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Adenosine Triphosphate and Guanosine Triphosphate Determinations in Intertidal Sediments

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ABSTRACT: Measurements were made of the spatial distributions of adenosine triphosphate (ATP) on a variety of sandy beaches. Samples were collected during ebb flow and extracted in the field using cold sulfuric acid and ethylenediamine tetraacetate (EDTA); the neutralized extracts were immediately frozen for subsequent analyses. ATP was measured using the firefly bioluminescent reaction; however, during these investigations, a disparity was observed between the concentrations of ATP that were calculated from peak height and integrated light flux determinations. Integral measurements were consistently higher. Examination of the kinetics of the light emission reaction revealed an altered pattern of reactivity (relative to ATP standards) for nearly all of the sediment extracts. Thin-layer chromatographic separation procedures indicated that the sediment extracts contained additional nucleotide triphosphates (that is, GTP, UTP) at levels high enough to significantly interfere with quantitative ATP determinations. A technique was devised to eliminate this altered kinetic pattern in order to calculate true ATP levels. Additional methods were developed for the quantitative determination of guanosine triphosphate (GTP) using a nucleoside diphosphate kinase-firefly luciferase coupled reaction. The role of GTP in microbial metabolism, especially in the process of protein synthesis, results in an increase in the [GTP]/[ATP] ratio as a function of increasing growth rate. The measurement of [GTP]/[ATP] ratios in cell extracts may be useful for determining the metabolic states and rates of growth of microbial populations in nature.

KEY WORDS: adenosine triphosphate, guanosine triphosphate, nucleotides, bioluminescence, sediments, growth rates, microorganisms

The central metabolic role of adenosine triphosphate (ATP) in nearly all cellular energy transductions, the apparent constancy of ATP to cellular

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organic carbon in a wide range of taxonomically diverse microorganisms, and the apparent ease of extraction and measurement of ATP from biological samples has stimulated research into the use of ATP as an indicator of biomass. The diverse applications of ATP measurements include: marine [*1–8*][2] and freshwater [*9–11*] ecosystems, soil [*12,13*] and sediments [*14–17*], exobiological testing [*18,19*], wastewater effluent and sewage treatments [*20–22*], analysis of potable water supplies [*21*], antibiotic susceptibility testing [*23*], and various clinical applications [*24*].

Even though ATP can be measured using any one of a number of chemical or enzymatic techniques, the preferred method of ATP analysis is based upon the firefly bioluminescent reaction. The kinetics, specificity, and mechanism of this enzyme catalyzed reaction have recently been reviewed in detail [*25,26*]. Since the publication of the now classic paper by Holm-Hansen and Booth [*1*], there has been an overwhelming selection of the firefly bioluminescent assay of ATP for all ecological studies; nevertheless, any further similarities among the various nucleotide extraction procedures, enzyme preparations, light detection instruments, and data reduction methods seem coincidental. During the past five years, there have been at least four international symposia [*27–30*] organized primarily for the presentation and discussion of new developments in the field of ATP methodology. Although it would certainly be an advantage to standardize the basic techniques employed for ecological studies, it seems to me at least that this would be a difficult task, especially since there still appears to be a significant flux of new ideas and techniques and additional questions and difficulties yet to be resolved.

This chapter discusses the analytical interference of non-adenine nucleotide triphosphates on intertidal sediment ATP determinations, presents data on the vertical and horizontal distributions of ATP and guanosine triphosphate (GTP) for several Southern California sandy beaches and introduces the application of [GTP]/[ATP] ratios for estimating bacterial growth and metabolism in environmental samples. A more extensive report emphasizing the ecological implications of these data and interpreted with respect to corroborative and ancillary data collected simultaneously will be published at a later date (Holm-Hansen et al, in preparation).

Experimental Work

Description of Sampling Sites

For the past 2½ years, the Bureau of Land Management (BLM) has been conducting a multidisciplinary environmental study of the Southern California Bight. The overall goal of this project is to gather enough

[2] The italic numbers in brackets refer to the list of references appended to this paper.

information on the structure of intertidal ecosystems to assist the BLM in predicting and eventually assessing the effects of offshore oil and natural gas development. The six beaches selected for this study were located at Government Point, Coal Oil Point, Corona Del Mar, Ocean Beach, Santa Catalina Island, and San Nicolas Island (Locations 1 through 6, respectively) (Fig. 1). All of the intertidal sample sites were characterized as sandy beaches; however, they ranged in character from populated, heavily used, contaminated regions (for example, Coal Oil Point and Ocean Beach) to relatively inaccessible locations (for example, Government Point and San Nicolas Island).

