhibition of the bioluminescence.

In spite of the inclusion of EDTA in the extraction reagent, the addition of Mg$^{2+}$ resulted in a significant disappearance of ATP within 1 h in 14 of the 150 different extracts. Heating of these 14 extracts at 100 C for 3 min prior to the addition of Mg$^{2+}$ considerably reduced the activity. This was presumably due to an effect of EDTA in combination with heat, since heat alone did not remove the activity in extracts of *E. coli* (Table 4). Results obtained with heated extracts are enclosed in brackets, since nucleotide levels may have been affected by the procedure. Furthermore, in some extracts enzymic activities possibly interfering with the assay of the total pool of adenine nucleotides were not

### Table 2. Linearity and specificity of the assay of mixtures of ATP, ADP, and AMP

| Nucleotide assayed | Nucleotides in the sample\(a\) | Observed concn of nucleotide (M) | Average over different nucleotide concn ± SE\(b\) |
|-------------------|---------------------------------|-------------------------------|-----------------|
| ATP               | ATP                             | $2.20 \times 10^{-6}$         | $2.19 \pm 0.01$ |
|                   | ATP, ADP, AMP                   | $2.22 \times 10^{-6}$         | $2.22 \pm 0.02$ |
| ADP               | ADP                             | $2.40 \times 10^{-6}$         | $2.27 \pm 0.05$ |
|                   | ATP, ADP, AMP                   | $2.26 \times 10^{-6}$         | $2.37 \pm 0.06$ |
| AMP               | AMP                             | $2.18 \times 10^{-6}$         | $2.09 \pm 0.04$ |
|                   | ATP, ADP, AMP                   | $2.14 \times 10^{-6}$         | $2.10 \pm 0.04$ |

\(a\) Three solutions containing approximately $2 \times 10^{-5}$ M ATP, ADP, or AMP were prepared, as well as a fourth solution, $2 \times 10^{-4}$ M, with respect to each of the three nucleotides. These four solutions were diluted 10, 10$^2$, 10$^3$, and 10$^4$ times and assayed for ATP, ADP, and AMP. Correction for the slight contamination (less than 1%) of each of the commercial nucleotide preparations with the other two nucleotides has been made.

\(b\) SE, Standard error.

\(^{c}\) Not determinable due to the low velocity of the adenylate kinase reaction.
| Extraction method | Sample volume (ml) | Extraction media before addition of sample | Further treatments of extracts after mixing | References |
|-------------------|-------------------|-------------------------------------------|-------------------------------------------|------------|
|                   |                   | Volume (ml) | Composition | Temp (°C) |                                      |             |
| Tris-EDTA         | 0.5               | 4.5         | 20 mM Tris + 2 mM EDTA, pH 7.75            | 100        | Heating<sup>a</sup> (90 s), adjustment for evaporation | 19, 24      |
| KOH               | 0.5               | 4.5         | 10 mM Tris + 10 mM KOH + 2 mM EDTA        | 100        | Same as with Tris-EDTA, but including neutralization | 25          |
| TAEB              | 1                 | 4           | 100 mM Tris + 10 mM arsenate + 10 mM EDTA + 6% n-butanol, pH 7.4 | 100        | Same as with Tris-EDTA, but including dilution | 5           |
| Ethanol           | 1                 | 5           | 96% Ethanol + 4 mM EDTA, pH 7             | 78         | Heating<sup>c</sup> (1 min), bubbling with N<sub>2</sub><sup>a</sup> (10 min), dilution | 21          |
| Butanol           | 1                 | 1.2         | 83% n-butanol + 17 mM EDTA, pH 7          | 20         | Extraction with water-saturated n-octanol<sup>d</sup> (8 ml, 3 min), dilution | 22          |
| Chloroform        | 1                 | 0.2<sup>e</sup> | 0.3 ml of CHCl<sub>3</sub> + 0.2 ml of 100 mM EDTA, pH 7 | 20         | Heating<sup>c</sup> (2 min), hot extract on Vortex mixer<sup>e</sup> (1 min), dilution | 7           |
| HCOOH             | 1                 | 1.2         | 0.38 M formic acid + 17 mM EDTA          | 0          | Ice bath (15 min), neutralization, dilution | 13          |
| H<sub>2</sub>SO<sub>4</sub> | 1          | 1.2         | 0.25 M sulfuric acid + 17 mM EDTA        | 0          | Same as HCOOH |                                    |
| Trichloroacetic acid | 1            | 1.2         | 0.51 M trichloroacetic acid + 17 mM EDTA | 0          | Ice bath (15 min), extraction with ether<sup>f</sup>, dilution | 2           |
| PCA               | 10                | 3           | 2.3 M PCA + 67 mM EDTA                    | 0          | Ice bath (15 min), centrifugation (20,000 × g, 0 C, 15 min), neutralization and removal of KClO<sub>4</sub>, dilution | 2, 9        |

