Uptake of adenosine in a marine bacterium is not an active transport process

Carmela Bengis-Garber

Dept. of Biology, Technion-Israel Institute of Technology, Haifa, Israel

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The uptake and intracellular interconversions of [8-^{14}C]adenosine in a marine bacterium *Vibrio harveyi* were investigated under varying physiological conditions. The results indicated that in contrast with the current views, translocation of adenosine across the cytoplasmic membrane in *Vibrio harveyi* was not driven by respiration. The uptake of adenosine was dependent upon its intracellular utilization and was inhibited under conditions preventing its metabolic conversions.

Adenosine transport *Marine bacteria* *Bacterial transport* *Adenosine salvage pathway* *Nucleoside metabolism*

1. INTRODUCTION

Bacteria can efficiently utilize exogenously supplied nucleosides using a number of interconversion pathways [1–3]. Due to a rapid metabolism inside the cells the data on the mechanism of nucleoside uptake are conflicting. In [4–8] it was proposed that the nucleoside uptake process consisted of phosphorolysis in the periplasmic space and transport of the resulting base by group-translocation involving membrane-bound phosphoribosyltransferase. The uptake experiments were performed in membrane vesicles [5,7,8] or in cells starved up to 48 h at 4°C [6]. In such cells, and in vesicles, membrane integrity and hence the transport properties were probably altered since the membrane became permeable to d′-5-phosphoribosyl-1-pyrophosphate [5–8] and to AMP [7]. Moreover, the information obtained from the purification procedure and properties of the phosphoribosyltransferase [4,5] was not sufficient to assume the transmembranal orientation of this enzyme. The group translocation hypothesis was disputed by a number of investigators who suggested that all nucleoside and base interconversion enzymes reside within the cell [9,13] and that transport occurs prior to metabolism [9–14]. Although nucleoside transport has been generally considered an energy-dependent process, existing experimental data did not prove this unequivocally. Results of the experiments described below indicated that in contrast with the current views, the nature of nucleoside uptake in at least one bacterial species seems to be similar to that in animal cells [15], where it involves consecutive operation of facilitated diffusion and intracellular salvage pathways.

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine--*N*-2-ethane-sulfonic acid; DNP, 2,4-dinitrophenol; PMS, *N*-methylphenazonium methosulfate; Ade, adenine; Ads, adenosine; Hyp, hypoxanthine; Ino, inosine

2. MATERIALS AND METHODS

[8-^{14}C]Adenosine (51 mCi/mmol) and [8-^{14}C]-adenine (55.6 mCi/mmol) were the products of New England Nuclear. Cellulose 13254 sheets (no. 6065) for thin-layer chromatography (TLC) were obtained from Eastman Kodak. All chemicals were of analytical grade.

Vibrio harveyi strain MAV was a gift from Professor S. Ulitzur of the Department of Food

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Engineering and Biotechnology, Technion (Haifa). The bacterium was grown in complex medium [16], harvested in late log phase and washed twice with the buffer containing 20 mM Hepes (pH 7.8), 2 mM $MgCl_2$, 0.5 M NaCl and 10 mM KCl. Uptake experiments were performed essentially as in [17]. The reaction mixture contained, in the final volume of 0.25 ml, 20 mM Hepes (pH 7.8), 2 mM $MgCl_2$, 0.5 M NaCl and cells containing 5 μg protein. When the effects of inhibitors and phosphate were tested, cells were preincubated in the reaction mixture for 10 min at 32°C. Reaction was started with the addition of substrate, carried out at 32°C and terminated by filtration through 0.45 μm cellulose nitrate membrane filters BA 85, 25 mm of Schleicher and Schull. For TLC of purine bases and nucleosides, filtered cells were extracted with 0.2 ml 0.33 M formic acid as in [13], and 50-μl samples of cell extracts were applied on thin-layer cellulose sheets. Bases and nucleosides were separated in distilled water, and nucleotides in saturated ammonium sulfate/1 M sodium acetate/ isopropranol (80:18:2), as in [18].

Intracellular ATP was assayed as in [19]. Protein concentration was determined as in [20]. Oxygen uptake in *Vibrio harveyi* cell suspension was assayed polarographically. Reduction of NAD^+ and of *b*-type cytochrome were followed in a DW-2a UV-Vis Aminco-Chance spectrophotometer at 360 nm and 555 nm, respectively, using the reference wavelength of 540 nm. Membrane potential and proton gradient were measured with fluorescent dyes 8-anilino-1-naphthalene sulfonic acid and 9-aminoacridine, respectively, as in [21] in a Perkin-Elmer MPF-44B fluorescence spectrophotometer.

