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Measurement and significance of ATP and adenine nucleotide pool turnover in microbial cells and environmental samples

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Summary

A method was devised to measure the turnover rates of adenosine 5'-triphosphate (ATP) and total adenine nucleotide (AN) pools in cultures and natural populations of microorganisms. The method relies on $^{32}PO_4$ as a tracer for P-flux through the α-, β- and γ-positions of cellular ATP. Following a timed incubation, cellular ATP is extracted and purified. The isolated AT^{32}P is subjected to enzymatic hydrolysis designed to selectively hydrolyze ATP to either ADP + Pi (ATPase reaction) or to AMP + 2 Pi (apyrase reaction). The released ^{32}Pi is separated from the parent nucleotides (ADP and AMP) and measured by liquid scintillation counting procedures. From time course information on the change in specific activity (i.e., nCi pmol^{-1}) of the γ-P and α-P positions of ATP, the turnover time of the total ATP and AN pools, respectively, can be determined. This kinetic information can be used to estimate total cellular energy flux (derived from the ATP turnover rate) and specific growth rate (derived from the AN turnover rate). The method is relatively simple, reproducible and accurate, and has the sensitivity required for successful application in even the most oligotrophic oceanic habitats.

Key words: *Adenine nucleotide pool - ATP-ADP turnover - Energy flux - Growth rate - Metabolism - Microorganisms*

Introduction

Nucleotides are essential intracellular intermediates which function in the storage and transfer of cellular energy, as activators and precursors for macromolecular biosynthesis and as regulators of metabolism. The central role of adenine nucleotides (AN), especially ATP, has been known since the pioneering research of Lipmann [1]. Numerous laboratory investigations have been conducted to define the steady-state intracellular

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concentrations (referred to as 'pools') of ATP, ADP and AMP [2–4]. In general, the ATP pool in viable microorganisms (prokaryotes and eukaryotes) appears to be regulated at a value of approximately 2–6 nmol ATP per mg dry weight, regardless of growth rate, culture conditions or mode of nutrition [2]. Consequently, measurements of static steady-state pools are insufficient to estimate total energy flux through the AN system.

Chapman and Atkinson [5] have recently reviewed the existing data base for estimates of in vivo ATP and AN fluxes. The turnover of ATP refers to the utilization of the γ-(terminal)-phosphate group of ATP (either through orthophosphate or pyrophosphate cleavage), and the subsequent regeneration of ATP via substrate-level, oxidative or photophosphorylation. Thus, ATP pool turnover reflects an ATP ⇌ ADP (or an ATP ⇌ AMP) cycle and does not result in net removal of the adenine moiety from the intracellular pool. Since ATP pool turnover times of < 1 s are not uncommon for rapidly growing bacteria, it should be obvious that static ATP pool measurements, by themselves, cannot be used to estimate the potential energy flux through a cell or population.

Under steady-state conditions (i.e., $d[ATP]/dt = 0$) the ATP turnover rate is equivalent to either the rate of ATP formation or the rate of ATP utilization and, in principle, could be estimated if either process was temporarily suspended without affecting the rate of the other. ATP turnover rates can also be estimated from combined measurements of the intracellular ATP pools and computations of the presumed rates of ATP synthesis derived from oxygen consumption (for aerobically growing heterotrophic microorganisms) and organic substrate utilization. These two approaches have been employed by several investigators using cultures of growing bacteria [5]. The ATP pool turnover rates thus obtained range from 0.13 to 180 s; however, the validity of the assumptions underlying these extrapolations, and hence the accuracy of the resultant data, has been questioned [5]. Furthermore, neither of these general approaches is applicable to natural microbial populations.

The AN turnover time is equivalent to the period which would be required to deplete the total AN pool as required for the biosynthesis of macromolecules (RNA, DNA and protein), if regeneration of AN was curtailed. As opposed to the turnover of ATP, the turnover of AN results in a net removal of material from the intracellular metabolite pool, which under balanced growth conditions must be replenished by de novo nucleotide biosynthesis or from the uptake of exogenous sources via salvage pathway mechanisms. To our knowledge, no direct measurements of AN turnover in microbial cells have been reported. Chapman and Atkinson [5] have calculated the biosynthetic requirements for AN from reported values of adenylate and deoxyadenylate residues in RNA and DNA and of histidine in protein. Their results from *Escherichia coli* and *Salmonella typhimurium* indicate that the rate of AN pool utilization is linearly, and positively, correlated with growth rate, and that the total AN pool turns over 30–50 times per generation. Consequently, at least in enteric bacteria, the AN turnover time is approximately 2.5% of the generation time [5]. Thus, the simultaneous measurement of ATP turnover and AN turnover in microbial cells, or in natural mixed populations of microorganisms, should provide useful information regarding both cellular/community bioenergetics and mean specific growth rate.

We have devised a method to measure, directly and simultaneously, the turnover of

both ATP and AN pools. In this report we describe: (1) the theoretical principles and analytical details of the method; (2) representative experimental data; and (3) a brief discussion of potential physiological and ecological applications. In two separate reports we will present detailed results of our field studies to date and ecological interpretations [6, 7].

