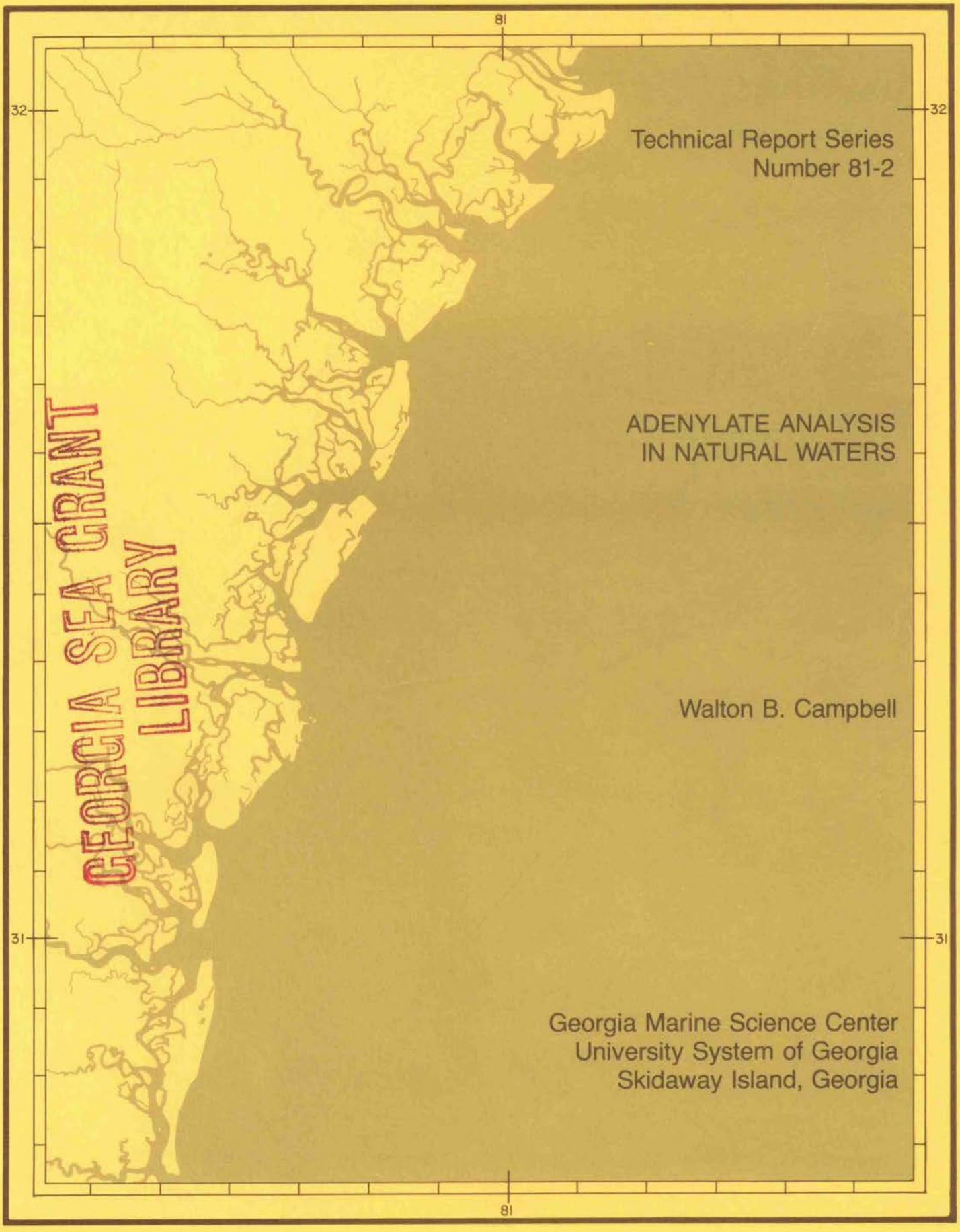

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ADENYLATE ANALYSIS IN NATURAL WATERS

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by

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ABSTRACT

The firefly luciferase assay is widely used by marine ecologists to measure both ATP and total adenylates. There are substantial variations in the procedures used in these analyses, some of which may lead to substantial errors. The preparation of reagents, analytical procedures, and standardization are discussed and sources of error evaluated. An extensive bibliography on the methods and their application is included.

INTRODUCTION

During the past decade marine ecologists have been using the firefly luciferase assay for adenosine 5' triphosphate in determinations of microbial biomass. According to Watson (1978),

> The ATP technique is a difficult technique and subject to many sources of error.

Judging from the number of conflicting reports in the literature, many investigators have this feeling. The introduction of total adenylate and adenylate pool energy charge ratio methods (Atkinson 1968) have both enhanced the information content of microbial adenylate pool measurements and increased the complexity of the assays with a concomitant increase in the sources of error.

This report is not intended to replace careful examination of the literature nor laboratory experience with these methods. The theoretical metabolic implications of adenylate pool measurements have been covered comprehensively by Knowles (1977). Methodological considerations are reviewed in this report toward the end that better measurements may be made by more investigators on diverse systems and measurements can be compared between labs and ecosystems. Our primary emphasis has been on problems associated with environmental sampling. Hopefully not all of the potential difficulties covered in this report will be manifest in a single set of samples. Each type of interference is discussed with respect to its site or mode of action. A variety of time and money saving experiments have been performed and are presented as an aid in adenylate pool analyses. Our experiments have been performed on both the JRB

model 1000 and SAI model 2000 ATP photometers. Most of the methodologies discussed could be adapted easily to another instrument.

I would like to express my gratitude to the Georgia Sea Grant Program for funding the publication of this report. Special thanks are due to co-workers who shared many long hours of assays and produced most of the discussions that provoked critical experiments covered in this document: T. R. Jacobsen, A. M. Wood, J. V. Nabholtz, R. A. Rabek. This research was funded by NIH grant 5-T01-GM01968 and DOE contract EY-76-S-09-639.

APPLICATIONS OF THE REACTION

Firefly luminescence has been studied for over a century (Harvey 1940). The dependence on ATP of the light output from crude firefly lantern preparations was elucidated by McElroy (1947), McElroy and Strehler (1949) and McElroy et al. (1969). Strehler and Totter (1952) first suggested an assay for ATP based upon this reaction. The absolute specificity of the enzyme, luciferase, for ATP was demonstrated by Green and McElroy (1956). Although assays of this type had been used in biochemistry, Holm-Hansen and Booth (1966) first suggested a biomass assay based upon the ATP content of microbes. Chapman et al. (1971) demonstrated a correspondence between the ratio of ATP, ADP and AMP pools and microbial metabolic condition in pure culture. Weibe and Bancroft (1975) proposed use of this pool ratio for ecological investigations of microbial community metabolism.

The firefly luciferein-luciferase assay is a very sensitive method of measuring ATP, other nucleotides, or other compounds which are enzymatically converted to ATP (e.g. cyclic AMP: Johnson et al. 1970, Shultz and Daly 1973; FMN and NAD: Stanley 1971a, b). Minimal detection ranges have been reported from 10^{-8} µg ATP (Chappelle and Levin 1968) to 10^{-10} mg ATP (Wise 1974) to 10^{-6} µg (Cheer et al. 1974) to 10^{5} bacteria/ml (Conn, et al. 1975).

The current hypothesis on the mechanism of the firefly enzyme (luciferase)-substrate (luciferin)-ATP-light reaction is that two steps are involved:

$$E(\text{luciferase}) + LH_2\ (\text{luciferin} + \text{ATP}) \xrightarrow{Mg+2} E\text{-}LH_2\text{-AMP} + \text{pyrophosphate}$$

(Chappelle and Picciolo 1975)

$$(E\text{-}LH_2\text{-AMP})_2 \xrightarrow{pH7.74} \text{oxyluciferin} + E + CO_2 + \text{AMP} + \text{light}$$

(Borun 1975)

Assuming all reactants except ATP are present in excess, the light output should be proportional to ATP concentration (Karl and Holm-Hansen 1976, Strehler and Totter 1952; Seliger and McElroy 1960). A quantum yield of 0.88 ± 0.25 photon per ATP molecule consumed has been calculated by Seliger and McElroy (1960) and this is the basis for the assay of ATP by this reaction (Strehler and McElroy 1957). The rate limiting step has been hypothesized to be the formation of the luciferyl-adenylate complex ($E\text{-}LH_2\text{-AMP}$) (McElroy and DeLuca 1973).

ASSUMPTIONS

The principal assumptions of both biomass and metabolic measurements of microbial adenylate pools are: 1) adenylates are made only by living organisms, 2) energy-containing adenylates (ATP and ADP) are rapidly degraded upon cessation of life, 3) adenylates can be quantitatively extracted and accurately assayed, 4) the amount of adenine nucleotides per unit biomass is predictable, and 5) non-cellular adenylates are not a significant source of interference. While these assumptions may be closely approached for some laboratory cultures, there are several pitfalls awaiting the unwary sampler of natural systems.

The assumption that ATP arises only from biological sources is not totally justified on the basis of work with simulations of the reducing conditions thought to exist in the primitive earth atmosphere. The nucleotide adenine has been produced in reasonable yields (100 mg/1 in 4 days at 27° C) without biological intervention (Oro 1960, Oro and Kimball 1962, Oro 1963, Lowe *et al.* 1963). Ultraviolet irradiation of dilute adenine and ribose solutions has been shown to produce the nucleoside adenosine by Ponnamperuma *et al.* (1963). The nucleoside phosphates ATP, ADP and AMP have been produced

abiologically in measurable quantities from ADP, AMP and adenosine respectively in primitive earth simulations (Ponnamperuma et al. 1963). The ATP produced was found to be active in producing light from purified firefly luciferin-luciferase enzyme system with time course curves identical to those from ATP extracted from a biological source. There is no reason to believe that interference from non-biogenic adenylate is a significant problem. However, the hypothesis that ATP is only associated with living organisms and is quickly hydrolyzed upon cessation of life processes (Hamilton and Holm-Hansen 1967) has been called into serious question. Hodson and Azam (1977) and Azam and Hodson (1977a) found waters from the aphotic zone off Southern California to contain ten times as much dissolved ATP as was found in organisms. According to the calculations of Hulett (1970), ATP dissolved in 21° C seawater at 21°C should have a half-life of 7-8 years in the absence of organisms. Azam and Hodson (1977a) have found concentrations of dissolved ATP in coastal seawater up to 500 ng/1. Their stability estimates for ATP in sterile seawater were for a half-life in excess of 100 days.