Collection of Samples and Extraction of Nucleotides

All samples were collected during ebb flow (that is, from high tide to low tide) according to the following protocol. At Government Point, Corona Del Mar, Ocean Beach, and Santa Catalina Island sites, vertical profiles (0 to 1, 4.5 to 5.5, and 9.5 to 10.5 cm) and integrated samples (0 to 10 cm, mixed thoroughly before subsampling) were obtained at mid-tide and low-tide positions of each beach. At Coal Oil Point and San Nicolas Island beaches, samples were obtained at high-, mid-, and low-tide levels, but only surface (0 to 1 cm) and integrated (0 to 10 cm) sediments were extracted. The sediments for surface and vertical profile samples were obtained with a large diameter plastic core tube (10 cm inside diameter), and the sand was extruded to the correct depth intervals with the aid of a rubber plunger. The integrated samples were obtained using a polyvinyl chloride (PVC) core tube (3 cm inside diameter). All samples were placed

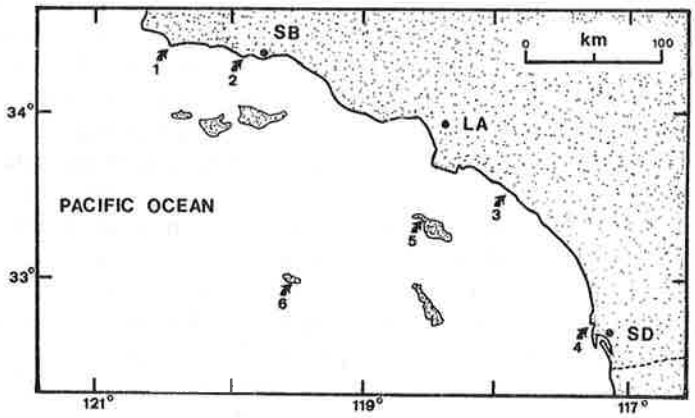


FIG. 1—*Map showing the geographical locations of the six beaches that were sampled during this study: (1) Government Point, (2) Coal Oil Point, (3) Corona Del Mar, (4) Ocean Beach, (5) Santa Catalina Island, (6) San Nicolas Island. SB, LA, and SD represent the locations of the cities of Santa Barbara, Los Angeles, and San Diego, California.*

into glass jars, mixed thoroughly with a spatula and subsampled in quadruplicate with a plastic syringe sampler designed to deliver 2 ml of wet sediment, and were extracted immediately thereafter using the sulfuric acid–ethylenediamine tetraacetate (EDTA) extraction procedure described by Karl and LaRock [*16*]. The sample extracts were neutralized in the field, diluted to 25 ml with tris buffer (0.02 *M*, pH 7.7), and stored frozen (−20°C) for subsequent laboratory analyses. No ATPase activity could be detected in any of the intertidal sample extracts, in contrast to recent results obtained in our laboratory for extracts of certain pure cultures of algae and bacteria and selected subtidal marine sediments (Karl, unpublished results).

Measurement of ATP and GTP

Light emission from the firefly bioluminescent reaction was measured using a commercially available ATP photometer. The instrument was equipped with an automatic injector (kinetics kit) that was designed to achieve rapid and reproducible mixing of the reagents while the vial is positioned in the photometer. The light emission kinetics of each reaction were recorded by interfacing a strip chart recorder with the photometer. This permits one to measure the peak height of light emission and the integrated light flux over a predetermined period of the subsequent reaction kinetics on the same sample or standard injection.

Commercially available luciferin–luciferase reagents (Sigma Chemical Company, FLE-50) were prepared by adding 5 ml distilled water, 10 ml sodium arsenate buffer (100 m*M*, pH 7.4), and 10 ml magnesium sulfate (40 m*M*) to each 50-mg vial. After an aging period of 4 to 5 h, the preparation was ready for use.