<sup>a</sup> Most of the extraction methods have been adopted from references in which they have been used for the assay of ATP in bacteria.
<sup>b</sup> Performed on a hot-plate with magnetic stirring.
<sup>c</sup> In a boiling water bath.
<sup>d</sup> To remove the organic solvent.
<sup>e</sup> Not including chloroform.
<sup>f</sup> Trichloroacetic acid was removed by extraction with water-saturated diethylether (3 × 4 ml). The first phase separation was facilitated by a short centrifugation also removing most of the cell debris. After the last ether extraction, water-saturated nitrogen gas was bubbled through the water phase for 5 to 10 min to remove solubilized ether.
<sup>g</sup> KClO<sub>4</sub> was removed from PCA extracts after neutralization with potassium hydroxide (3 ml) containing 150 μmol of Tris-H<sub>2</sub>SO<sub>4</sub> buffer and 250 μmol of K<sub>2</sub>SO<sub>4</sub>. After precipitation (1 h, 0 C) precipitate matter was removed by centrifugation (27,000 × g, 0 C, 15 min).
completely removed as described in footnotes d and f in Table 6.

A low energy charge most likely indicates a poor extraction and was found in extracts prepared with butanol, chloroform, formic acid, and sulfuric acid. The acids containing strongly chaotrophic anions, i.e., trichloroacetic acid and PCA, resulted in a high energy charge and a complete and irreversible inactivation of nucleotide converting enzymes. Extraction with trichloroacetic acid also resulted in a high yield of ATP, and no method resulted in a significantly higher yield. However, with all methods except chloroform, a comparable yield of ATP was obtained in one or two bacterial strains.

Extracts prepared with trichloroacetic acid, ethanol, and TAEB were free of enzymes interfering with the assay and values on the total pool of adenine nucleotides did not differ by more than 9%. A high value on the total pool was obtained particularly with KOH, but also with, e.g., sulfuric acid in some bacterial strains. Although extraction time and PCA concentration were sufficient (2), extracts prepared with PCA had low levels of all three nucleotides in several bacterial strains.

From results on yield of ATP and interference with the assay the Tris-EDTA, ethanol, trichloroacetic acid, and PCA methods were selected for further studies. These studies included the extraction of several aliquots of a stationary culture of E. coli to determine the reproducibility of the methods. Extracts were also stored under various conditions to study the stability of the extracts. Results are shown in Table 7. The reproducibility in the determination (extraction and assay) of all three nucleotides was within a few percent for all extraction methods.

The stability of the extracts was excellent and, with the exception of Tris-EDTA extracts, the decay rate was less than 4% per day for all nucleotides, even at room temperature. Decomposition of nucleotides resulted in a decreased level of ATP and, in some extracts, in an increased level of the total pool of adenine nucleotides. It should be noted that Mg$^{2+}$ was not present in the extracts during the storage.