The process of nucleotide extraction seeks to inactivate ATPases and ADPases by denaturation of enzyme proteins. All ATP-ase activity is not associated with proteins (Lowenstein 1958,1960). Nucleotide tri- and di-phosphates have been found to bind to apatite crystals (Taqui, et al. 1962; Cohn and Hughes 1962). This not only causes an adsorptive loss of nucleotides from solution but also promotes, in the presence of inorganic phosphate and bivalent metal ions, trans-phosphorylations of the terminal nucleotide phosphate at physiological temperature and pH in the absence of proteins (Krane and Glimcher 1962). ATP hydrolysis is catalyzed by members of the 3d group, especially copper (Schneider and Britzinger, 1964; Tetas and Lowenstein, 1963 and Buisson and Sigel, 1972), but use of 2, 2' bipyridyl is reported to complex these ions effectively out of solution by means of mixed-ligand metal ion complexes.

FIREFLY ENZYME

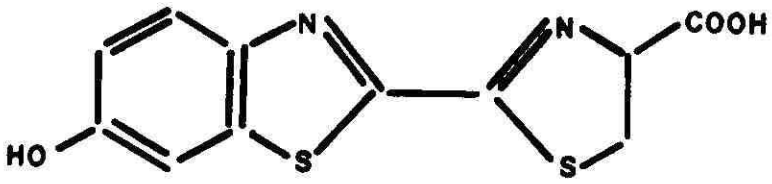
The enzyme and substrate for the ATP to light reaction may be obtained in a variety of forms. The substrate, luciferin, can be synthesized or purchased

either as a synthetic product or in a partially purified mixture with its enzyme. The enzyme, luciferase, can be purified chromatographically (Green and McElroy 1956, Nielsen and Rasmussen 1968, Bekhor et al. 1977), by isoelectric focusing (Lundin 1977), purchased as a purified product or as a crude mixture. The commonly used and relatively inexpensive Sigma preparations, FLE 50 and FLE 250 have been found to be undersaturated in luciferin. Thus additional linearity and sensitivity can be obtained by supplementing the crude FLE with purified or synthetic D-luciferin (Jones and Simon 1977, Karl and Holm-Hansen 1976, Bekhor et al. 1977, Lundin et al. 1976, Neufeld et al. 1975, Chappelle and Levin 1968, Schram et al. 1970). These authors arrive at the rather different minimal values of luciferin necessary for maximum sensitivity of 100 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.0 µg/ml and 0.112 µg/ml respectively. All do agree that additional luciferin increases assay sensitivity up to some concentration, beyond which it becomes inhibitory. Such an increase in sensitivity of 100 fold (Karl and Holm-Hansen 1976) allows dilution of the preparation to such an extent that contaminating endogenous ATP within the preparation becomes so dilute as to substantially lower its contribution to the background light emission.

Sensitivity can also be increased by using the much more highly purified DuPont reagent (#760-145-902), either alone or as a mixture, with less refined preparations available from Sigma, Calbiochem, Worthington, Antonik or Lumac. This blending of enzymes has the advantage of enhancing the sensitivity of the crude preparations while increasing the stability of the highly purified enzyme substrate (usually less than 4 hours at room temperature unless made up in 17% glycerol as recommended by Chapman et al. 1971). Addition of 0.1% bovine serum albumin has also been reported to increase both enzyme stability and sensitivity (Jones and Simon 1977). Purified luciferase is available for custom blending with luciferin or any of the mixture preparations for the desired combination of sensitivity, background stability, and economy.

LUCIFERIN

The substrate for the enzyme luciferase, D-luciferin, was first synthesized and characterized by Seliger et al. (1961) as $C_{13}H_{10}N_2S_2O_3$:



It may be synthesized by the methods of Bitler and McElroy 1957, Seto et al. (1963) or White et al. (1963). It has a molecular weight of 280.33, a flourescence peak at 535 nm (Seliger et al. 1961) and is quite stable to storage if frozen in the crystalline form.

The oxyluciferin (dehydroluciferin) produced during the assay reaction or during prolonged storage in aqueous solution is a potent competitive inhibitor of luciferin (80% inhibition at 3.2 μM) (DeLuca et al. 1964; Airth et al. 1958). This oxyluciferin catalyzes the breakdown of ATP in the presence of pyrophosphate without the emission of light (DeLuca and McElroy 1965). Natural firefly lanterns have a very high pyrophosphatase activity which counteracts the inhibition in vivo (Hastings 1968). Competely synthetic enzyme preparations lack this activity. The metabolic cofactor, Co-A (5×10^{-5}M), has been reported to remove oxyluciferin from its binding site on the luciferase enzyme surface by forming oxyluciferyl-Co-A. In the presence of cysteine this complex reportedly formed a stable N-oxyluciferyl cysteine which did not interfere with the light producing reaction (Airth et al. 1958).

LUCIFERASE

The enzyme, luciferase, approximately 50,000 mw., has been purified from natural sources and characterized by Green and McElroy (1956), Nielsen and Rasmussen (1968) and Bekhor et al. (1977). The temperature optimum for this enzyme has been found to be 23° C (Seliger and McElroy 1964; Green and McElroy

1957; Chappelle and Levin 1968). The emission peak of yellow-green light at 562nm is shifted toward the red and attenuated by a pH lower than 7.6 (Seliger and McElroy 1960, McElroy and Seliger 1962, McElroy and DeLuca 1973) and is adversely affected the presence of metallic ions (Zn^{+2}, Cd^{+2}, Hg^{+2}) or halogens (Seliger and McElroy 1964, Aledort et al. 1966).

The quantum efficiency of the reaction has been reported to be decreased by a wide variety of natural and artificial chemicals. The herbicides isopropyl-3-chlorocarbanilate, isopropyl-5-chloro-2-hydroxycarbanilate and isopropyl-3-chloro-4-hydroxycarbanilate have been reported as competitive inhibitors with respect to D-luciferin (Rusness and Still 1974). Both 0.1 μM phosphoenol pyruvate and 2 μg/ml pyruvate kinase (which are used in energy charge assays) have been reported to be inhibitory to the luciferase light yield (Roberton and Wolfe 1970). In addition, the chlorine ion has been found to be inhibitory (65%) to the production of light at 97 mM concentration, and 20% ethanol has been reported inhibitory (50%) (Holmsen et al. 1966). Heavy metals, such as mercury, also inhibit the light yield from the luciferase assay by direct action on the enzyme-substrate complex (Patterson et al. 1970). Samples collected from near shore, swamp, or forest regions subject to leaching of water-soluble phenolic compounds may inhibit significantly the luciferin-luciferase reaction. These phenolic compounds have been reported to be sorbed from solution after extraction but prior to assay, by treatment with an insoluble polyvinylpyrrolidone (Guinn and Eidenbock 1972) but there has been some question about how complete this adsorption is in some systems (Cunningham and Wetzel 1978).

The presence of inorganic salts changes the ionic strength of the reaction mixture and can be inhibitory to the quantum light yield. Sodium chloride (10mM) has been found to be highly inhibitory (50%) (Strehler and Totter 1952, Holmson et al. 1966). Magnesium ion concentration has been reported to have an optimal concentration of approximately 0.01 M and was found to be inhibitory both above and below this concentration (Green and McElroy 1956, Chappelle and Levin 1968). Measured with respect to 97 mM sodium chloride the cations Ca^{+2}, K^{+2}, Rb^{+2}, and Li^{+2} have been shown to be inhibitory (100% to 73%) to the light yield at concentrations of 97 mM (Aldedort et al. 1966). The salts, NaBr, $KClO_3$, and KH_2PO_4, have been found to be inhibitory 53% to

93%; iodide ion was (100%) inhibitory and $NaHCO_3$, NaF, and KF salts produced stimulations of 127%, 222% at 97 mM with respect to the reaction yield in 97 mM NaCl (Aledort et al. 1966).

ENERGY CHARGE CONVERSION ENZYMES

The estimation of total adenylates and energy charge involve measurement of ATP as described and also ADP and AMP. The most common method of doing this is by performing enzymatic conversion on subsamples which are then processed as ATP assays. After enzymatic (pyruvate kinase) conversion of the ADP to ATP, the ADP concentration is obtained by subtraction. A second subsample is processed in the same manner as the ADP sample except that it also contains the enzyme, adenylate kinase (myokinase) to convert AMP to ATP and ADP. AMP concentration is then determined by another subtraction. A brief description of the interconversion enzymes should facilitate understanding both the mechanism of sample processing and precautions associated with their use on diverse types of samples.

PYRUVATE KINASE

Pyruvate kinase (ATP:pyruvate kinase EC.2.7.1.40) reversibly catalyzes the transfer of the phosphoryl group from phosphoenolpyruvate to ADP in the presence of magnesium and potassium to yield pyruvic acid and ATP (Lohmann and Meyerhof 1934). The mammalian enzyme has been reported to exist as several distinct isozymes (Osterman and Fritz 1972, Jimenez et al. 1971). Pyruvate kinase is probably a constituent of all living cells (Boyer 1962). It is easily obtained in good yields from skeletal muscle and is stable for over one year in high purity preparations (Kayne 1973). Preparation and purification are described by Boyer (1962) and Kayne (1973). This enzyme has preferential specificity of purine di- and tri- phosphates (the activity with inosine diphosphate is 75% and with guanosine di-phosphate being 60% of that with ADP (Davidson 1959). Maximum reaction velocities at pH 7.5 have been found to be ADP $\cong$ GDP > IDP > ADP > UDP > CDP > dCDP (CDP being only 30% of that of ADP)

(Plowmann and Krall, 1965; Weber, 1969), while the Km for ADP was one fourth that for GDP.

Pyruvate kinase is inhibited competitively by ATP (Meyerhof and Oesper 1949; Reynard et al. 1961). As with other kinases, pyruvate kinase has a requirement for the divalent cation, magnesium (Lohman and Meyerhof 1934), and additionally requires potassium or a related monovalent cation for activity (Boyer et al. 1942) such as NH_4^+ or Rb^{+2} (Kachmar and Boyer 1953). It is strongly inhibited by calcium (Kachmar and Boyer 1953), copper, zinc, mercury (Boyer 1962) and p-mercuribenzoate (Solvonuk and Collier 1955). Diethyl-stilbesterol (Kimberg and Yielding 1962) and phenylethylbiguanide (Mildvan and Cohn 1965) have also been found to be competitively inhibitory. Fluoride, ferricyanide, iodoacetate, phenothazine, alloxan, urethan arsenate and 2,4-dinitrophenol were reported as having little or no inhibitory activity (Boyer 1962).