All sediment extracts were diluted 3:1 with tris buffer (0.02 *M*, pH 7.7) to reduce the concentration of SO_4^{2-} in the extracts. This ensures maximal activity of UDP-glucose pyrophosphorylase (see Table 1). The methods used for GTP analysis have been described in detail by Karl [*31,32*] and are presented in abbreviated form in Table 1. The technique is based upon selective enzymatic hydrolysis of ATP and uridine triphosphate (UTP) contained within the cell extracts, followed by a quantitative determination of the GTP concentration based on light emission resulting from a nucleoside diphosphate kinase–firefly luciferase coupled reaction. During this reaction, the GTP is enzymatically converted to ATP, thereby resulting in ATP-dependent light emission. The GTP-dependent light emission reaction kinetics and a typical GTP standard curve are presented in Fig. 2. The methods are simple, reproducible, and extremely sensitive, and require no preparatory chromatographic separation or purification procedures. A complete report describing the enzymatic principles, reaction specificities, assay limitations, and potential sources of analytical interference has been published elsewhere [*32*].

TABLE 1—*Summary of the procedure used for quantitative GTP determinations.*

	Procedure	Reaction	Result
I.	Incubate the nucleotide extracts with D-glucose, NADP, α-D-glucose-1-P, HK, G6P-DH, and UDPG-PP	$ATP + glu \xrightarrow{HK} glu\text{-}6\text{-}P + ADP$ $glu\text{-}6\text{-}P \xrightarrow[NADP \quad NADPH]{G6P\text{-}DH} gluconate\text{-}6\text{-}P$ $\alpha\text{-D-}glu\text{-}1\text{-}P + UTP \xrightarrow{UDPG\text{-}PP} UDP\text{-}glu + PP_i$	Complete enzymatic hydrolysis of ATP and UTP
II.	Place samples into a boiling water bath	2 min, 100°C	enzymatic reactions terminated
III.	Inject samples into FLE-50 containing: FL, Luciferin, excess ADP, and NDPK; measure the initial rise of light emission	$GTP + ADP \xrightarrow{NDPK} ATP + GDP$ $ATP + LH_2 \xrightarrow{FL} light$	GTP is enzymatically converted into ATP; ATP reacts with FLE-50 to produce light
IV.	Standardize with known GTP solutions	(*x*) GTP → (*y*) ATP → (*z*) light	construct a standard curve and calculate the GTP concentrations in the sample extracts

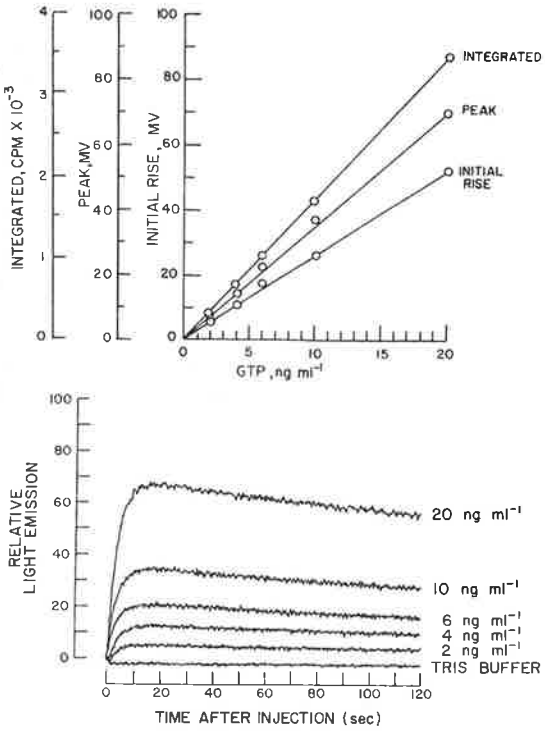


FIG. 2—*Kinetics of GTP-dependent light emission and standardization of the GTP assay. The upper graph depicts the concentration-dependent light emission reactions for samples ranging from 0 to 20 ng GTP per millilitre. The lower graph presents these light emission data in the form of* [GTP] *versus light emission standard curves. Either the initial rise of the luminescent curve, the peak height of light emission, or an integrated portion of the subsequent decay curve (15 to 75 s) may be used to measure the concentration of GTP in sample extracts (figure taken from Karl* [*32*]).