3. RESULTS AND DISCUSSION

Adenosine metabolism within the cell may proceed via two alternative pathways [3]. One involves conversion to AMP either through phosphorolysis and phosphoribosylation, or by direct phosphorylation of adenosine. In an alternative pathway the first enzymatic attack on the incoming adenosine is deamination. If adenosine translocation across the cytoplasmic membrane is limited by the rate of its intracellular conversion, then the effects of cellular phosphate and ATP pools on the rate of adenosine uptake would depend on the utilization pathway. Phosphate limitation and ATP depletion would inhibit initial rates of adenosine uptake if the latter is metabolized via the phosphorolysis pathway and would be without effect if deamination is predominant.

Addition of inorganic phosphate significantly stimulated the uptake of adenosine in cell suspensions of *Vibrio harveyi*. The extent of the phosphate effect varied with the adenosine concentrations. As illustrated in fig.1, 10 min preincubation of cells with 1 mM P_i resulted in 2–3-fold stimulation of the initial rates of adenosine uptake at 0.2–2 μM external [8-^{14}C]adenosine. No stimulation was observed at >4 μM adenosine. The site of the phosphate effect was most likely within the cell since inhibition of adenosine uptake by glucose could be overcome by addition of P_i and thus probably resulted from trapping of the intracellular phosphate (fig.2). Furthermore, [^{32}P]-phosphate was taken up at a higher rate than [8-^{14}C]adenosine when assayed in the same experiment (submitted). In addition, adenine was taken up at a lower rate and with lower affinity than adenosine under similar conditions (not shown). These data spoke against the possibility of prior phosphorolysis of adenosine in periplasmic space.

Cellular ATP pools also affected rates of

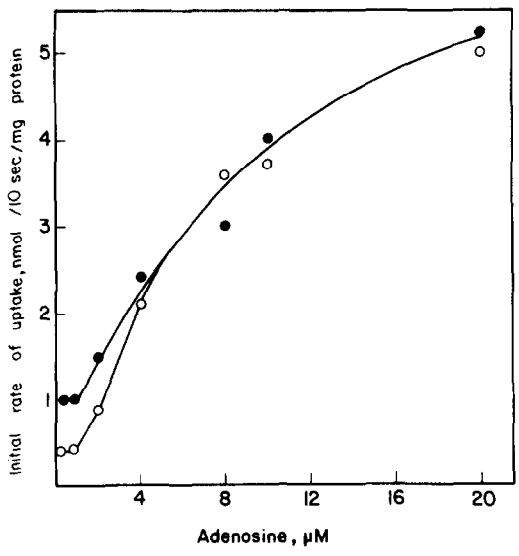

Fig.1. Effect of phosphate on the initial rates of adenosine uptake: (○) no phosphate; (●) preincubation with 1 mM K_2HPO_4.

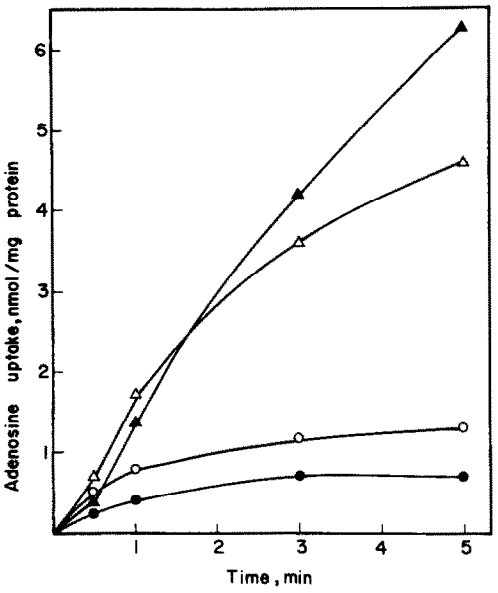

Fig.2. Effects of phosphate and glucose on the time course of adenosine uptake. [8-^{14}C]Adenosine was 0.4 μM; glucose and phosphate were added at time zero; (○) no additions; (●) + 10 mM glucose; (△) + 0.1 mM K_2HPO_4; (▲) + 0.1 mM K_2HPO_4 + 10 mM glucose.

Cellular ATP pools also affected rates of adenosine uptake. Cells harvested in logarithmic stage contained higher ATP pools and exhibited higher adenosine uptake rates in comparison with cells grown to stationary phase (not shown). The effect of cellular ATP levels on the uptake of adenosine and its fate within the cell were investigated at 0.4 μM and 10 μM of external adenosine which were, respectively, the conditions of maximal and negligible phosphate stimulation (fig.1). As illustrated in table 1, complete exhaustion of cellular ATP was achieved by preincubation with 1 mM arsenate for 10 min. Fig.3A shows that the uptake of 0.4 μM adenosine was severely inhibited in arsenate-treated cells even at the shortest uptake time (10 s). In contrast, the initial uptake rate at 10 μM of exogenous adenosine was slightly stimulated by arsenate (fig.3B). At longer incubation times the uptake was progressively inhibited in ATP-starved cells. Results of the analysis of intracellular-labeled products of [8-^{14}C]adenosine are summarized in table 2. 10 s incubation of a cell suspension with 10 μM [8-^{14}C]adenosine resulted in intracellular ac-