ADENYLATE KINASE

Adenylate kinase (myokinase, ATP:AMP phosphotransferase, EC.2.7.4.3) reversibly catalyzes the transfer of one phosphoryl group from ATP onto AMP to produce two molecules of ATP in the presence of magnesium as described by Kalckar (1943). Adenylate kinase also catalyzes the conversion of IDP to ITP by the same mechanism (Kalckar 1943). The foreward reaction of adenylate kinase to produce ATP and AMP is inhibited by AMP, but no inhibition has been noted from IMP or adenosine (Colowick and Kalckar 1943). Karl and Holm-Hansen (1978a) have reported an inverse relationship between total adenylate concentration and the apparent Km of adenylate kinase when used in a coupled reaction with pyruvate kinase and low total adenylate concentrations (<500ng/mg). For rabbit muscle adenylate kinase, a bivalent cation is required and the order of reactivity is $Mg^{+2} > Ca^{+2} > Mn^{+2} > Ba^{+2}$ (O'Sullivan and Noda 1968; Mildvan 1970). Adenylate kinase is ubiquitous in living cells (Noda 1973). It's preferences for nucleotide triphosphates are ATP > 2'dATP > CTP > GTP > UTP > ITP (O'Sullivan and Noda 1968). The pH optimum is 7.5 (Kalckar 1943). Adenylate kinase is found in high concentrations in rabbit and frog muscle and is only reduced to 79% of it original activity after being subjected to 100° C

for 10 minutes in 0.1 N HCl (Colowick and Kalckar 1943). Electrophoretic resolution of adenylate kinase has revealed the existence of several isozymes from each source (Giblett 1969; Khoo and Russell 1972). Its preparations and purification are described by Colowick and Kalckar (1943) and Kalckar (1943).

TYPES OF ADENYLATE EXTRACTION

The principal function of adenylate extraction methods is to cause leakage of cellular nucleotide pools, usually through cell lysis, accompanied by a rapid inactiviation of all enzyme systems which could produce, degrade or interchange (by means of transphosphorylations) of nucleotide phosphate bonds. A second aspect of adenylate extractions is to stabilize the extracted nucleotide phosphates against losses from solution by non-enzymatic hydrolysis, adsorption or precipitation while maintaining an environment which is compatible with subsequent assay techniques. Surveys of adenylate extraction techniques for different conditions of the medium have yielded different optimum methods for each matrix (Lundin and Thorne 1975a,b; Knust et al. 1975; Cunningham and Wetzel 1978). Three approaches to extraction are commonly employed. First are the organic solvent extractions which are usually carried out at or above room temperature; these include ionizing solvents such as dimethyl sulfoxide (Chappelle and Levin 1968; Searle 1975; Levin et al 1967); and formamide (Knust et al. 1968; Knust et al. 1975) and nonionizing organic solvents such as chloroform (Paul and Johnson 1977; Dhople et al 1971; Dhople and Hanks 1973a,b), boiling ethanol (St.John 1970; 1971) acetone (Chappelle and Levin 1968), acetone with ether (Clark et al. 1978), methylene chloride (Knust et al. 1975), butanol-octanol (Conklin and MacGregor 1972; D'Eustachio and Johnson 1968), n-butanol (Chappelle and Levin 1968) and n-bromosuccinimide (MacLeod et al. 1969). The hydrophobic organic solvents disrupt cell walls causing leakage and inactiviation of enzymes through dehydration denaturation.

A second type of extraction uses acids and is generally performed at or near freezing temperatures. The types of acids used include perchloric (Picciolo et al. 1971; Albaum and Lipshitz 1950; Albaum et al. 1951; Abood and Goldman 1956; Cheng 1961; Chapman et al. 1971; Cole et al. 1967; Bagnara and

Finch 1972; Bächi and Ettlinger 1973; Cocucci et al. 1973; Ball and Atkinson 1975; Parkinson and Medley 1972); trichloroacetic (Brooks and Lutwak-Maunn 1971; Brooks 1970; Bauchop and Elsden 1960; Bomsel and Pradet 1968; Chalauatnatol and Haesungcharern 1977); sulfuric (Ausmus 1971, 1973; Hodson et al. 1976; Forrest and Walker 1965a, b; Belaich 1966; Lee et al. 1971a,b; Karl and LaRock 1975); formic (Klofat et al. 1969, Bomsel and Pradet 1968), and nitric (Picciolo et al. 1975b). Low temperatures are generally required (Azam and Hodson (1977a) to minimize acidic hydrolysis of the nucleotides while allowing oxidiative denaturation of the enzymes and other cellular components.

The third type of extraction involves boiling aqueous buffered solutions such as those using TRIS [tris-hydroxymethyl-amino-methane] (Greengard et al. 1954; Beutler and Baluda 1964; Holm-Hansen and Booth 1966; Chappelle and Levin 1968; Bamstedt and Skjoldal 1976) boiling glycine buffer (Bedell and Govindjee 1973), boiling glycyglycine (Ching and Kronstad 1972), boiling water (Burn 1962; Aledort et al. 1966; Ching and Ching 1972; Cockburn 1974), boiling sodium bicarbonate (Bancroft et al. 1976), boiling phosphate (Bulleid 1978), boiling phosphate-citrate buffer (Bulleid 1978), or just boiling buffered growth medium (Billen et al. 1953).

Not all the above extractions are optimal for all systems and some of them may be considerably less than optimal for most systems (Lundin and Thore 1975b). According to Bulleid (1978) the two major requirements of extraction are: 1) the maximum amount of ATP is extracted from the sample and 2) substances interfering with the later stages of the assay are removed, minimized, or not extracted. As noted above, perchloric acid extractions seem to have remained quite popular even after reports that ATP is coprecipitated in a non-predictable manner when the perchloric acid is neutralized with base (Wiener et al. 1974).

EXTRACTION EFFICIENCY

The most common method of evaluating the efficiency of adenylate extraction methods is to measure the yield from each method as a percentage of the one giving the highest total yield. This is valid only so long as the tech-

nique giving the highest yield is doing so without changing the adenylate concentration from the other cellular components during extraction. The quantity of adenylates extracted from E. coli was found to be very sensitive to pH in that at a pH greater than 7.0 metabolic processes which generate ATP ADP, and UTP may proceed during the extraction processes (Kaplan et al. 1951). Thus alkaline extractions such as boiling bicarbonate (Bancroft et al. 1976), boiling phosphate-citrate (Bulleid 1978), and boiling glycylglycine (Ching and Kronst 1972) may produce artifically elevated ATP and ADP yields since these can both be produced from AMP or UDP by NADH under alkaline extraction conditions (Lundin and Thore 1975).

Soils and sediments may contain phosphate binding sites which make adenosine phosphate extractions less efficient the greater the concentration of sites (Williams et al. 1970). This adsorption can be minimized by using 0.6 N sulfuric acid extraction (Lee et al. 1971b). Unfortunately, this solublizes many metal ions which then must be removed by cation exchange resins since metal ions are inhibitory to the luciferin-luciferase assay reaction (Seliger and McElroy 1960). Metal ions such as Cu^{+2}, Zn^{+2}, Ca^{+2}, and Ce^{+3} have been shown to hydrolyze ATP and ADP in aqueous solution above pH 7 (Tetas and Lowenstein 1963). According to Karl and LaRock (1975) some of the ionic interferences may be reduced by the addition of EDTA to the extractant, but Lee et al. (1971b) dispute this. Addition of potassium phosphate buffer to a boiling TRIS buffer extraction has been reported to reduce ATP binding and give an increase in ATP extraction yield from the sediments (Moriarty 1975). Fulvic acids (>1000, <10,000 mw) have been reported to form irreversible complexes with ATP, preventing its interaction with the firely enzyme luciferase (Cunningham and Wetzel 1978). These complexes were found to be dependent only upon the concentration of fulvic acids.

CONCENTRATION OF AQUATIC SAMPLES

Aquatic samples often require concentration before extraction. In open ocean samples Sutcliff et al. (1976) found total ATP recovery from filtered sea water to be directly proportional to the volume of water filtered, but for inshore waters they noted an apparent loss of up to 50% to some undefined

absorption phenomenon associated with the increased particle content of inshore water. Chapman et al. (1971) found that filtration of pure cultures caused a drop in energy charge of 0.5 (the entire energy charge scale spans from 0 to 1) and loss of 50% of total adenylates, while Jones and Simon (1977) reported 44-97% loss of ATP as a result of filtration concentration. Direct extraction in acid without filtering can be done to avoid loss of ATP or change in energy charge, but it must then be concentrated before assay. You can extract directly in acid without filtering to avoid losses of ATP or lowering of the adenylate energy charge - then concentrate. The speed with which adenine nucleotide pools can change has been found to be as little as one minute for an 80% decrease in ATP (Klofat et al. 1969), or energy charge may drop 50% in less than one minute (Cocucci et al. 1973). Karl and Holm-Hansen (1978a,b) found an asymptotic relationship between the decrease in energy charge and the volume of water filter-concentrated. They also noted the higher the starting energy charge, the greater the effect of filtration. The total adenylate pool remained constant while the individual nucleotides rearranged their respective proportions. The difference between leaving a small film of water on the filter as opposed to pulling it dry has been reported to cause a loss of up to 98.6% of the ATP pool (Cheer et al. 1974). Lundin and Thore (1975b) found that centrifugation harvesting resulted in a significant decrease of the total pool of adenine nucleotides and of the energy charge.

The potentially rapid response of cellular adenine nucleotide pools to even minor stress makes the measurement of ATP alone a rather questionable procedure. The theory of adenylate-pool energy charge reflecting cellular metabolic condition dictates that the proportions of the pool constituents be free to change in response to physiological needs (Bamstedt and Skjoldal 1976, Skjoldal and Bamstedt 1976). This has been repeatly demonstrated (Strange et al. 1963; Forrest 1965; Klofat et al. 1969; Chapman et al. 1971a,b; Chapman et al. 1971; Wiebe and Bancroft 1975; Miovic and Gibson 1973). Cole et al. (1967) measured very large fluctuations in ATP levels in E. coli subjected to stress by cooling or abrupt changes in oxygen partial pressure. Shifting the conditions of the medium in E. coli cultures were followed by such large decreases in ATP levels that Cole et al. (1967) concluded "these results throw doubt on methods for assaying ATP in which a large lag exists between harvest-

ing and extracting for assay." This is not at all uncommon in field ecological sampling regimes. If filtered samples were frozen and extracted several days later, up to 70-80% of the ATP was lost, but if frozen in liquid nitrogen after extraction, little if any ATP was degraded (Cheer et al. 1974). The possibility of extracting unconcentrated samples and then concentrating the extract on activiated charocoal (Rasmussen and Nielsen 1968; Hodson et al. 1976) has been proposed as a method of collecting adenylate data from minimally perturbed aquatic samples. Karl and Holm-Hansen (1978a) have reported nonquantitative recoveries of AMP, ADP, and ATP from such columns. Azam and Hodson (1977c) report 50-60% recovery on columns. They also report 13X higher recovery of ATP from water samples using acid extraction instead of TRIS. Other means of increasing sensitivity rely on making the luciferin-luciferase system more sensitive. This is discussed in the section covering luciferase.

The above observations have lead to the conclusions that: 1) adenylate samples should be extracted as soon as possible to limit sampling effects on the nucleotide pool proportions (Holm-Hansen et al. 1975; Hodson and Azam 1977; Knowles 1978), and 2) total adenylate pools are more likely to give a realistic estimate of biomass than any single component of the pools, much less the most labile pool constituent, ATP.