Materials and Methods

Theory and outline of the method

Each molecule of ATP contains three phosphate groups in a linear arrangement designated the α, β and γ- (terminal)-positions, respectively. The β-P and γ-P both exist as anhydride-bound PO_4 groups with characteristically high standard free energies of hydrolysis ($\Delta G'^{\circ}_{ATP} = -7.3$ kcal), and have been termed 'high energy phosphate (~P) bonds' [8]. Due to rapid and complete interconversion of the γ- and β-positions during cellular energy transductions (i.e., A-P-P-P* + A-P $\rightleftharpoons$ A-P-P + A-P-P* $\rightleftharpoons$ A-P-P*-P + A-P), the kinetic constants for short-term labeling of the γ- and β-phosphate groups are expected to be similar, if not identical. In comparison to the anticipated rapid and uniform isotopic equilibration expected for the γ- and β-groups, the specific radioactivity of the α-P position might be expected to lag behind the ~P-phosphates since the α-P is non-exchangeable and would become labeled only during de novo synthesis (or exogenous salvage) of adenine nucleotides. When radioactive $^{32}PO_4$ (or $^{33}PO_4$) is added to a culture of microorganisms or to a naturally occurring microbial population, the rise in the specific radioactivity of the γ- (or β)-positioned P of ATP (expressed as nCi ^{32}P pmol^{-1} ATP) will reflect the rate of ATP turnover (i.e., hydrolysis and/or phosphorylation), and the rise in specific radioactivity of the α-P position will reflect the rate of adenine nucleotide turnover (i.e., removal for biosynthesis and/or de novo synthesis). These data, used either separately or in concert, should provide meaningful estimates of total metabolic activity, energy flux and rates of macromolecular biosynthesis. The method described herein requires a short-term incubation with $^{32}PO_4$ followed by ATP extraction and purification by ascending thin-layer chromatography (TLC). The purified ATP is subjected to specific enzymatic hydrolysis designed to selectively release either the γ- (ATPase reaction) or [$\beta+\gamma$]-phosphate groups (apyrase reaction). The $^{32}PO_4$ released during the selective enzymatic hydrolysis is separated from the parent adenine nucleotide moiety by activated charcoal treatment and is radioassayed. From time course measurements, the rates of α-, β- and γ-P incorporation can be measured directly and the ATP and AN turnover rates calculated.

Sample collection and $^{32}PO_4$ labeling

Field collections analyzed in this study include freshwater samples from Krauss Pond (on the University of Hawaii campus) and seawater samples from a station 6 km NE of the island of Oahu, Hawaii (21°29.8′ N; 157°45.5′ W). The water samples were collected manually in acid-washed polycarbonate bottles, and were prefiltered through a 90 μm Nitex® screen prior to the start of the experiment in order to remove large organisms. The incubation began with the addition of $^{32}PO_4$ (carrier free; New England Nuclear Corp.) to a final activity of between 0.1 and 0.5 μCi ml^{-1} depending on the desired

sensitivity and anticipated ambient concentration of total dissolved phosphorus. All incubations were performed in the laboratory under conditions of ambient light and constant temperature (23°C).

ATP extraction and purification

Subsamples were extracted either by concentrating particulate materials onto glass fiber filters (GF/F, Whatman®) with subsequent immersion of the filters into boiling phosphate (pH 7.4, 60 mM) buffer ('slow procedure'), or by acidification of the total water sample with concentrated H_3PO_4 to yield a final concentration of 1 M H_3PO_4 ('fast extraction'). The more conventional boiling buffer extraction was generally used except when information on the initial (0–2 min) labeling of ATP was desired. The aqueous extracts were concentrated in vacuo and assayed for total ATP as described previously [9]. The acid extracts were concentrated and desalted using the charcoal adsorption procedure of Hodson et al. [10]. No consistent differences were observed for the total ATP pool specific radioactivity between the two methods of ATP extraction and concentration.