The recovery of known amounts of the nucleotides subjected to the extraction procedures in the absence and presence of bacterial culture was also determined as shown in Table 8. Recoveries were generally close to 100% for all three nucleotides. However, extraction with PCA resulted in some loss of all three nucleotides in the presence of bacteria. This effect may be due to coprecipitation of nucleotides with potassium perchlorate (26) or binding of nucleotides to cell components.

**DISCUSSION**

Interference with the assay of adenine nucleotides in biological material may arise during extraction or analysis. This may be due to enzymic or chemical degradation of the nucleotides themselves, or cell components containing these nucleotides, e.g., nicotinamide adenine dinucleotide (NAD$^+$), flavine adenine dinucleotide, and ribonucleic acid.

With the present enzymatic assay procedure, a major problem was found to be nucleotide converting enzymes not inactivated by the extraction. Nucleotide converting enzymes generally require Mg$^{2+}$ or other divalent metal ion as a necessary cofactor. The presence of EDTA in the extraction reagent thus will eliminate enzyme activity immediately after disruption of bacterial membranes. If, however, disruption is
slow, nucleotide levels may be affected. Furthermore, in prepared extracts the enzymic activity may return after the addition of Mg$^{2+}$, a necessary cofactor in the enzymatic assay of adenine nucleotides.

Enzymes resulting in the disappearance of ATP after the addition of Mg$^{2+}$ could be irreversibly inactivated by EDTA in combination with heat, trichloroacetic acid or PCA (Tables 4 and 6). EDTA presumably acts by destabilizing the enzymes by complexing metal cofactors and has previously been included in methods for nucleotide extraction in plants (9), blood platelets (26), and bacteria (5, 13, 24).

Chemical degradation of NAD$^+$ results in the formation of ADP and AMP (12). Levels of NAD$^+$ in bacteria (15, 27) and rates of chemical degradation (12, 16) may be large enough to result in increased total pools of adenine nucleotides. This might explain the high total pool obtained with KOH since alkaline conditions promote the decomposition of NAD$^+$.

In addition to effects on levels of adenine nucleotides by chemical or enzymic degradation reactions, there may be an incomplete release of nucleotides from the bacteria or losses during subsequent steps in the extraction. All these effects will most likely result in an underestimation of ATP, whereas the estimation of the total pool of adenine nucleotides may be either increased or decreased. A high yield of ATP is thus the main criterion of a successful extraction.

Extraction with trichloroacetic acid resulted in yields of ATP that in all bacterial strains were higher than other extraction methods, or very close to the highest yields. Furthermore, extraction with trichloroacetic acid resulted in complete recovery of added nucleotides (Table 8). Nucleotide converting enzymes possibly interfering with the assay were detectable in only one bacterial strain (K. pneumoniae). Ethanol and TAEB, also resulting in extracts essentially free of nucleotide converting enzymes, gave values on the total pool of adenine nucleotides that were similar to those obtained with trichloroacetic acid (Table 6). Finally, chemical or enzymic degradation of cell components containing adenine nucleotides, e.g., NAD$^+$, is not likely to appear in cold trichloroacetic acid containing EDTA. Several results indicate that trichloroacetic acid extraction is the method most closely reflecting actual levels of adenine nucleotides in the intact bacterial cell.

Basic characteristics of the various extraction methods as performed by us are summarized in Table 9. Assuming that trichloroacetic acid extraction gives the physiologically most...
Table 6. Amount of adenine nucleotides extracted by different methods