If filter-concentrated samples are harvested by extracting the filter, it can be excluded from the extract or allowed to be frozen with the concentrate. The main reason for including the filter in with the extract is that all of the extract should be present to be later assayed. This includes both the liquid absorbed into the filter itself (as much as 0.8 ml for a 47 mm cellulose acetate filter) and the extract captured by capillary-action between the walls of the extraction tube and the filter membrane (as much as 0.3 ml for the apparatus used in this laboratory. The logic for excluding the filter is the possibility of some variable adsorption or dephosphorylations of adenylates by the filter or the materials (eg. metals) concentrated thereon.

Samples frozen with the filter show slightly higher ATP and total adenylate contentration (Table 1). The energy charges from the samples frozen with the filter removed are approximately 10% higher than those with the filter

Table 1. Replicate samples frozen with and without concentrating filter included. Coefficients of variation for replicate ATP and total adenylate extraction were less than 3% and for energy charge less than 8%.

	Frozen with Filter			Filter Removed		
Depth	nM ATP	nM Total	E.C.	nM ATP	nM Total	E.C.
8 m	0.48	1.54	0.55	0.50	1.41	0.62
18 m	0.87	1.83	0.66	0.54	1.34	0.65
21 m	1.09	2.42	0.65	1.11	2.27	0.73
26 m	1.58	4.32	0.64	1.13	2.59	0.67

included. The reason for the lower energy charges in the samples was a consistently higher AMP concentration. If the included filter and contents were adsorbing adenylates, there would seem to be a slight preference for ATP over AMP, or else the removed filter retain more AMP than ATP. Possibly the filter and contents were leaching an inhibitor of adenylate kinase which interfered with the conversion reaction at the time of assay. Although these data are not extensive, workers using filtration of samples should consider the possibility of this discrepancy with respect to their own filtered samples.

PROCEDURE

Cleaning Glassware

Great care must be taken with the preparation of standards and chemicals for use in the luciferin-luciferase assay. Metallic ion residues left behind from chromic acid cleaning cling tenaciously to glass surfaces and are slowly released into solution (Laug 1934; Henry and Smith 1946; Richards 1936; Ames and Nesbett 1960). Metallic ions interfere with the enzyme-substrate reactions, as discussed under the description of luciferase, and can also cause hydrolysis of ATP in solution, as discussed under the section on extraction methods. Cleaning of glassware and utensiles with dichromate solutions should be avoided. Soaking or rinsing in 0.1 N, HCl, followed by thorough rinsing and oven drying, has produced satisfactory results.

Experimental Protocol

The experimental protocol used for energy charge determinations is summarized in Table 2 and pictorially outlined in Figure 1. It is essentially a modification of that described by Chapman et al. (1971) for use with an SAI model 2000 ATP photometer, or any other photometer which will accommodate 1.5 ml sample volumes and use peak detection mode. A total of 600 enzyme units of pyruvate kinase and 1800 enzyme units of myokinase are dialized separately for a minimum of four hours against 800 ml of 0.1 M potassium phosphate buffer pH 7.4 at 4° C.

The ATP determinations are performed on aliquots of a mixture of 100 µl of a mixture A which contains 16 mM $MgSO_4$ and 84 mM pH 7.4 potassium phosphate buffer incubated 15 min. at 35°C. This is kept on ice until time of assay (less than 60 min.) whereupon two 200 µl subsamples are placed in 1.3 ml 7.5 mM pH 7.74 glycyglycine buffer contained in 20 ml scintillation vial. This is mixed by rapid swirling, lowered into the assay chamber of the photometer and mixed with an injected volume of 200 µl firefly lantern extract. The assay count is begun at the instant of injection of FLE by an automatic switch and only the first major peak within the first 6 sec. is measured.

The ADP determinations are identical to those of the ATP except that 100 µl of the sample is incubated with mixture A and a mixture B containing 16 mM $MgSO_4$, 76 mM pH 7.4 phosphate buffer, 0.8 mM phosphoenol pyruvate and 600 enzyme units of pyruvate kinase. The ATP concentration determined above is then substracted from the ADP + ATP concentration determined in this step to yield the ADP concentration.

The measurement of AMP is identical to that of ADP except that 1800 enzyme units of myokinase are added to the pyruvate kinase, phosphoenol pyruvate, phosphate buffer and $MgSO_4$ reaction mixture. The ADP + ATP concentration determined above is then subtracted from this ATP + ADP + AMP value to obtain the AMP concentration.

AMP Correction

Occasionally a negative value for AMP will be calculated when dilute adenylate samples are assayed (Bamstedt and Skjoldal 1976). This is caused by the dependence on total adenylate concentration for the coupled adenylate kinase-pyruvate kinase reaction. The apparent adenylate kinase K_m for AMP is raised by lowering the concentration of ATP (Karl and Holm-Hansen 1978). This is not a problem in samples containing several micromolar total adenylates and can be corrected in samples in the nannomolar range by performing conversion efficiency assays on each sample. This is done by adding some unknown quantity (0.4-0.04 ng/µl or approximately 1-10 nM) of ATP to a duplicate AMP determination, incubation sample, and standard. A second standard curve for the ATP + ADP + AMP measurements will be generated by which more accurate AMP determinations may be made. Since AMP is usually less than 10% of the total adenylate pool of living cells, this additional processing may not be desirable for large-quantity, routine assays.

For adenylate samples near the lower limits of detection (less than 500 ng/ml), the impure nature of the inexpensive firefly lantern extracts necessitates a heat inactivation step of incubated ATP, ADP and AMP subsamples. A 2 min treatment before assay at 100° C has been suggested (Karl and Holm-Hansen 1978) as sufficient to denature the added pyruvate kinase and prevent instantaneous production of ATP from ADP introduced in the crude firefly reagent. This procedure has been found to increase the linearity and lower detection limits of the ATP vs light curve.

EVALUATION OF EXPERIMENTAL PROTOCOL

A series of experimental evaluations of several practical considerations to routine adenylate pool measurements are discussed below. They involve those aspects of adenylate measurement as they pertain to the above protocol.

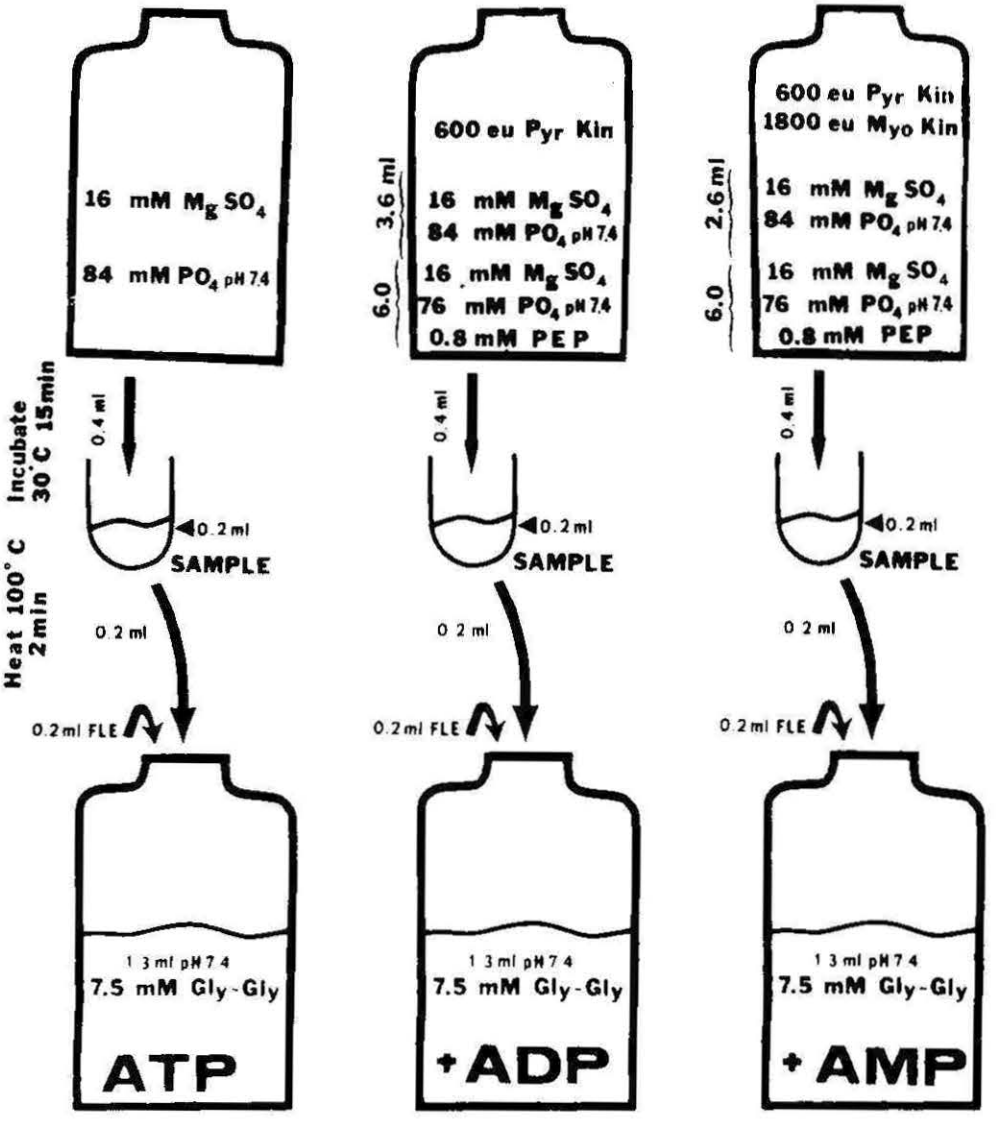


Figure 1. Schematic of the procedure for adenylate analysis.

Stability of Adenine Nucleotides

The stability of dilute solutions of ATP at room temperature was measured to ascertain the necessity of maintaining samples at low temperatures during the assay. The concentration of a 1.0×10^{-7} M ATP (Fig. 2) standard decreased 10% within the first 3 hrs and fell 20% within 6 hrs at 25°C. The energy charge of the standard was down 28% by 6 hr and after 8 hr at room temperature the energy charge had dropped 0.35 units of its 1.0 span. A drop of 93% in ATP concentration occurred after 24 hr at 25° C, with an accompanying decrease of 0.8 energy charge units. Thus a dilute solution of nucleotide deteriorates over intervals as short as 3 hr at room temperature. Each data point was the mean of 10 replicates. Standard deviations were smaller than the point depicting the mean.

Changes over time of dilute aqueous solutions at 4° C were determined in order to evaluate the maximum time that samples can be held after thawing. Shown in Figure 3 is the deterioration curve for a 1.0×10^{-7} M ATP standard held in a 4° C ice bath for up to 65 hours after being thawed. In the first 24 hr the ATP had decreased 12% and energy charge had decreased 22%. At 65 hr ATP concentration was still within 69% of its original value while energy charge had dropped to 61%. Each data point represents the mean of 10 replicate samples, and one standard deviation for the ATP measurements is shown (brackets), while those for the energy charge were smaller than the symbols depicting the means.