ATP purification was achieved by a two step one-dimensional separation on plastic backed (20×20 cm) sheets coated with polyethyleneimine (PEI) impregnated cellulose (MN 300 PEI; Brinkman Instrument Co., Westbury, NY). The commercially available TLC plates were soaked in a bath of 10% NaCl, air dried at room temperature and subjected to an ascending development with distilled water [11]. The pre-treated TLC plates were dried and stored at 4°C until needed. Samples were applied ~3 cm from the bottom edge of the plate (usually 10 μl per sample but occasionally up to 50 μl; eight samples per plate) with a known amount of non-radioactive ATP added to serve as an optical tracer and to evaluate the overall recovery of the TLC procedure. After sample application the plate is washed in two consecutive distilled water baths (5 min each) to remove salts and residual $^{32}PO_4$, air dried, and developed in 0.2 M formic acid to the top of the plate [12]. Under these conditions, the nucleotide triphosphates remain at the origin. The plate is then washed in a distilled water bath and air dried before final development in the same direction with a phosphate solution (pH 3.4, 0.85 M), washed in two successive distilled water baths and air dried. Development with phosphate greatly diminishes $^{32}PO_4$ tailing and comigration [13] and, in effect, represents an important purification step for separating AT^{32}P from residual $^{32}PO_4$. The final wash is required to remove residual non-radioactive PO_4 (i.e., the developing solvent) which, if present, will inhibit the ATPase reaction. Both ascending TLC development procedures are performed at room temperature in closed glass tanks. Total separation time is approximately 4–5 h. The ATP spots are localized by short wavelength UV light, circled with a pencil line, cut out with a pair of scissors or carefully scraped with a blunt spatula, and eluted in 1 ml of 0.35 M $MgCl_2$ for 1 h with continuous shaking. A 10 μl aliquot of the eluate is diluted into 1 ml of Tris buffer (pH 7.4, 0.02 M) and assayed for total ATP by the firefly bioluminescence reaction [14]. This value is compared to a measurement of the total ATP applied to the plate to determine the overall recovery of ATP during the purification procedure, which was generally >90%. The remainder of the purified ATP (or a portion thereof) is transferred to a clean vial in preparation for the ATPase (or apyrase) reaction (see below).

ATPase reaction

Stock solutions of porcine cerebral cortex ATPase, EC 3.6.1.3 (Sigma Chemical Co., A-7510) are prepared in distilled water to a concentration of 1 unit ml^{-1} and stored frozen (-20°C) until needed. Under these conditions the ATPase reagent is stable for at least 1 month without appreciable loss of activity. For short-term storage (<2-3 days) the stock ATPase reagent should be stored at 4°C, rather than -20°C, in order to maximize stability. Storage at -20°C, however, eliminates bacterial contamination of the stock solutions when long-term storage (>3 days) is required. To perform the ATPase hydrolysis reaction, 750 μl of the 'TLC-purified' ATP solution (in 0.35 M $MgCl_2$) is placed into a 12×75 mm culture tube containing 250 μl distilled water, 10 μl of a 1 M KCl solution, 20 μl of a 5 M NaCl solution, and 25 μl of a 3 mM AMP solution, followed by the addition of 50 μl of the stock ATPase reagent. The AMP is added to repress the ADP dismutase reaction ($2ADP \rightleftharpoons AMP + ATP$) which is also catalyzed by ATPase (see Results section). The contents of the tube are mixed thoroughly and a 10 μl subsample is immediately removed, diluted in 1 ml of 0.02 M Tris (pH 7.4) buffer to quench the reaction and assayed for determination of 'time zero' ATP concentration using the firefly bioluminescence reaction. The ATP assay procedure is repeated after approximately 10–15 min with one or two representative samples in order to establish the extent of ATP hydrolysis. Once the initial rate of hydrolysis is known one can estimate the time required for complete hydrolysis of ATP to ADP, which in our experience ranges from 20 to 40 min. Since all samples contain identical concentrations of carrier ATP (from the TLC procedure) and equivalent amounts of enzyme, the rates of hydrolysis are expected to be similar. However, complete ATP hydrolysis should be confirmed for each individual sample before proceeding with the separation and purification of products. Following ATP hydrolysis, a 250 μl aliquot of the sample is removed for 'TOTAL RADIOACTIVITY' (i.e., α-$^{32}P + \beta$-$^{32}P + \gamma$-^{32}P). A second 500 μl aliquot is added to a small plastic microfuge tube containing 500 μl of an activated charcoal slurry (25 mg charcoal per ml of 0.1 M H_3PO_4), mixed thoroughly and centrifuged for 10 min at 1300 × *g*. A portion of the supernatant (usually 750 μl) is removed for liquid scintillation counting for determination of '$^{32}PO_4$ RADIOACTIVITY' (i.e., γ-^{32}P). The phosphoric acid-charcoal treatment removes >99.5% of the ADP from solution and catalyzes a <1% non-specific binding of $^{32}PO_4$ (see Results section). Since γ-^{32}P and β-^{32}P specific activities are expected to be equal over all time periods (experimental evidence for this assumption is presented later), the α-^{32}P activity can be calculated by difference (i.e., α-$^{32}P = [TOTAL\text{-}^{32}P] - [2 \times \gamma\text{-}^{32}P]$.