Results and Discussion

At the onset of these experiments, it was presumed that the ATP concentrations calculated for an environmental sample extract would be identical (within the analytical error) regardless of whether the sample was assayed using peak light emission or integrated light flux determinations, provided the two sets of data were related to the appropriate ATP standard curve. The results, however, indicated that the ATP concentrations based on integrated light flux determinations were always greater than the values calculated from peak emission data; moreover, the difference between the two measured ATP values displayed considerable variation between the samples of various beaches. A summary of the results obtained is presented in Table 2. The value of ΔATP/peak [ATP] (that is, integrated [ATP] − peak [ATP]/peak [ATP] × 100 percent) ranged from 44 to 226 percent; however, the majority of the samples averaged between 70 and 90 percent (overall $\bar{X}$ = 86 percent). The value,

TABLE 2—*Comparison of intertidal sediment ATP concentrations as determined by two methods.*

Sample Location			ATP Concentration[a]			
Beach	Tide	Depth (cm)	Integrated	Peak	ΔATP[a] (Int-P)	ΔATP/peak (%)
Corona Del Mar	mid	0 – 1	146	78	68	87
		5	135	94	41	44
		10	114	66	48	73
		1 – 10	136	82	54	65
	low	0 – 1	191	117	74	63
		5	188	126	62	49
		10	126	77	49	64
		0 – 10	173	105	67	64
Catalina Island	mid	0 – 1	1125	533	592	111
		5	182	104	77	74
		10	160	104	56	53
		0 – 10	407	229	177	78
	low	0 – 1	825	452	373	83
		5	78	47	34	76
		10	26	12	15	127
		0 – 10	320	197	123	62
Ocean Beach	mid	0 – 1	302	171	131	77
		5	195	116	80	69
		10	219	119	101	85
		0 – 10	258	159	99	62
	low	0 – 1	316	180	137	76
		5	331	182	149	83
		10	166	95	72	76
		0 – 10	247	145	102	70
Government Point	mid	0 – 1	288	171	117	68
		5	197	122	75	62
		10	179	101	78	76
		0 – 10	220	125	96	77
	low	0 – 1	425	238	187	79
		5	581	348	234	67
		10	241	116	125	108
		0 – 10	456	261	196	75
Coal Oil Point	high	0 – 1	88	32	56	172
		0 – 10	98	55	42	77
	mid	0 – 1	420	119	301	254
		0 – 10	443	136	307	226
	low	0 – 1	368	202	165	81
		0 – 10	324	177	147	83
San Nicolas Island	high	0 – 1	76	45	31	69
		0 – 10	86	60	27	45
	mid	0 – 1	150	103	47	45
		0 – 10	68	44	25	57
	low	0 – 1	172	109	63	58
		0 – 10	102	66	36	55

[a] All ATP concentrations are in nanograms per millilitre.

ΔATP/peak [ATP] showed no consistent pattern with sample location on a particular beach, tide level, or sediment depth (Table 2). Furthermore, Spearman's rank difference correlation analysis indicated that the values for ΔATP/peak [ATP] were not significantly correlated with the absolute concentrations of ATP for these intertidal extracts (that is, $r = 0.23$) (Fig. 3). It was apparent, however, that the samples collected at Coal Oil Point exhibited consistently higher ΔATP/peak [ATP] values and the samples from San Nicolas Island consistently lower ΔATP/peak [ATP] values than the mean values from the other intertidal stations ($\bar{X}$ ΔATP/peak [ATP] = 72 percent, Corona Del Mar; 92 percent, Santa Catalina Island; 84 percent, Ocean Beach; 77 percent, Government Point; 149 percent, Coal Oil Point; and 55 percent, San Nicolas Island).

In an attempt to resolve the observed disparity between the two methods of ATP determination (that is, integral versus peak), an analog recorder was attached to the photometer in order to examine the kinetics of the light emission reactions. Figure 4 displays the results that were obtained for several selected intertidal extracts. In addition to the various environmental samples, ATP standards were prepared at concentrations that resulted in peak height emission values that were identical to the