It should be borne in mind that the two experiments shown above were performed with ATP standards in deionized pH 7.0 water and thus would indicate only an upper limit of holding characteristics. Real samples, containing cellular debris and possible inorganic materials would potentially contain adsorption and reaction sites which could seriously shorten the permissible holding time. If samples must be held for prolonged periods after thawing, the speed and degree of their deterioration at 4° C should be determined individually. Substantial decreases in the ATP content of ATP standards held in an ice bath for as little as 6 hours have been reported (Patterson et al. 1970).

Table 2. Procedure for adenylate assay.

Reagents

1000 ml potassium phosphate buffer 0.1 M pH 7.4; 1800 enzyme units myokinase (Sigma grade III); 1200 enzyme units pyruvate kinase (Sigma type II); 1.0 ml 0.01 M phosphoenolpyruvate; 100 ml 0.1 M $MgSO_4$; 2000 ml 0.075 M glycylglycine (10 g/l + 60 ml 0.1 M $MgSO_4$; adjust to pH 7.74 with NaOH)

	$MgSO_4$	PO_4 Buffer	PEP
20.0 ml Mix A	3.2	16.8	-
12.5 ml Mix B	2.0	9.5	1.0

	Mix A	Mix B	Pyruv. Kinase	Myokinase	HOH
10 ml ADP mix	3.13	6.25	0.15 (approx)	-	0.52
10 ml AMP mix	3.13	6.25	0.15 (approx)	0.52(approx)	-

120 ml firefly enzyme: 2 FLE-250 rehydrated to 30 ml each + 1.2 mg synthetic luciferin + 60 ml arsenate buffer (12 ml 0.1 M $MgSO_4$ + 0.54 g KH_2AsO_4, adjust to pH 7.74 with NaOH, bring up to 60 ml)

Incubation

0.4 ml sample extract with 0.1 ml AMP, ADP, or Mix A (for AMP, ADP, or ATP). Agitate every 5 min and maintain at 30-35 C for 15 min, then hold on ice until assay.

Assay

0.2 ml of incubated mixture + 1.3 ml glycylglycine; inject 0.2 ml firefly enzyme and begin peak count.

Optimization of Light-Producing Reactants

Batch preparations of ATP standard solutions may be divided into several aliquots and frozen for later use. Storage times of up to 6 months (Aledent

et al. 1960), and one year (Cheer et al. 1974) have been reported with no measureable change in ATP standards and we have stored frozen standards for over three years with no loss in ATP content. We do not recommend refreezing a thawed standard because some deterioration in ATP content may occur during the interval the standard is in use (Patterson et al. 1970). One must also ensure that frozen standards are well sealed to preclude freeze-dry concentration of the solution during storage. When ATP standards are thawed for calibration purposes they must not be brought to room temperature by heating above room temperature as this will accelerate the rate of ATP deterioration in a dilute solution. Frozen standards must be completely thawed (e.g., no remaining ice) before being sampled due to the likelihood of differential freeze exclusion of ATP from the ice. Depending upon the rate the standards was initially frozen, the rate that the ATP molecules come out of the ice may be different than the rate water molecules leave the ice crystals.

The optimal quantities of reagents for the firefly ATP assay depend upon the interactions between the optical geometry of the instrument and sample, the mixing characteristics of the reactants, the effect of these on reaction kinetics, and the total pH buffering ability of the reaction system. An experiment was conducted to determine the optimal glycylglycine buffer volume for 200 µl of sample to which 200 µl of firefly lantern extract was injected. The results (Fig. 4) imply that the buffer volume is rather critical to obtain good reproducibility. The extremely wide error bars around all quantities of glycylglycine less than 1.3 ml make these unacceptable. The range of 1.3 to 1.4 ml had much smaller standard errors and the highest sensitivity. The range of 1.5 to 2.0 fell on a broad plateau of acceptable standard errors but was less sensitive by 5.2%. Proportions other than those tested would require reevaluation.

FLE Enzyme Optimization

To optimize the economics, sensitivity, and low background counts of the firefly lantern enzyme assay of ATP, the crude preparation can be diluted (Chappelle and Levin 1969; Schram 1970; Karl and Holm-Hansen 1976). Different concentrations of FLE extract without additional luciferin were compared

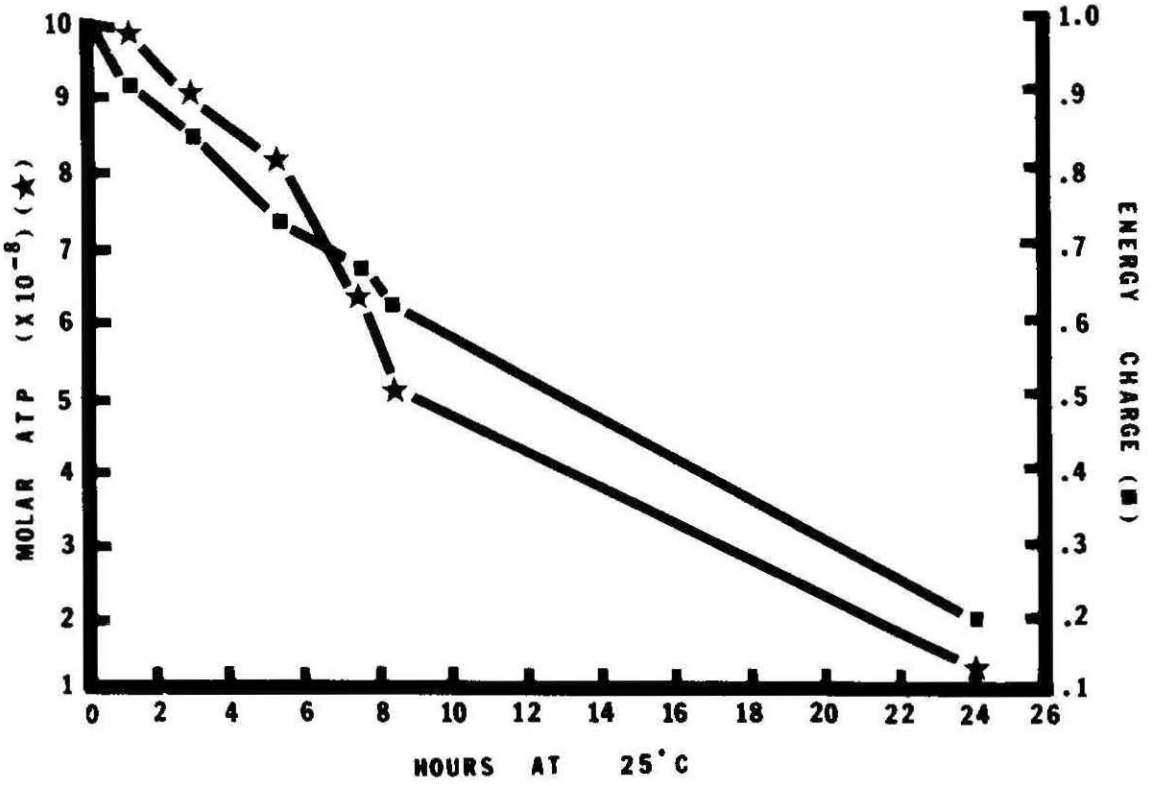


Figure 2. Stability of dilute solutions of ATP at room temperature.

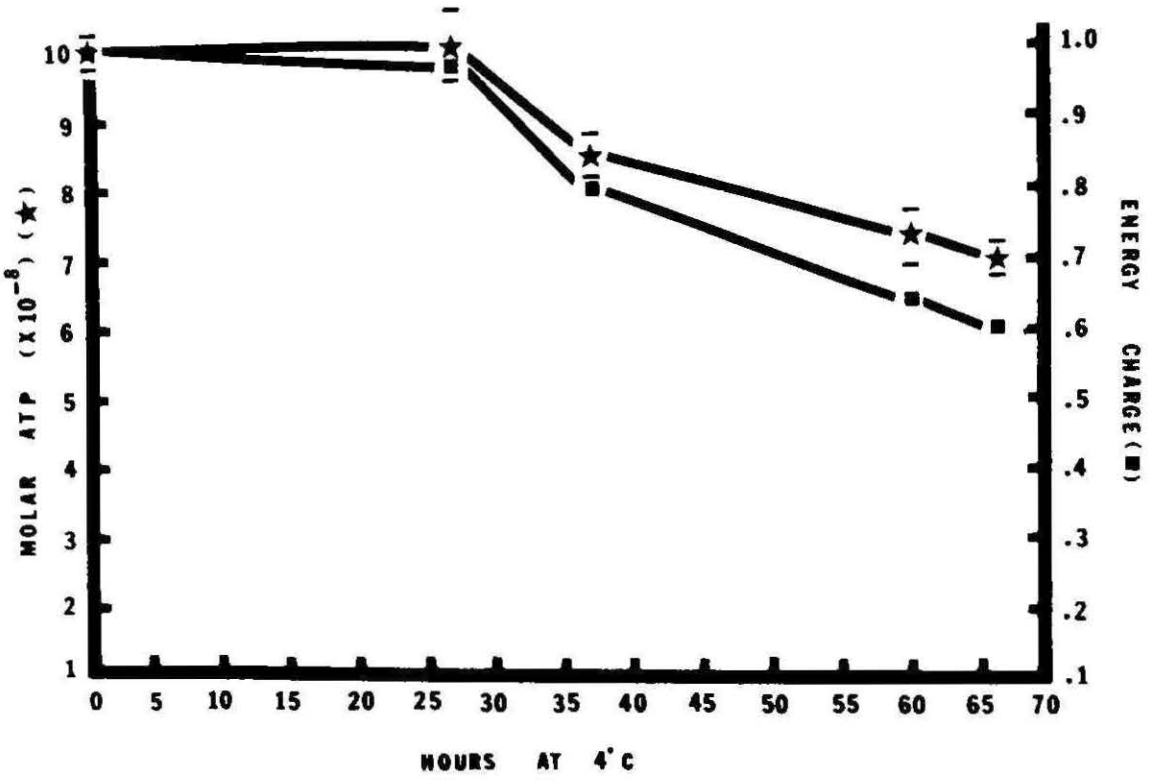


Figure 3. Stablility of ATP at 4°C.

(Figure 5). When counts from 1.0 x 10^{-7} M ATP standard were assayed in 66% Sigma FLE (1:3 in 0.05 M arsenate buffer), the background was within 33% of the counts of the standard, while full strength FLE preparation had a background within 69% of the standard.