Apyrase reaction

Stock solutions of potato apyrase (Sigma Chemical Co., A-6132) were prepared in distilled water at a concentration of 10 units ml^{-1}, dispensed into small vials (~200 μl each) and stored frozen (-20°C) until needed. Stability under these storage conditions is excellent with an observed decrease in activity of less than 10% over a period of 1 month. To perform the apyrase hydrolysis reaction, 750 μl of the TLC-purified ATP solution (in 0.35 M $MgCl_2$) is placed into a 12×75 mm culture tube containing 250 μl of distilled water and 20 μl of a 1 M $CaCl_2$ solution, followed by the addition of 25 μl of the stock (10 units ml^{-1}) apyrase reagent. The contents of the tube are mixed thoroughly

and a 10 μl subsample, immediately removed, diluted in 1 ml of 0.02 M Tris (pH 7.4) buffer to stop the reaction, and assayed for determination of time zero ATP concentration using the firefly bioluminescence reaction. The ATP assay procedure is repeated after approximately 10–15 min with one or two representative samples in order to establish the kinetics of ATP hydrolysis. Once the initial rate is known, one can estimate the time required for complete hydrolysis of ATP and ADP to AMP. Since potato apyrase does exhibit a nominal level of AMPase activity (usually 1–3% of the ATPase and ADPase activity), it is important that the incubation period does not extend beyond the time required to completely hydrolyze ATP and ADP. Generally, the incubation period is between 30 and 45 min (at 25°C); however, since we detected a significant level of variability among different apyrase reagents, we recommend that the extent of ATP hydrolysis be monitored directly during the incubation period. Typically, we terminated the incubation when $\geqslant$99.5% of the initial ATP in the sample had been hydrolyzed. At this point, ADP hydrolysis is also complete and AMP hydrolysis is minimal (data presented in Results section). Following hydrolysis, a 250 μl aliquot of the sample is removed for liquid scintillation counting for determination of the TOTAL RADIOACTIVITY (i.e., α-^{32}P + β-^{32}P + γ-^{32}P). A second 500 μl aliquot is added to a small plastic microfuge tube containing 500 μl of the activated charcoal slurry, mixed thoroughly and centrifuged for 10 min at 1 300 $\times$ *g*. A portion of the supernatant (usually 750 μl) is removed for liquid scintillation counting for determination of $^{32}PO_4$ RADIOACTIVITY (i.e., β-^{32}P + γ-^{32}P). The α-^{32}P is calculated by difference (i.e., α^{32}P = [TOTAL-^{32}P] - [β-^{32}P + γ-^{32}P]) and is corrected for the partial AMP hydrolysis (4–5%) which inevitably occurs during the apyrase reaction (see Results section). It should be re-emphasized that the primary function of the apyrase reaction is to provide a mechanism to test the assumption regarding the rapid isotopic equilibration of the β- and γ-positions of ATP. Consequently, the use of the apyrase reaction is not absolutely essential for estimating either the α-, β- or γ-P specific radioactivities, or for calculating the turnover of ATP and total AN pools (see Results and Discussion section for more details).

Results and Discussion

Evaluation of ATP purification and elution procedures

In order to estimate the specific radioactivity of the α-, β- and γ-positioned phosphate groups on ATP, the molecule must first be separated from all other radiolabeled small molecular weight intermediates in the cell and from the residual $^{32}PO_4$. The one-dimensional TLC procedure previously used to separate [^{3}H]adenine labeled ATP, ADP and AMP [15] was unacceptable for experiments with $^{32}PO_4$ due to substantial co-migration of AT^{32}P with other phosphorylated metabolites. Furthermore, tailing and comigration of $^{32}PO_4$ with ATP resulted in unacceptably high blanks in the subsequent enzymatic $^{32}PO_4$ release procedures. In order to avoid the necessity for an additional purification step to separate AT^{32}P from $^{32}PO_4$ a sequential two step single-dimension separation procedure was employed (see Materials and Methods). Under these specified reaction conditions, deoxyadenosine triphosphate (dATP) comigrates with ATP, but since the intracellular concentration of dATP in bacteria is generally ~5% that of ATP

TABLE I

EFFECTS OF $MgCl_2$ CONCENTRATION ON THE EFFICIENCY OF ELUTION OF ATP FROM PEI PLATES AND ON THE RELATIVE ACTIVITY OF ATPASE

$MgCl_2$ concentration (M)	ATP elution efficiency[a] (%)	ATPase activity[b] (relative units)	Apyrase activity[b] (relative units)
0	4	100	100
0.05	7	75	100
0.07	15	71	100
0.10	30	68	99
0.25	94	55	96
0.35	99	38	83
0.50	98	18	75
0.70	100	5	61

[a] ATP released from PEI plate after a 1 h elution period.

[b] Calculated from initial rate of ATP hydrolysis.

[16], and since the specific activity of dATP would not be expected to exceed that of ATP, we consider this source of error to be negligible. If desired, this potential interference can be evaluated quantitatively and eliminated using the periodate treatment of Reynolds and Finch [17], but this was not attempted in the present study.

The ATP spots were identified using short wavelength UV light, and eluted from the PEI cellulose plates with $MgCl_2$. The effects of $MgCl_2$ concentration on the elution efficiency of ATP and on the subsequent activity of the ATPase and apyrase enzyme reactions was evaluated (Table 1). Since the efficiency of ATP elution is unaffected by $MgCl_2$ concentrations in the range of 0.35–0.70 M, and since $MgCl_2$ inhibits both ATPase and apyrase activities, we selected 0.35 M $MgCl_2$ as a compromise concentration.