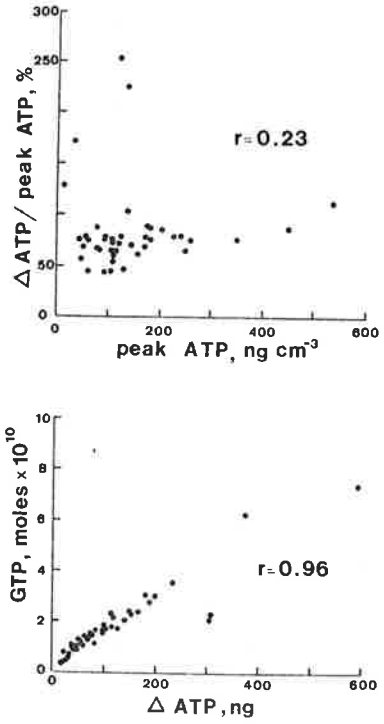


FIG. 3—*Spearman's rank difference correlation analyses* (n = *43) of peak* [*ATP*] *versus* Δ*ATP/peak* [*ATP*] (upper graph), *and* Δ*ATP versus* [*GTP*] (lower graph).

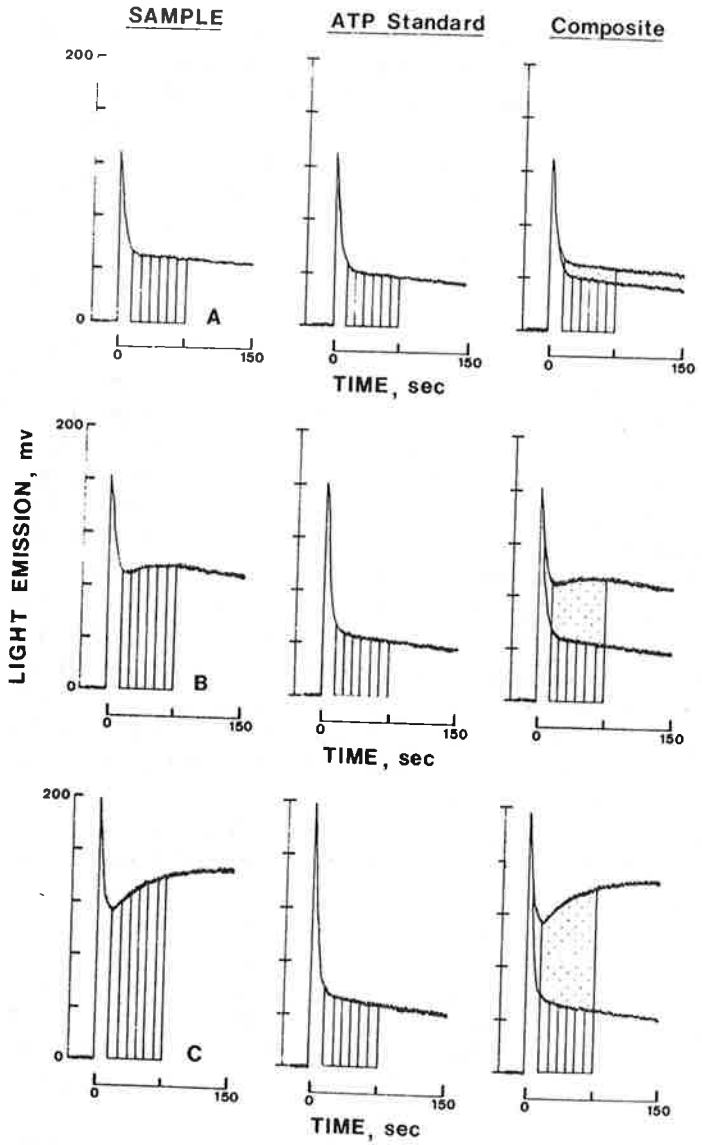


FIG. 4—*Kinetics of light emission resulting from the injection of several selected intertidal extracts into a vial containing the firefly luciferase enzyme preparation. The* Δ *ATP/peak [ATP] (percent) values were: Sample A, 52 percent; Sample B, 82 percent; Sample C, 220 percent.*

sample extracts; these ATP-dependent light emission kinetics are also presented in Fig. 4 for visual comparison. It is now apparent that the discrepancies that were observed between the two methods of ATP determination (that is, peak and integral light emission) can be explained, at least qualitatively, in terms of an altered pattern of light emission. The

experimental sample extracts catalyzed a light emission reaction that differed significantly from that of standard ATP solutions, particularly in regard to the reaction kinetics following the peak height of light emission (that is, 15 to 45 s after sample injection). Moreover, the three data sets presented in Fig. 4 depict the extreme variation that can occur among samples collected from otherwise relatively comparable habitats (that is, sandy beaches). Since the altered kinetics affect the integrated light flux determinations to a greater extent than the peak emission values, integrated light measurements cannot be used for estimating intertidal ATP concentrations under these reaction conditions.