Figure 6 demonstrates the effects of added luciferin and dilution on crude FLE extract. For 100% FLE synthetic D-luciferin the 1 x 10^{-7} M ATP sample was 68% over background, and at 66%, the same standard was eight times the background. The total counts for this 1/3 dilution were still over 300% of those from the 100% unsupplemented FLE extract (Figure 4) while the background was 1500 counts lower with the luciferin-fortified, diluted FLE extract.

Effect of Freezing FLE Extracts

The utility of being able to prepare, in advance, a large quantity of firefly lantern extract, using only as much as is needed for an assay series, and refreezing the remainder, lead to an investigation of the stability of FLE extract to repeated cycles of refreezing. Shown in Figure 7 is the typical response of a oneliter volume of FLE extract used for routine assays and refrozen several times. The initial sensitivity of the enzyme is almost halved after the first freeze-thaw cycle, which is accompanied by a slight drop in background. The overall trend is for a generally stable response with a slight decline in background counts for each additional thawing. At some time, in this example the sixteenth cycle, the enzyme activity drops drastically and does not recover with further cycles of freezing. The exact time of this decline is not always sixteen but is usually more than ten cycles. Attempts to reactiviate the enzyme preparation by addition of more luciferin produced no measureable increase in activity. Attempts to reactiviate the enzyme activity through additions of cofactor Co-A either alone or with cysteine produced no measureable change (Airth et al. 1958). Addition of a small quantity (1 ml) of this inactive enzyme to 100 ml of freshly prepared FLE resulted in total inactivation of all of the new FLE extract.

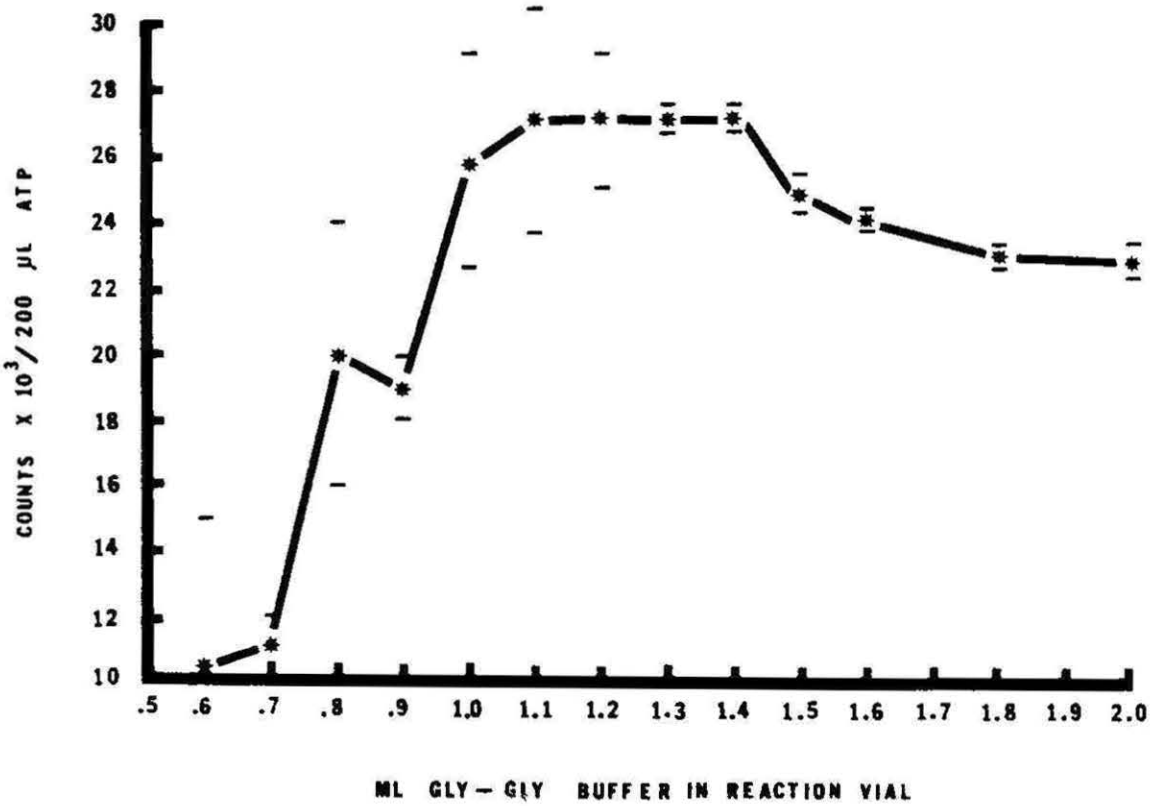


Figure 4. Effect of amount of glyclyglycine on ATP assay.

There is apparently a very potent inhibitor produced during freezing, thawing and storage. The FLE enzyme solution for the data presented in Figure 7 was prepared as discussed earlier. The standard errors for samples are shown as bars while the standard error intervals for the background count were smaller than the symbol depicting the point. This FLE was used in routine assays, frozen to -30° C for from 12 hours to several days, thawed for 2-3 hours in a 25° C water bath and this process repeated until loss of activity occurred. While the FLE was thawed and being used, an aliquot of approximately 100 ml was allowed to come to room temperature for the actual assays, since the temperature optimum for the reaction is 23° C (Green and McElroy 1956; Chappelle and Levin 1968). The remainder of the thawed enzyme was held in an ice bath until more was needed during the day. Any FLE remaining at the

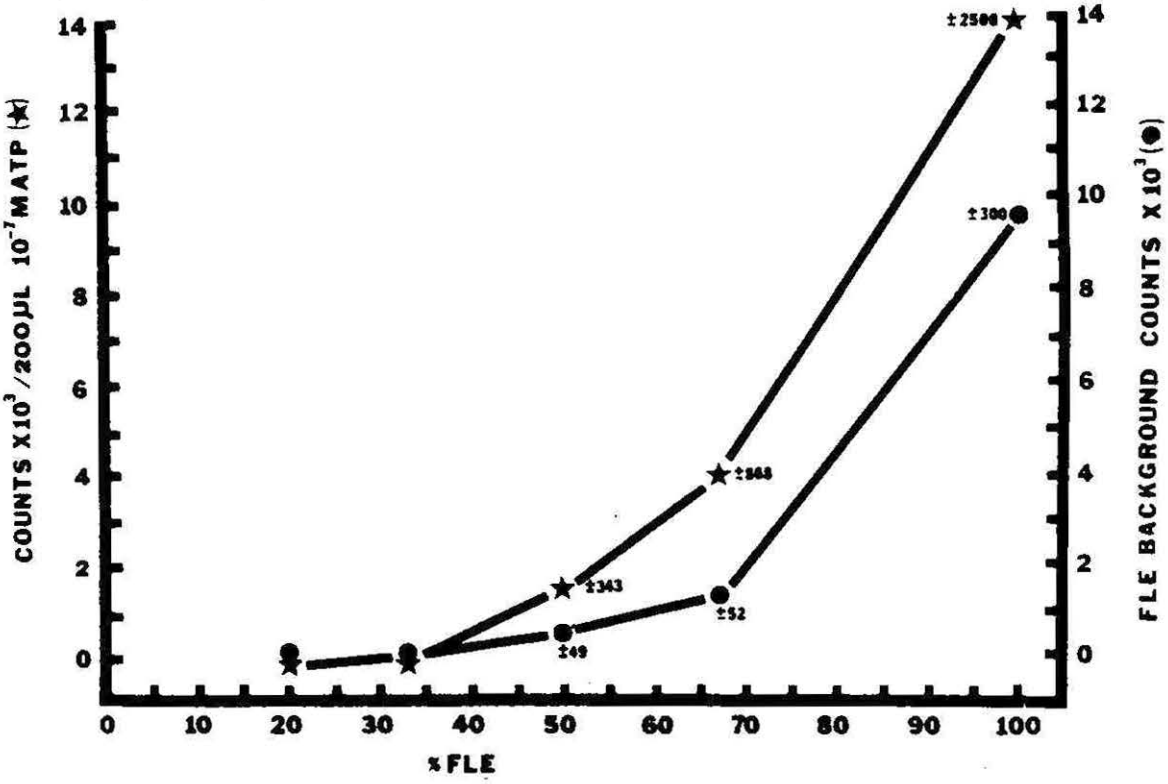


Figure 5. FLE extract dilution without added luciferin.

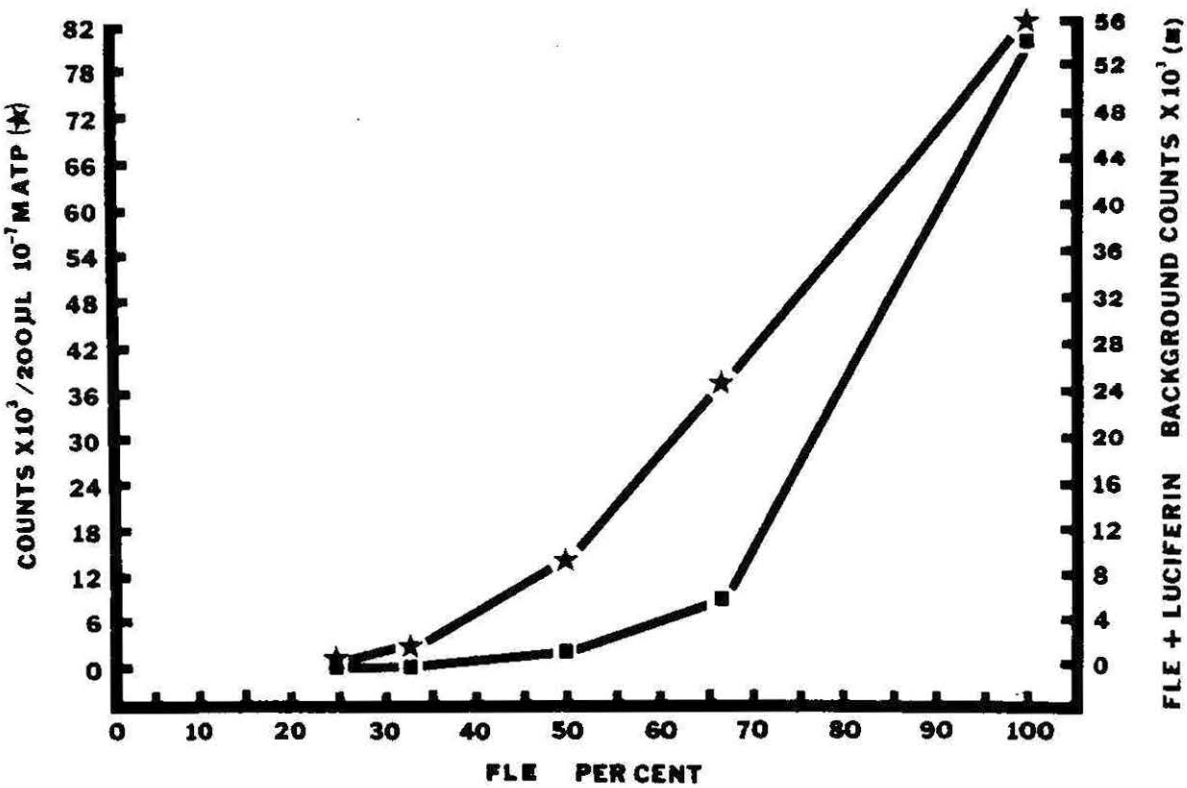


Figure 6. FLE extract dilution with added luciferin.

end of a day was returned to the main volume and refrozen. The inhibitor could have been produced during any or all of the above steps and no evaluation was made to ascertain the relative importance of each of these. Freezing FLE in small batches, to eliminate the need for repeated thawing, is thus recommended.