Separation of hydrolysis products

In order to estimate the ^{32}P activity in the α-, β- and γ-positions of ATP using the enzymatic hydrolysis reactions described herein an effective separation must be achieved between the $^{32}PO_4$ released from the purified $AM^{32}P$-^{32}P-^{32}P and the corresponding adenine nucleotide end-products of the reaction (i.e., $AM^{32}P$-^{32}P for ATPase reaction, and $AM^{32}P$ for apyrase reaction). We investigated the use of activated charcoal for this purpose. In preliminary experiments using aqueous charcoal preparations (charcoal slurry prepared in distilled water) we observed a significant loss of $^{32}PO_4$ from solution. This resulted in an underestimate of the γ-P activity and an overestimate of the α-positioned ^{32}P. In subsequent experiments we determined that the removal of $^{32}PO_4$ was the result of non-specific adsorption onto the activated charcoal, and that this analytical interference could be eliminated by preparing the charcoal slurries in a solution containing non-radioactive PO_4. Furthermore, the partitioning between $^{32}PO_4$ and the labeled adenine nucleotide end-products was most efficient at an acidic pH. However, caution is advised at excessively low pH (<1), where the possibility of acid-catalyzed ATP, ADP

TABLE 2

EFFECTS OF CHARCOAL CONCENTRATION ON THE SEPARATION OF $^{32}PO_4$ FROM PARENT ADENINE NUCLEOTIDE FOLLOWING ATP HYDROLYSIS BY ATPase AND APYRASE

The charcoal concentrate was prepared in 0.1 M H_3PO_4 and added in a 1:1 ratio with sample as described in Materials and Methods. For the reactions a mixture of [2,8-^{3}H]ATP and $^{32}PO_4$ was subjected to ATPase or apyrase hydrolysis as described in Materials and Methods. When <0.5% of the initial ATP remained, charcoal was added, the samples were centrifuged and the supernatant solution assayed for ^{3}H and ^{32}P activities.

Charcoal concentration (mg ml^{-1})	ATPase reaction		Apyrase reaction	
	^{3}H-ATP hydrolysis product (% of control)	$^{32}PO_4$ (% of control)	^{3}H-ATP hydrolysis product (% of control)	$^{32}PO_4$ (% of control)
0.1	89.4	100.7	67.8	99.7
0.25	56.2	100.6	32.9	100
0.5	29.4	99.9	12.5	99.3
0.75	15.2	101.3	1.2	100
1	9.6	100.4	0	100.5
2.5	1.3	99.2	0	101
5	0	96.5	0	101
7.5	0	100.4	0	100.3
10	0	98.8	0	100.4
25	0	98.4	0	94.7
50	0	98.2	0	97.9
75	0	96.7	0	97.2
100	0	96.7	0	97.2

and AMP hydrolysis might also occur. After testing several concentrations we selected 0.1 M H_3PO_4 as the optimum reaction condition. We also evaluated the effects of charcoal concentration on the separation of AT^{32}P hydrolysis products (Table 2). At final charcoal concentrations of less than ~1 mg ml^{-1} the removal of the adenine nucleotides (ADP and AMP) was incomplete. At higher charcoal concentrations, adenine nucleotide removal was essentially 100%. Non-specific binding and removal of $^{32}PO_4$ was independent of charcoal concentration over the range tested, and averaged <0.9% for the ATPase reaction and <0.8% for the apyrase reaction (Table 2). On the basis of these experimental results, we selected 25 mg charcoal ml^{-1} in 0.1 M H_3PO_4.

Selectivity and kinetics of ATPase reaction

The selectivity of ATPase in the removal of the γ-positioned phosphate group of ATP was evaluated by monitoring the kinetics of (2,8-^{3}H)-labeled ATP hydrolysis (Fig. 1). The reaction end-products were identified by PEI-cellulose TLC [15]. Contrary to our initial expectations, we observed the production of labeled AMP during the ATPase reaction (Fig. 1A), presumably the result of a reversible adenylate kinase-like dismutase (2 ADP $\rightleftharpoons$ ATP + AMP) previously reported to occur in purified preparations of ATPase [18]. However, in subsequent experiments we were successful in selectively inhibiting the dismutase activity without affecting the primary ATPase activity (i.e., ATP $\rightleftharpoons$ ADP + Pi) simply by adding exogenous AMP to shift the dismutase

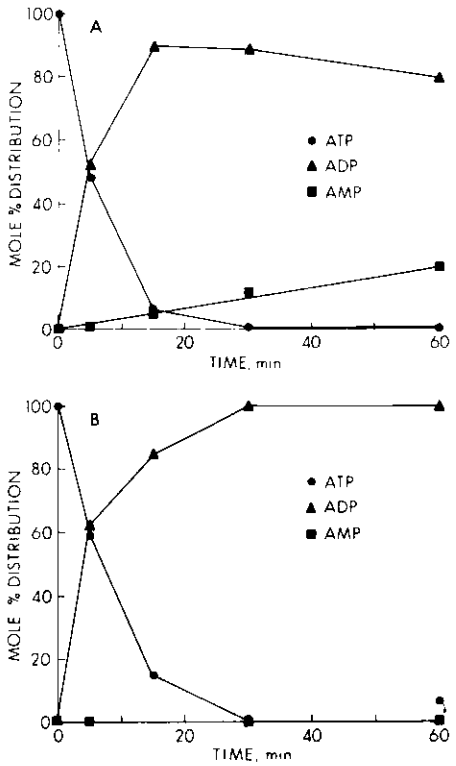


Fig. 1. Time-dependent mole percent distribution of adenine nucleotides during the hydrolysis of ATP by ATPase. (A) Reaction products without added AMP; (B) reaction products with added AMP. See text for details.