| Extract | Escherichia coli | Staphylococcus aureus | Bacillus cereus | Pseudomonas aeruginosa | Klebsiella pneumoniae |
|---------|------------------|----------------------|----------------|------------------------|----------------------|
|         | A   | AA  | AAA | ECV | A   | AA  | AAA | ECV | A   | AA  | AAA | ECV | A   | AA  | AAA | ECV | A   | AA  | AAA | ECV |
| Tris-EDTA | 16  | 28  | 42  | 0.54 | 47  | (81)^a | (84) | 0.77 | 22  | 42  | 47^a | 0.68 | 29  | 53  | 94  | 0.73 | 26  | (33) | (41)^a | 0.73 |
| KOH    | 16  | 38  | 61  | 0.38 | 45  | 87  | 108^d | 0.61 | 20^* | 41  | 58  | 0.53 | 27  | 46  | 93  | 0.66 | 23  | (31) | (39)^a | 0.70 |
| TAEB   | 16  | 28  | 37  | 0.61 | 50  | 76  | 76^a | 0.83 | 25  | 40  | 43  | 0.76 | 27  | 42  | 84  | 0.74 | 23  | 32  | 37  | 0.75 |
| Ethanol | 17  | 26  | 40  | 0.55 | 57  | 76  | 84^a | 0.80 | 27  | 35  | 39  | 0.80 | 32  | 44  | 81  | 0.88 | 21  | 31  | 36^d | 0.73 |
| Butanol | 14^a | (30) | (38) | 0.60 | 66  | 84  | 110^d | 0.68 | 22  | (38) | (40) | 0.75 | 30  | (42) | (93) | 0.65 | 15  | (26) | (28)^a | 0.72 |
| Chloroform | 8   | 19  | 38  | 0.36 | 37  | 70  | 95^d | 0.56 | 14  | 31  | 47  | 0.48 | 21  | 38  | 91  | 0.55 | 9   | 21  | 39^a | 0.39 |
| HCOOH  | 10  | 21  | 63^f | 0.25 | 8   | (15) | (37)^d | 0.31 | 33  | (38) | (42) | 0.85 | 35  | (43) | (94) | 0.69 | 18  | (20) | (27)^a | 0.71 |
| H$_2$SO$_4$ | 14  | 23  | 55^e | 0.34 | 44  | (63) | (64)^d | 0.84 | 34  | (38) | (42) | 0.86 | 25  | 43  | 129^f | 0.38 | 26  | (29) | (36)^a | 0.80 |
| TCA    | 16  | 26  | 37  | 0.59 | 69  | 82  | 83  | 0.92 | 33  | 38  | 40  | 0.89 | 36  | 40  | 77  | 0.97 | 26  | 29  | 35^g | 0.80 |
| PCA    | 16  | 22  | 30  | 0.66 | 53  | 68  | 71  | 0.86 | 24  | 29  | 32  | 0.83 | 38  | 45  | 96  | 0.71 | 23  | 27  | 32^h | 0.79 |

^a As shown in Table 3.
^b cf. Table 5, footnote a. Nucleotide levels in whole cultures. Energy charge values corrected for nucleotides in the medium.
^c Values enclosed in parentheses could only be determined after inactivation of enzymic activities in the extracts (100 °C, 3 min).
^d Incubation to the following day of the complete assay mixture containing extract, pyruvate kinase, adenylate kinase, and necessary cofactors resulted in a decrease of the presented value by more than 20%.
^e Standard error of the mean > 10%. Average standard error of the mean in entire material 2.7%.
^f Prolonged incubation resulted in an increase of the presented value by more than 20%.
Tris-EDTA, ethanol, trichloroacetic acid and PCA extraction methods

| Extraction method | Standard deviation (% average) | Initial rate of decay (%) per day | -20°C | 0°C | +20°C |
|-------------------|--------------------------------|----------------------------------|-------|----|-------|
|                   | A | AA | AAA | A | AA | AAA | A | AA | AAA |
| Tris-EDTA         | 3.2 | 2.0 | 2.4 | <0.2 | <0.2 | <0.2 | 5.6 | 3.5 | <0.2 |
| Ethanol           | 2.6 | 3.1 | 4.5 | <0.2 | <0.2 | <0.2 | 1.4 | <0.4 | <0.5 |
| Trichloroacetic acid | 3.0 | 2.5 | 1.4 | <0.2 | <0.2 | <0.2 | <0.5 | <0.2 | <1 |
| PCA               | 3.4 | 3.2 | 3.4 | <0.2 | <0.2 | <0.2 | <0.2 | <0.2 | <0.2 |

* See Table 3.