Although crystalline firefly luciferase is specific for ATP [*26,33*], it is well known that commercial reagents (both crude and "purified") contain contaminating enzymes that can catalyze the production of ATP from a variety of precursors. An extensive investigation of the nonspecificity of crude firefly reagents has already been presented by Rasmussen and Nielsen [*34*], and several additional reports have appeared since then confirming these results [*35,36*]. All of these experiments, however, were conducted using pure solutions of nucleotides rather than representative cell extracts; therefore, the analytical implications and consequences of this nonspecificity were not impressed upon the general audience. To my knowledge, this is the first report of the quantitative significance of the nonspecificity of firefly luciferase when assaying extracts of naturally occurring microorganisms. It is evident from the data presented in Table 2 and the reaction kinetics displayed in Fig. 4 that this level of error cannot be neglected, regardless of the application.

If one wishes to obtain more reliable estimates of the ATP concentration in environmental sample extracts, a number of procedures may be employed to reduce or eliminate the analytical interference resulting from reactive compounds other than ATP. The most effective method, but not necessarily the most practical, is to utilize an ATP-specific crystalline luciferase enzyme preparation such as the one described by McElroy and Green [*33*]. Measurement of either the initial rise of the luminescent curve [*35*] or the peak height of light emission or the use of partially purified reagents [*34*] will significantly reduce but will not totally eliminate this altered reactivity.

As discussed previously (Table 2, Fig. 4), the extent of this interference was quite variable between the various environmental samples, which implies that the concentration(s) of the additional reactive molecule(s) was also quite variable. Even though the first impulse is to design procedures to eliminate the effect of these compounds [*34–36*], it may be more valuable to identify the molecules responsible for this altered reactivity and, if possible, to determine their concentration(s) as well. Since ATP must be enzymatically produced within the crude enzyme extracts through the addition of these unknown compounds (that is, firefly luciferase is specific for ATP), the interfering molecules must contain a

~P bond. Of the various compounds tested in preliminary experiments (for example, α-D-glucose 1-phosphate, D-glucose 6-phosphate, phosphoenolpyruvate, adenosine disphosphate (ADP), and various non-adenine-containing nucleotides), only the nucleotide triphosphates (NTPs = GTP, UTP, ITP, and CTP) resulted in altered light emission kinetics when assayed with equimolar concentrations of ATP. It was also evident that the patterns of light emission resulting from these nucleotide mixtures were similar to the kinetics displayed in Fig. 4 for the various intertidal sample extracts.

An experiment was conducted in an attempt to obtain a qualitative analysis of the entire suite of NTPs contained within the environmental cell extracts. The NTPs contained within several representative extracts were concentrated approximately 100-fold using the activated charcoal adsorption procedure described by Cashel et al [*37*]. The eluates were evaporated to dryness and reconstituted in phosphate buffer (0.85 *M*, pH 3.4). Subsamples were immediately applied to PEI-cellulose thin layers with plastic backing (Brinkman Instrument Co.) and were developed in one dimension using phosphate buffer [*37*]. It should also be mentioned that the kinetics of the light emission reaction observed with dilute (100-fold in tris buffer) preparations of these concentrated extracts were similar to the reaction kinetics of the original samples, indicating that all of the compounds responsible for the light emission had similar adsorption, elution, and stability characteristics. Standard solutions of the various NTPs were also developed on the same thin-layers, and their mobilities (expressed as R_f values) were determined using high intensity short-wavelength ultraviolet irradiation of the air-dried plates. Following separation and air drying, the entire cellulose plate was cut into 1-cm fractions ranging from the origin (0 cm) to the solvent front (15 cm), and the material contained within each interval was eluted with magnesium chloride ($MgCl_2$, 0.6 *M*), diluted with tris buffer (0.02 *M*, pH 7.7), and reassayed using the firefly bioluminescent reaction. In this way, the mobility of the interfering compounds could be determined, and these results could be directly compared to the R_f values of the nucleotide standards. The results of these investigations revealed that the altered kinetic patterns could be resolved into two major regions on the plate; one corresponding to GTP and the other corresponding to UTP.