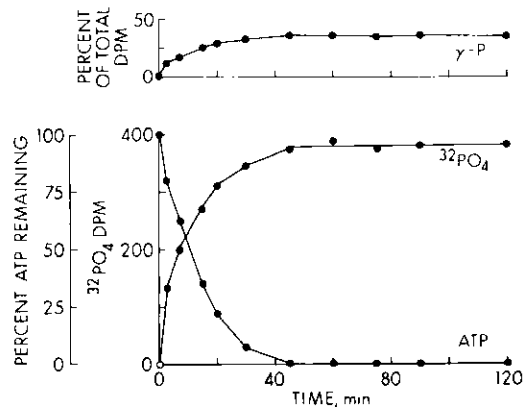


Fig. 2. Time-dependent hydrolysis of ATP and release of $^{32}PO_4$ from uniformly labeled AT^{32}P. Top frame shows percentage of total radioactivity which appears as γ-P. The theoretical percentage is 33.3%.

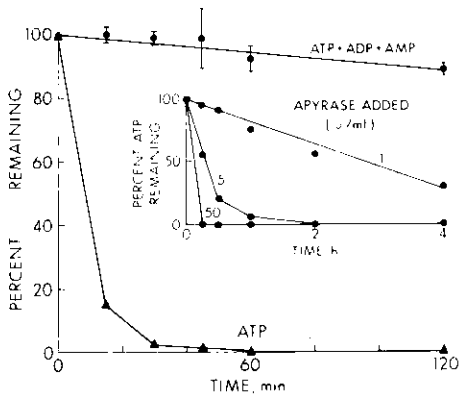


Fig. 3. Time-dependent hydrolysis of ATP and inventory of total adenine nucleotides ([ATP] + [ADP] + [AMP]) during the apyrase reaction. Inset shows ATP hydrolysis rate at three different concentrations of apyrase.

reaction equilibrium (Fig. 1B). In the presence of exogenous AMP (Fig. 1B) there is a quantitative and complete conversion of ATP to ADP which appears to be stable even during extended incubation periods. In marked contrast to the samples incubated without exogenous AMP, no ^{3}H-AMP was detected (compare Fig. 1A and B). Finally, the ATPase reaction kinetics and selectivity were evaluated with a sample of uniformly labeled ATP derived from a freshwater pond microbial community. The data, summarized in Fig. 2, again demonstrate the correspondence between $^{32}PO_4$ release and ATP hydrolysis (ADP production) and the apparent stability of the AM^{32}P-^{32}P (i.e., ADP) during extended incubation periods. The percent of the TOTAL RADIOACTIVITY recovered from the hydrolysis of the γ-positioned P ranged from 32 to 35% (Fig. 2, top) which is in excellent agreement with the theoretical value of 33.3%.

Selectivity and kinetics of the apyrase reaction

The selectivity of the apyrase dependent hydrolysis of purified AT^{32}P was evaluated by monitoring the hydrolysis of ATP and by monitoring the changes in the relative molar concentrations of the individual adenine nucleotides (i.e., [ATP], [ADP], [AMP]). In principle, the total AN concentration ([AN] = [ATP] + [ADP] + [AMP]) should remain constant (i.e., ATP $\rightharpoonup$ ADP + Pi; ADP $\rightharpoonup$ AMP + Pi) in the absence of AMPase activity. The rate of ATP hydrolysis was proportional to the apyrase activity added to the sample (see Fig. 3, inset). However, at all concentrations of enzyme tested we observed a small (<5%), but measurable decrease in total AN concentration, a result which we interpret as evidence for AMPase activity. The results, summarized in Table 3, indicate that the rate of hydrolysis of AMP is extremely constant, and therefore predictable, over a wide range of enzyme concentrations and hence total ATP hydrolysis (incubation) times. If ATP hydrolysis is monitored in the sample (as recommended in Materials and Methods) and if the incubation is terminated when ATP hydrolysis is ~99% complete, the amount of AMP hydrolyzed will always be $\leqslant$5% (4.45$\pm$0.26%; Table 3). This small amount of AMP hydrolyzed can easily be corrected for in the calculation of α-, β- and γ-labeling (see next section).