† Eight to 10 samples.

‡ Reported values refer to the initial rate of decay of nucleotides. In extracts prepared with trichloroacetic acid and PCA, there was a more than 10-fold increase with respect to ATP (A) and ATP + ADP (AA) after 1 or 2 weeks at room temperature. Prior to this there was a sudden increase of the ATP + ADP + AMP (AAA) level.

Table 8. Recovery of nucleotides added during extraction with Tris-EDTA, ethanol, trichloroacetic acid, and PCA

| Extraction method | Recovery (% ± SEM) | Culture absentc | Culture presentc | Culture absentc | Culture presentc |
|-------------------|--------------------|----------------|-----------------|---------------|-----------------|
|                   | A | AA | AAA | A | AA | AAA | A | AA | AAA |
| Tris-EDTA         | 95 ± 2 | 96 ± 2 | 97 ± 2 | 94 ± 6 | 99 ± 5 | 102 ± 3 |
| Ethanol           | 96 ± 1 | 99 ± 3 | 99 ± 3 | 106 ± 8 | 102 ± 3 | 100 ± 5 |
| Trichloroacetic acid | 100 ± 4 | 102 ± 6 | 103 ± 6 | 106 ± 6 | 103 ± 6 | 102 ± 6 |
| PCA               | 96 ± 1 | 95 ± 3 | 102 ± 2 | 80 ± 5 | 85 ± 3 | 93 ± 2 |

* See Table 3.

† A mixture of ATP, ADP, and AMP (2 × 10⁻⁴ M) was extracted alone or together with a stationary culture of E. coli. The culture had approximately the same level of adenine nucleotides and was added to the extraction media immediately after the nucleotide mixture. SEM, Standard error of the mean.

‡ See Table 7 for abbreviations.

Table 9. Characteristics of the various extraction procedures

| Extraction method | Yield of adenine nucleotides (as compared to trichloroacetic acid) | Inhibition of bioluminescence | Interference with the assay by enzymes | Reproducibility | Stability of extracts |
|-------------------|---------------------------------------------------------------|-----------------------------|---------------------------------------|-----------------|----------------------|
|                   | ATP | Total pool | Equal or higher | Higher | Slight | Slight | Variable | Good | Good |
| Tris-EDTA         | Less or equal | Equal or higher | Slight | Slight | Good | Good |
| KOH               | Less or equal | Equal | Slight | Slight | Good | Good |
| TAEI              | Less or equal | Equal | Slight | Slight | Good | Good |
| Ethanol           | Less or equal | Equal | Slight | Slight | Good | Good |
| Butanol           | Less or equal | Equal | Slight | Slight | Good | Good |
| Chloroform        | Less or equal | Equal | Slight | Slight | Good | Good |
| HCOOH             | Less or equal | Equal | Slight | Slight | Good | Good |
| H₂SO₄             | Less or equal | Equal | Slight | Slight | Good | Good |
| Trichloroacetic acid | Less or equal | Equal | Slight | Slight | Good | Good |
| PCA               | Less or equal | Less | Strong | Strong | Good | Good |

* See Table 3.

† Average standard deviation taken over all bacterial strains in Table 6 varied between 1.9 and 5.1%.

‡ Only ATP degradation determined and found to be <0.2%/day at -20°C and <10%/day at 0°C.

§ With the exception of P. aeruginosa.
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relevant results, it may be concluded that no other method is suitable for the assay of all three nucleotides in all bacterial strains. However, fairly similar results were obtained in one or two bacterial strains for all three nucleotides with TAEB, ethanol, formic acid, or sulfuric acid, and for ATP with all extraction methods except chloroform.

The choice of extraction obviously has to be governed by the purpose of the investigation and may also be influenced by practical considerations. Thus, before the final choice is made, it is probably useful to compare a few methods in each particular system using trichloroacetic acid extraction as a reference.

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