Although the majority of energy-requiring and energy-producing reactions within a cell require direct coupling with the adenine nucleotides (especially ATP), many cellular processes proceed only indirectly as a result of the adenylate system. Many important biosynthetic reactions associated with microbial growth require energy derived from NTPs other than ATP. For example, GTP is required for the activation and interconversion of precursors for cell wall biosynthesis, for DNA replication (as dGTP) and RNA transcription, and for the initiation, aminoacyl tRNA binding and translocation processes of protein synthesis. This increased

demand for GTP (as well as the other intracellular NTPs) with increasing growth rate results in an increase in the intracellular concentrations of GTP relative to the cellular levels of ATP. Figure 5 presents data obtained from the literature to illustrate the growth-rate-dependent fluctuations that occur in the nucleotide triphosphate pools of microorganisms. Therefore, the determination of GTP, or more precisely, the [GTP] / [ATP] ratio in environmental samples, may provide valuable information regarding the metabolic or growth status of naturally occurring microbial populations.

Table 3 presents the results that were obtained for quantitative determinations of GTP in the various intertidal sample extracts. A Spearman's rank difference correlation analysis indicated a significant correlation for the plot of ΔATP versus GTP (that is, $r = 0.96$) (Fig. 3), suggesting that measurements of ΔATP may be used as relative estimates of the GTP concentrations in intertidal extracts.

The [GTP] / [ATP] ratios (on a pmolar basis) ranged from 0.4 to 1.86, with an overall average of 0.69. These results are comparable to the [GTP] / [ATP] ratios measured in extracts of logarithmically growing marine bacteria (Karl, unpublished results). By comparison, unicellular algae [*40*], fungi [*41*], and multicellular organisms such as copepods and annelids (Karl, unpublished results) have much lower [GTP] / [ATP] ratios, presumably due to the lower specific rates of cellular biosynthesis. The highest ratios in this present study were measured for the sediment extracts at Coal Oil Point, and the lowest ratios were found at San Nicolas Island (Table 3). These results may reflect the relative rates of community biosynthesis or may be due to variations in the structure of the two intertidal ecosystems (that is, bacterial biomass/total biomass).

Summary

In summary, the significant results of these current investigations are: (1) the measurement of ATP in intertidal sediment extracts using integrated light flux determinations results in variable overestimates of the true ATP concentrations; (2) the value ΔATP (that is, integrated [ATP] − peak [ATP]) can be used as a relative measure of the GTP concentration in intertidal sediment extracts ($r = 0.96$ for ΔATP versus [GTP]); (3) quantitative ATP and GTP determinations have been made on sediment extracts from a variety of Southern California sandy beaches, and the corresponding [GTP] / [ATP] ratios have been calculated and compared to the results obtained with growing microorganisms and eukaryotes; and (4) since the intracellular [GTP] / [ATP] ratio in bacterial and algal cultures has been shown to increase with increasing growth rate, the determination of [GTP] / [ATP] ratios in environmental sample extracts may be useful for estimating microbial growth rates in nature.