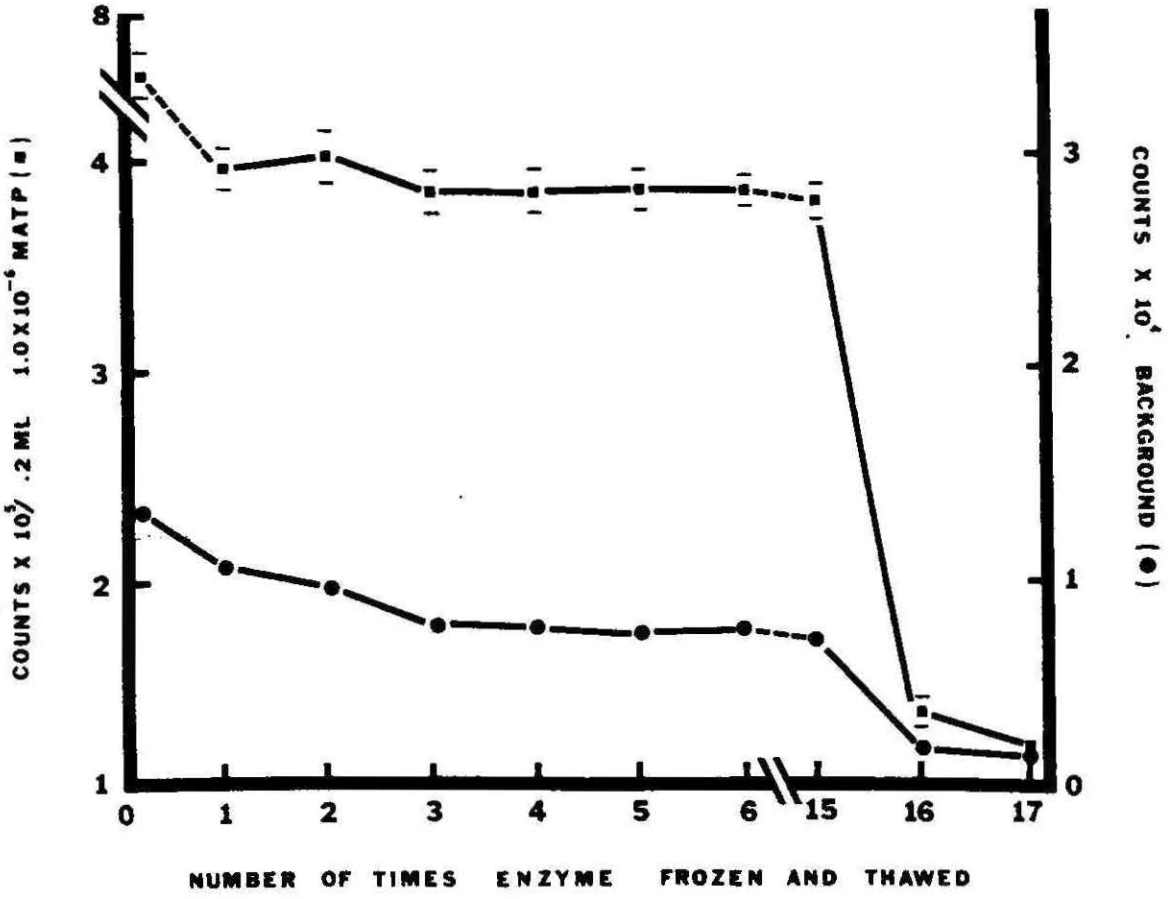


Figure 7. Freeze-thaw experiment with the FLE extract.

Handling of Energy Charge Reagents

Additional observations on the handling of energy charge reagents have been made toward the end of easier routine sampling processing. Both pyruvate kinase and myokinase should be dialyzed a minimum of four hours (for high

concentrations of adenine nucleotide the contamination introduced by omitting dialysis is apparently negligible) but can be dialyzed for 6 days and retain 97.8% ± 0.06% of original activity. When maintaining the dialysis solution at 0-4°C it is possible to have ice formation on the surface of the solution. This has not been found harmful to the kinases as long as the portion of dialysis tubing containing the enzymes is not allowed to become frozen. After dialysis, the kinases can lose up to 96.1% ± 0.21% of their activity if allowed to become frozen.

Aqueous (pH 7) solutions of phosphoenol pyruvate can be stored at 0-4°C for up to 32 days and retain 98.2% ± 0.51% of original activity, but if allowed to freeze were found to drop to 0.1% of previous activity levels. The enzyme mixtures for ADP and AMP conversions dropped 73.2% ± 0.33% and 70.1% ± 0.23% respectively when stored at 0-4°C for 24 hours. Taking into account the above findings, maintenance of all adenylate standards and samples at or below 4°C at all times is advisable to minimize sample deterioration and freezing of reaction enzymes should be avoided.

Preparation of Standards

Very highly purified adenosine phosphate standards may be prepared from commercial preparations of "pure" ATP, ADP, and AMP by chromatographic repurification on DOWEX-1,10% crosslinked, 200-400 mesh anion exchange resin (Cohn and Carter 1950). Since the resin is supplied in the chloride form, it must be regenerated initially to remove the luciferase-inhibitory ion (Aledort et al. 1966) by exchange with 10% NaOH through the 1 cm^2, 1 cm long bed. A solution of commercial adenosine nucleotide should be made up approximately 10^{-2} M and adjusted to pH 11 with NaOH. With a flow rate of 3 ml min^{-1} the pH then should be dropped in steps by use of acetic acid. Adenine should elute at approximately pH 7, 0.01 M NH_4 acetate; AMP at pH 3, 0.003 M acetic acid; ADP at pH 2.5 with 0.2 M Na acetate; and ATP at pH 2.5 with 0.2 M Na acetate. The pH of the resolved peaks should then be adjusted to pH 7.0 with NaOH and the concentrations precisely determined spectrophotometrically. Experience with purified adenine nucleotides from Sigma, Calbiochem and Boehringer would indicate that this step is superflous. This procedure is not recommended for

use except in cases where there is some doubt about the purity of the standard ATP, ADP, or AMP.

Prepare a 10^{-2} M solution disodium adenosine 5'-triphosphate (m.w.= 551.2) in deionized, autoclaved water at pH 7 ± 0.1. This should be serially diluted to give a 1 X 10^{-4} M solution. One further dilution will give a 1 X 10^{-5} M solution (this is roughly the equivalent of 10^{10} cells of Enterobacter aerogenes in logarithmic growth phase). The concentration of this 1 X 10^{-5} M ATP solution should next be measured spectrophotometrically. Be sure to use 1 cm quartz spectrophotometric cells. The molar extinction coefficient of adenine nucleotides at pH 7.0 for the wavelength 259 nm is 1.54 X 10^{4} Kalckar (1947). Using the equation $O.D._{259}/E = C$ where $O.D._{259}$ is the optical density of the solution at 259 nm (a dimensionless quantity), C is the molar concentration of the solution and E is the molar extinction coefficient (moles^{-1} cm^{-1}) the actual concentration can be determined and noted before further serial dilutions are made. If the above manipulations have been carried out precisely, the $O.D._{259}$ of a 1 X 10^{-5}M solution of ATP at pH 7.0 will be 0.154.

An extensive standard series of ATP solutions may be prepared by either of two different dilution methods from spectrophotometrically validated standards. Solutions of ATP should be diluted (Figure 8) to obtain the series of 1 through 9 X 10^{-5} M in increments of 1.0 X 10^{-5} M. These solutions are used as the stock for further dilutions. A 1 : 10 serial dilution is performed on each of these nine stock solutions and each again is checked for accuracy by optical density measurement at 259 nm. A different set of these (Figure 9) 1:10, 2:20, 3:10 etc. dilutions is carried out on the 1 X 10^{-5} M standard created above and then measured spectrophotometrically in a 10.0 cm cell. This second set of standards is serially diluted 1:10 for each unit step in molarity to generate a continuous series from 1.0 x 10^{-4} M ATP to 1.0 x 10^{-8} M.

Either of the two different dilution series may be used to bracket unknown sample values and intersperse several values between the extreme upper and lower boundaries. The original stock solutions of the dilution series should be assayed for ADP by reaction with pyruvate kinase and for AMP by

reaction with pyruvate kinase and myokinase and phosphoenol pyruvate (see Experimental Protocol section). This is performed as a check that the spectrophotometric measurements were on ATP and not ADP or AMP which have the same extinction coefficients as ATP. ADP and AMP standard curves are prepared following the same procedure as ATP preparations. Standards from 5×10^{-7} to 1×10^{-8} M range have been used by us to bracket the concentration range found in the continental shelf waters of the Southeastern U. S. and Gulf of Mexico when filtered concentrated 100-fold.

Once the standard concentrations of ATP are prepared, an instrumentenzyme standardization procedure must be undertaken. Standards close to the concentration of the unknown solutions should be run under the same assay conditions (temperature, pH and ionic strength) as the samples to which they are to be compared. The derivation of the standard curve cannot be performed after all the unknowns have been processed, because both the firefly enzyme activity and the residual light emitted by the enzyme preparation change with time (Booth 1975). Standards must be used periodically throughout the course of sample assays. The corrections for this enzyme drift will be smaller, the shorter the time necessary to process the unknown samples. It is recommended that all samples to be run at one time be ready for immediate assay to keep the run time as short as possible. Since the enzyme will continue to drift with time, if delays of hours are encountered during an assay run, standards should be checked periodically throughout the delay period to ensure a knowledge of the rate of change when the assay is resumed.

Since the firefly lantern extract changes its light yield characteristics with age, storage conditions, and physical handling, the assay for ATP must be calibrated by means of standard ATP solutions. The standard curve is prepared by plotting log ATP concentration against log counts from the photometer over the range of intended sample assays. The curve is not strictly linear and does not necessarily regress through an origin, so the approximate range of the unknown solutions should be bracketed by standard measurements periodically to ascertain the changing slope and intercept for accurate back calculation of unknown concentrations.