TABLE 3

MOLE PERCENT DISTRIBUTION OF ADENINE NUCLEOTIDES DERIVED FROM ATP FOLLOWING AN INCUBATION WITH VARYING CONCENTRATIONS OF APYRASE

Apyrase concentration (units ml $^{-1}$)	Incubation time[a] (min)	Mole % distribution			AMP hydrolysis (%)
		ATP	ADP	AMP	
2	180	0.26	1.6	94.05	4.09
3	100	0.18	<0.2	95.19	4.43
4	76	0.30	<0.2	95.23	4.27
5	60	0.25	<0.2	94.97	4.58
6	48	0.24	<0.2	94.88	4.68
7	36	0.27	<0.2	95.07	4.46
8	33	0.20	<0.2	94.84	4.76
9	27	0.36	<0.2	94.73	4.71
10	20	0.59	1.1	94.22	4.09
					$\bar{X} = 4.45$ (± 0.26)

[a] Time determined by initial rate of ATP hydrolysis, relative to control sample without apyrase addition.

Calculation of α-, β- and γ-labeling of ATP and comparison of methods

We have described two independent methods for the determination of the distribution of ^{32}P activity in the α-, β- and γ-positioned phosphate of ATP. For routine measurements we recommend the ATPase reaction. In our experience, this reaction is the most predictable, reproducible and straightforward procedure to perform on purified ATP samples. We will, however, discuss the application of the apyrase reaction as an alternative approach to evaluate the distribution of ^{32}P in ATP.

The standard ATPase reaction (as described in Materials and Methods) yields information on the total ^{32}P activity in ATP and on the activity of the γ-positioned P group. From time course data of γ-P incorporation (i.e., change in specific radioactivity [nCi ^{32}P pmol^{-1} ATP pool]) the turnover time of the cellular or microbial community ATP pool can be calculated. In addition to the direct measurement of the γ-P radioactivity, the distribution among the α-, β- and γ-positioned P groups can also be estimated if one assumes that the β-P specific activity is equal to the γ-P specific activity. Hence, β-^{32}P is equal to the measured γ-^{32}P activity, and α-^{32}P is equal to the measured TOTAL RADIOACTIVITY minus twice the measured γ-^{32}P value (i.e., α-^{32}P = [TOTAL-^{32}P] - [2 × γ-^{32}P]). Consequently, from a single enzymatic reaction one can derive the α-, β- and γ-P specific radioactivities and can estimate the turnover time of ATP and total AN pools. If the working assumption of uniform labeling of β- and γ-positions of ATP is shown to be universally applicable, then the measurement of ATP and AN pool turnover becomes a relatively straightforward procedure. Lutkenhaus et al. [19] have performed a set of experiments designed to evaluate the kinetics of P incorporation into the α-, β- and γ-positions of ATP *Escherichia coli*. Their results indicate that the β-P and γ-P positions of ATP are in isotopic equilibrium over all time periods measured from 25 to 3 048 sec.

However as discussed previously, the assumption of β and γ isotopic equilibration need not be taken on faith. We have developed an additional enzymatic procedure (i.e., the apyrase reaction) to evaluate quantitatively the distribution of ^{32}P among the three phosphate positions of ATP (as described above). The $^{32}PO_4$ released during the apyrase reaction is equivalent to the amount contained in the $[\beta+\gamma]$ positions of ATP, plus $4.45 \pm 0.26\%$ release from the α-P at the point of complete ATP and ADP hydrolysis (Table 3). This reaction, by itself can yield α-^{32}P and $[\beta + \gamma]$-^{32}P, as follows:

$$\alpha\text{-}^{32}P = \frac{(\text{TOTAL } ^{32}P) - (^{32}PO_4 \text{ released})}{0.955}$$

$$[\gamma\text{-}^{32}P + \beta\text{-}^{32}P] = [\text{TOTAL } ^{32}P] - \alpha\text{-}^{32}P$$

When used in conjunction with the ATPase reaction (which yields γ-^{32}P) the apyrase reaction results can be used to test the assumption of uniform labeling of the β- and γ-positions of ATP by comparing the results obtained from the combined reactions (i.e., $[\beta\text{-}^{32}P + \gamma\text{-}^{32}P]$ from apyrase reaction minus $[\gamma\text{-}^{32}P]$ from ATPase reaction which yields an independent estimate of $[\beta\text{-}^{32}P]$ to compare with the measured $[\gamma\text{-}^{32}P]$ value). Table 4 summarizes a typical set of results derived from such a comparison. The β-P and γ-P specific radioactivities of ATP were in equilibrium in naturally occurring microbial communities over the time scales measured, a result which is consistent with the pure culture studies of Lukenhaus et al. [19]. This result is predicted on theoretical grounds as well, since the two terminal (~P) phosphate groups of ATP are expected to have equally rapid rates of hydrolysis and turnover in living cells. Once the assumption

TABLE 4

COMPARISON OF THE INCUBATION TIME-DEPENDENT DISTRIBUTION OF ^{32}P IN THE β AND γ POSITIONS OF ATP ISOLATED FROM A SURFACE SEAWATER MICROBIAL COMMUNITY

Incubation time (min)	Specific radioactivity		γ/β
	γ-^{32}P (μCi M^{-1} ATP)[a]	β-^{32}P (μCi M^{-1} ATP)[b]	
4	65	68	0.96
8	175	166	1.05
16	203	218	0.93
60	804	800	1.01
150	1251	1262	0.99
220	1474	1434	1.03
285	1503	1486	1.01
			$X = 1.00$ ± 0.04

[a] As measured by the ATPase reaction

[b] As determined by difference from the apyrase and ATPase reactions (i.e., $\beta = [\gamma + \beta] - [\gamma]$)

of equal β-P and γ-P labeling kinetics is either accepted or experimentally verified, the mole distribution of ^{32}P among the α-, β- and γ-positions of ATP can be accurately and routinely measured using the single ATPase reaction protocol described above.