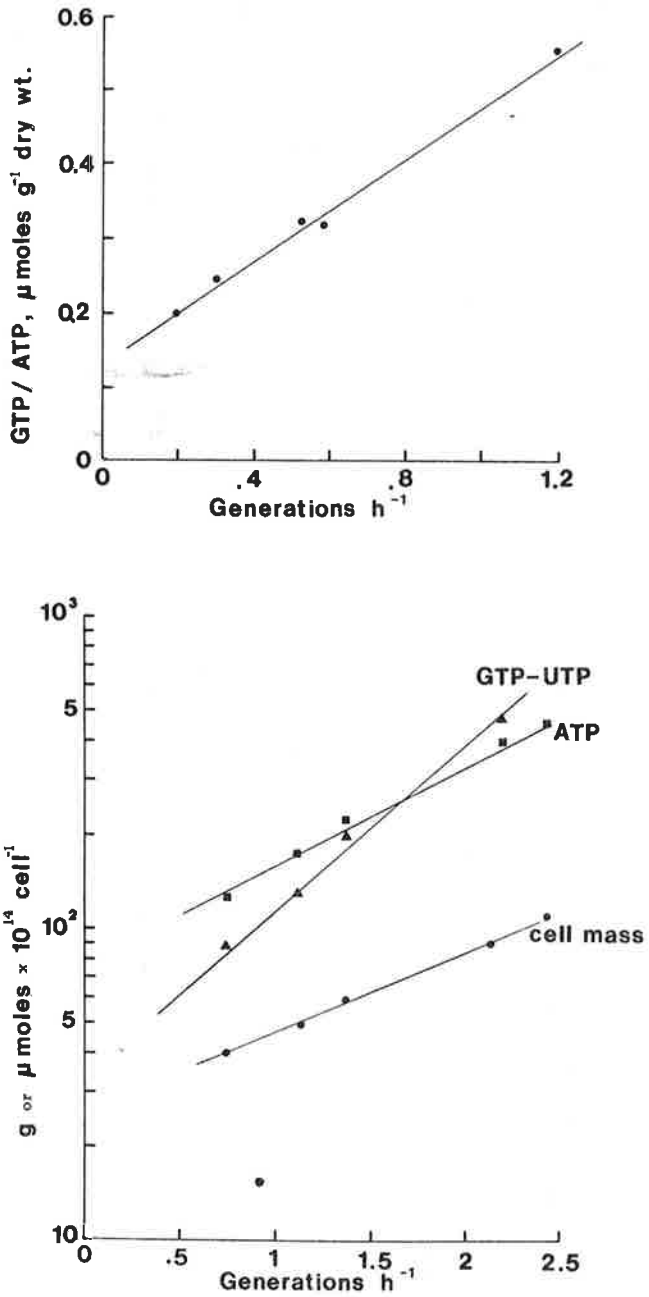


FIG. 5—*Variations in the [GTP] / [ATP] and [GTP + UTP] / [ATP] ratios as a function of growth rate. The data in the upper graph were redrawn from data presented by Smith and Maaløe* [38] *for the bacterium* Salmonella typhimurium. *The data in the lower graph were redrawn from data presented by Franzen and Binkley* [39] *for the bacterium* Escherichia coli *(cell mass expressed in grams; GTP + UTP and ATP expressed in micromoles).*

TABLE 3—*Intertidal sediment ATP and GTP concentrations and [GTP] / [ATP] ratios.*

Sample Location			[ATP] (pmoles/ml)	[GTP] (pmoles/ml)	[GTP]/[ATP]
Beach	Tide	Depth (cm)			
Corona Del Mar	mid	0 – 1	154	. . .	. . .
		5	185	108	0.58
		10	130	84	0.65
		1 – 10	162	108	0.67
	low	0 – 1	231	153	0.66
		5	249	139	0.56
		10	152	103	0.68
		1 – 10	207	128	0.62
Catalina Island	mid	0 – 1	1050	742	0.71
		5	205	149	0.74
		10	205	120	0.59
		0 – 10	452	303	0.67
	low	0 – 1	892	620	0.70
		5	93	96	1.03
		10	24	38	1.58
		0 – 10	398	230	0.59
Ocean Beach	mid	0 – 1	337	213	0.63
		5	229	149	0.65
		10	235	174	0.74
		0 – 10	314	162	0.52
	low	0 – 1	355	208	0.59
		5	359	247	0.69
		10	187	142	0.76
		0 – 10	286	174	0.61
Government Point	mid	0 – 1	337	181	0.54
		5	241	145	0.60
		10	199	116	0.58
		0 – 10	247	153	0.62
	low	0 – 1	469	274	0.58
		5	686	365	0.52
		10	229	176	0.77
		0 – 10	515	298	0.58
Coal Oil Point	high	0 – 1	63	117	1.86
		0 – 10	108	86	0.80
	mid	0 – 1	235	230	0.98
		0 – 10	268	218	0.81
	low	0 – 1	398	244	0.61
		0 – 10	349	238	0.68
San Nicolas Island	high	0 – 1	89	57	0.64
		0 – 10	118	58	0.49
	mid	0 – 1	203	81	0.40
		0 – 10	87	43	0.49
	low	0 – 1	215	103	0.48
		0 – 10	130	63	0.48

Addendum

Since this paper was originally presented at the ASTM symposium in Ft. Lauderdale, Fla., Jan. 1978, additional reports have appeared in the literature confirming the general occurrence and analytical significance of GTP and UTP in marine [*42*] and freshwater [*43*] ecosystems.

Acknowledgments

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