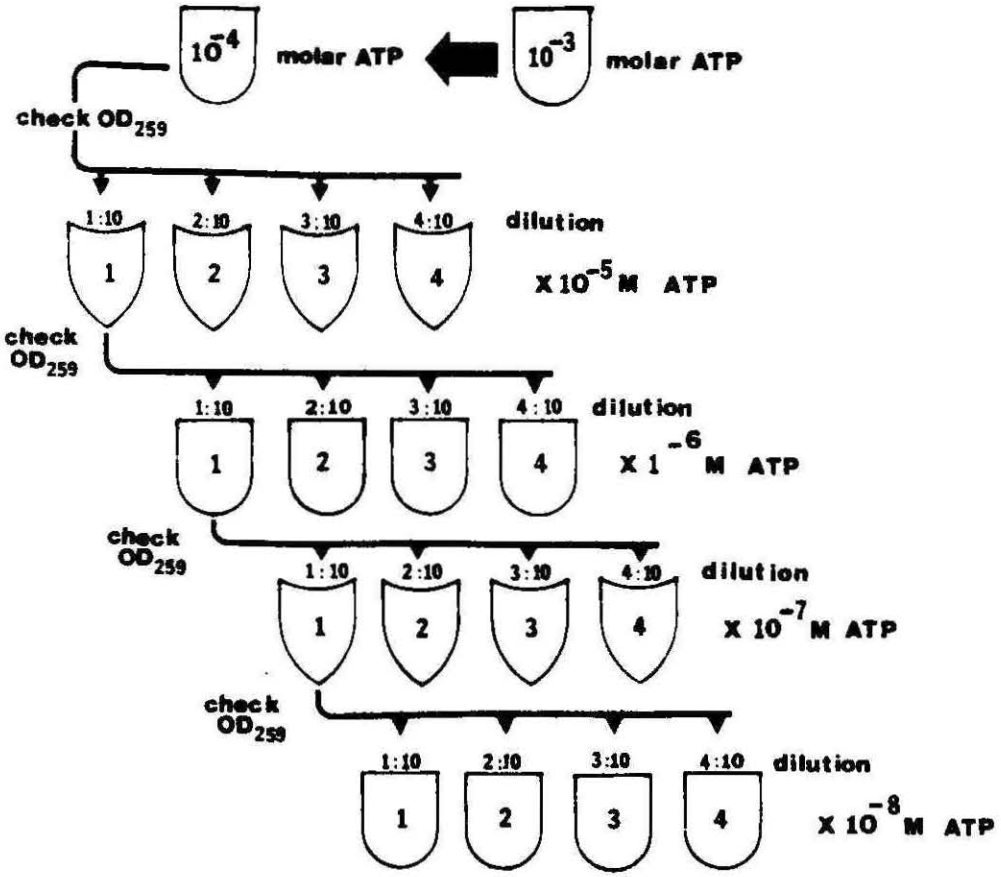


Figure 8. Dilution series for ATP standards.

The most common method of standard curve preparation is by means of log-log transformation. It should be emphasized that the logarithm of the ATP concentration is not really a linear function of the logarithm of the measured light output. Even cursory examination of the references cited below will reveal that this non-linearity is readily apparent for less than single-decade concentration ranges. The mechanical disturbance of injection will evoke light production for firefly lantern extracts in distilled, deionized, autoclaved water. Although the assumption that zero ATP concentration should give zero light output (as measured above background light from the enzyme preparation itself) is true, the functional converse of this statement is not necessarily true. That is, photometric devices have finite lower limits of sensi-

tivity for a particular wavelength. Zero-measured light output can occur at non-zero concentrations of ATP. Thus computations cannot assume that the line through the standard points also passes through the origin. Strictly speaking, a log-log plot has an undefined origin. As discussed in the section

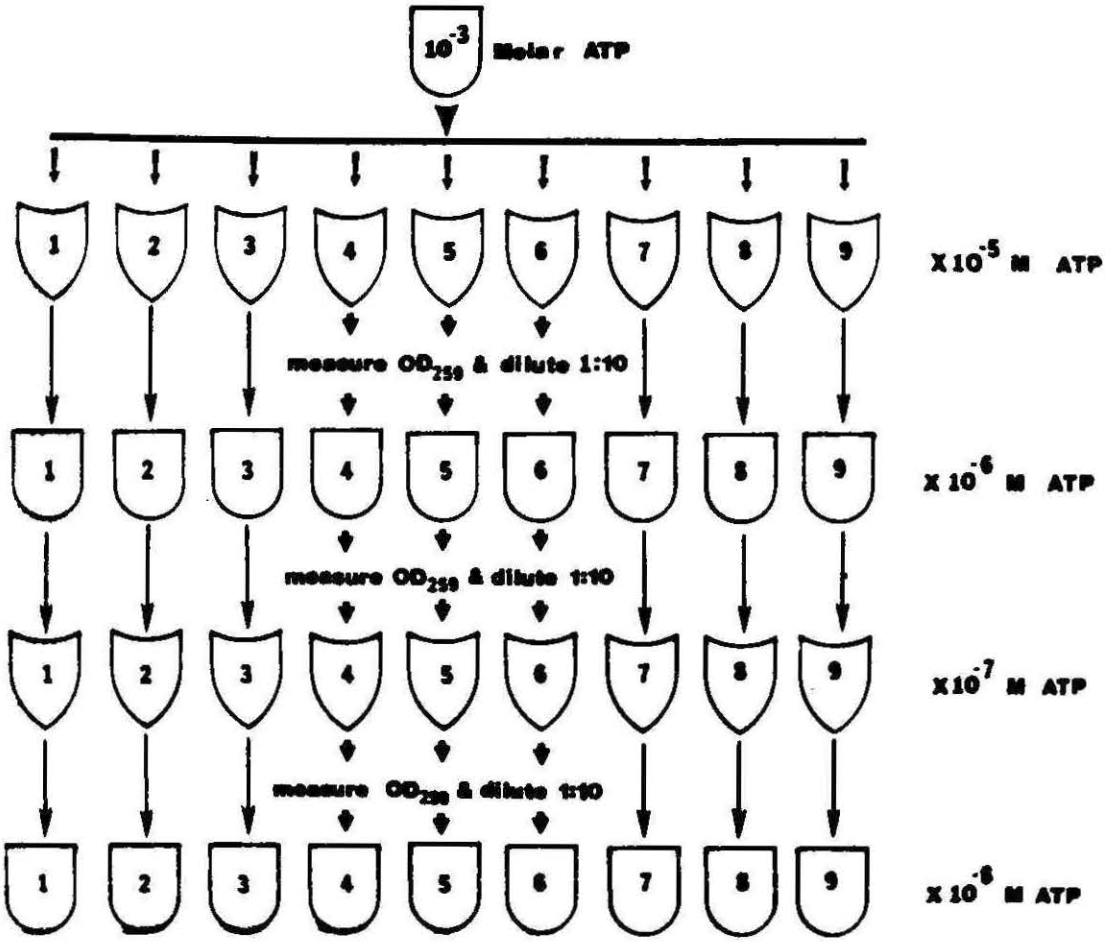


Figure 9. Dilution series for ATP standard curve.

on enzyme preparation and purification, a change in the proportions of reagents or a different photodetector system may detect a smaller non-zero concentration.

Many authors utilize a linear fit either by a least-squares regression or best fit by eye. This is very simple and is the most rapid method of deter-

mining the approximate relative magnitudes of differences between samples. The most convincing standard curves are prepared sufficiently small that non-linearities are not apparent (Karl and Holm-Hansen 1976; Lundin and Thore 1975; Law and Button 1977). Less compelling but realistic straight lines can be drawn through standard error bars or between several data points to produce the best-fit line (Chappelle and Levin 1968; Patterson et al. 1970; Neufield et al. 1975; McLeod et al. 1969; Parkinson and Medley 1972; Lazdunski and Belaich 1972; Addanki et al. 1966; Schram 1970). The most accurate graphic portrayals of this non-linear correspondence is the method of linear interpolation between actual standard points (Strehler and Totter 1952; Strehler 1968); Picciolo et al. 1975, Cheer et al. 1974).

Attempts have been made to eliminate drawing the actual standard curve and rely on regression analysis commonly available on automatic computing devices. This may produce the appearance of increased precision without actually doing so. The use of a simple proportional relationship between the measured light output of an unknown and a standard (St. John 1975) assumes a linear relationship between the light and ATP without the benefit of a log-log transformation or any knolwedge of the shape of the curve between determined points. This could be useful only over a very small concentration ranges for qualitative comparative purposes to determine which samples had more ATP without regard to actual quantity. Other workers have applied polynomial regressions for their standard curves (Booth 1975). Fitting a curve to the data will produce a smooth line among the data points, but would not be as accurate as individual short straight lines drawn between accurately prepared standard points.

To summarize the above points; too few data points in the standard curve can produce an apparent linearity which may appreciably affect the absolute accuracy of the results. This accuracy is a function of the span of the data points. A log-log transformation of the data will produce the smoothest, most continuous distribution of the standard points. The standard curve cannot be assumed to pass through the origin. A linear interpolation between actual data points will produce more accurate data than will an attempt to smooth a curve to compensate for presumed poor quality control of the standard solutions.

Each time an ATP standard curve is rerun during the course of a total adenylate or energy charge determination, and AMP standard curve of the same concentrations should be rerun so that any changes in the conversion efficiency for AMP, either with time or with concentration will be monitored and taken into account during calculations. The AMP+ADP+ATP samples can then be calculated using this curve, if it has a different slope at different concentrations.

Calculation of Samples

The simplest method of data reduction uses a log transformation of both machine counts and ATP concentrations for the standard curve. Several, usually three or more, short two-point straight-line sections should be constructed between standard concentrations and mean values of each set of machine counts for each of four or more half powers of ten increments bracketing the expected range of the sample concentrations. Slope and intercepts are calculated as follows:

$$\text{Slope}_{1,2} = \frac{\log \text{ATP}_1 - \log \text{ATP}_2}{\log(\text{counts}_1 - \text{background}) - (\log(\text{counts}_2 - \text{background})}$$

$$\text{Intercept}_{1,2} = \log \text{ATP}_1 - [\text{Slope}_1 \times \log (\text{counts}_1 - \text{background})]$$

For each set of counts between any standard values (1) and (2) the sample ATP concentration is then calculated:

$$\text{ATP} = \text{ANTILOG} (\text{Slope}_{1,2}) \text{ X } (\text{counts-background}) + \text{Intercept}_{1,2})$$

where counts is the machine counts produced from an unknown sample. If the sample was concentrated and/or diluted, ATP must then be multiplied by the inverse of these concentration factors to yield the concentration in desired units. The units of the standards (nM, mg/l etc.) will be the units of the results unless subsequently transformed. Representative values of slope (1.01 ± 0.2) and intercept -10.89 ± 1.22) were determined from 87 separate standard curves. Standard curves with background determinations should be performed

often (every 60-100 assays) to ensure accuracy and comparability of data. This is necessary, because all firefly enzyme mixtures have different ATP vs light curves and this will change with time (2-4 hours) enough to be measurable and thus affect the absolute accuracy of the determination.

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