Model for ATP labeling and calculation of ATP pool and AN pool turnover times

If we assume that the change in the α-P or γ-P specific activity (SA; nCi pmol^{-1}) of ATP is an exponential function of incubation time (t), and that the initial SA (at t_0) = 0 and the maximum SA (at isotopic equilibrium) = 1.00, then the decimal (0–1.00) equivalent of the SA of the α- or γ-P of ATP at any time (SA_t) is given by the equation:

$$SA_t = 1 - (2^{-N})$$

where N = the number of turnover cycles (i.e., dimensionless number) observed during the incubation period. ATP pool turnover time (from the γ-positioned P SA data) and AN pool turnover time (from the α-positioned P SA data) can be calculated from the expression:

$$T = t/N$$

where T is the turnover time in s, min or h. This equation holds for incubation periods (t) less than or equal to $5N$.

This model predicts that after one turnover cycle, the SA of the α-P or γ-P will be equal to 0.5 SA_{max}, after two cycles 0.75 SA_{max}, after three cycles 0.875 SA_{max}, and so on. The pools will achieve an SA which is analytically indistinguishable from SA_{max} after an incubation period equal to five turnover cycles (i.e., 0.97 ± 0.04). Exponential labeling of the ATP pool further assumes that the ATP pool is homogeneous (i.e., well mixed). These assumptions are consistent with existing metabolic models and our own limited laboratory and field data [6, 7]. A final assumption is that the specific activity of the precursor P pool (i.e., μCi $^{32}PO_4$ per μmol of total inorganic plus organic phosphorus) is constant for the duration of the labeling period. Since the time intervals selected for these studies are relatively short for most environmental samples (generally <1 h) this assumption appears to be valid. If, under unusual circumstances, the turnover time of the exogenous P-pool is equal to or shorter than the turnover time for the ATP or AN pool, this exponential ^{32}P-labeling model is unacceptable in its present form.

Physiological and ecological applications of the ATP and AN turnover methods

Previous studies of ATP pools in microorganisms and in environmental samples [2] have emphasized the futility of extrapolating these static measurements to estimates of metabolic activity or respiration. Although ATP turnover rate is expected to be positively correlated with metabolic activity, it is well documented that turnover rates are independent of steady-state intracellular ATP concentrations. Consequently, the direct measurement of ATP turnover when coupled with independent measurements of pool size, should provide useful information on biological energy flux in cells, populations or natural microbial communities. If we assign a value of –10 to –12 kcal mol^{-1} for the free energy of ATP hydrolysis under in vivo conditions [20], the measured ATP turnover

can be extrapolated to energy flux for comparison to other indirect estimates such as O_2 respirometry (for aerobically growing heterotrophs), O_2 production or CO_2 reduction (for photoautotrophs), or microcalorimetry (for total microbial communities). Direct measurements of ATP turnover should also be useful in pure culture studies to evaluate the efficiency of substrate utilization or the degree of energy coupling during the growth of microorganisms. Furthermore, because of the central role of adenine nucleotides in cellular energetics the ATP turnover method described herein might eventually be used to study the short-term response of microorganisms to environmental perturbations or to detect and monitor sublethal stress induced by the addition of toxic substances or xenobiotics.

The measurement of the turnover rate of the α-positioned P of ATP is, in and of itself, an independent estimate of the growth rate of a cell (or mean growth rate of a population or community), since the α-P becomes labeled only during net biosynthesis of macromolecules. As summarized by Chapman and Atkinson [5], the total AN pool is expected to turnover, on the average, 40 times per generation. Consequently, mean doubling time (t_d), and hence specific growth rate (μ) can be estimated directly from α-P turnover time ($T_{\alpha\text{-P}}$):

$$t_d = \frac{T_{\alpha\text{-P}}}{0.025}$$

$$\mu = \frac{0.693}{t_d}$$

While this relationship between μ and α-P turnover certainly requires additional calibration with pure and mixed cultures of bacteria and unicellular algae, our preliminary laboratory results estimating μ from α-P turnover are consistent with independent measurements of cell growth and biosynthesis [6, 7]. Finally, measurement of the α-P specific radioactivity when used in conjunction with the rate of incorporation of ^{32}P into RNA or DNA can be used to calculate actual rates of total microbial nucleic acid synthesis. A detailed discussion of the application of this method and field results comparing it to the [^{3}H]adenine technique will be presented elsewhere [21].

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