Table 1

Comparative effects of metabolic inhibitors and phosphate on adenosine uptake rates and on cellular ATP content

Additions		Initial rates of adenosine uptake		ATP content[b]	
		0.4 μM (% of control)	10 μM	nmol/mg	% of control
None		100	100	6.2	100
K_2HPO_4	1 mM	200	115	10.6	170
Na_2HAsO_4	1 mM	15	160	0.4	6
KCN	4 mM	20	10	2.5	40
PMS	5 μM	100	50	5.3	85
KCN, PMS		100	48	2.5	40
DNP[c]	0.5 mM	70	75	3.5	56
DNP, K_2HPO_4		100	85	6.1	57[a]

[a] Cellular ATP content in the presence of phosphate (10.6 nmol/mg) was taken as 100%

[b] The reaction mixtures for the assays of ATP content were identical with those for the uptake experiments except the amounts of cells in the ATP assays which were equivalent to 0.2 mg of protein/ml

[c] 1 mM DNP produced similar effects

For both the uptake and ATP assays cell suspensions were incubated with all additions as described in section 2. The uptake time was 10 s, and the initial rates were expressed as nmol.10 s^{-1}.mg $protein^{-1}$

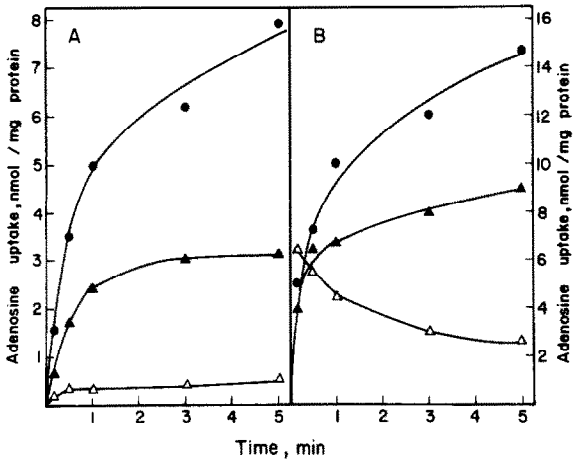

Fig.3. Inhibition of adenosine uptake by arsenate. Cells were preincubated with either 1 mM Na_2HAsO_4 or 1 mM K_2HPO_4: (A) 0.4 μM [8-^{14}C]adenosine; (B) 10 μM [8-^{14}C]adenosine; (▲) no additions; (●) + K_2HPO_4; (△) + Na_2HAsO_4.

cumulation of labeled inosine, hypoxanthine and IMP which at longer incubation times gave rise to adenine and guanine nucleotides. At 0.4 μM exogenous adenosine only phosphorylated compounds were labeled at all tested incubation times. In a different set of experiments cellular labeled products were analysed after 30 s and 45 s incubation with 0.4 μM adenosine, and here too only nucleotides were labeled (not shown). Shorter times than 30 s were not analysed because of low total counts on TLC sheets. These results indicated that the incoming adenosine was metabolized via the phosphorolysis pathway at lower concentrations and via deamination at higher concentrations. Adenosine did not accumulate within the cell against the concentration gradient, and its utilization was rate-limiting for uptake.

Analysis of the mode of action of DNP and KCN on the uptake of adenosine gave additional evidence in favour of a respiration-independent translocation mechanism. The effect of DNP on adenosine uptake could be generally correlated with the intracellular ATP concentrations. As illustrated in table 1, in cells harvested in the logarithmic stage and preincubated with phosphate, DNP treatment caused virtually no inhibition of adenosine uptake. Some inhibition was observed in cells which were not incubated with phosphate and contained lower ATP levels. In stationary stage cells where ATP levels were 2–3-times lower than in cells harvested in the logarithmic growth stage, DNP caused up to 50% inhibition of the adenosine uptake (not shown). Cyanide, on the other hand, which depleted cellular ATP pools to an extent comparable with DNP, caused severe inhibition of the adenosine uptake, which was released in the presence of PMS (table 1). Cyanide also completely inhibited the electron flow in the respiratory chain, which was

Table 2

Cellular 8-^{14}C-labeled products of the uptake of [8-^{14}C]adenosine

Conditions	Uptake time	Intracellular ^{14}C-labeled products (% of total radioactivity in the cell)									
		Ade	Ads	Hyp	Ino	AMP	ADP	ATP	GMP	IMP	GTP
Adenosine, 10 μM											
Control	10 s	0	3	67	15	0	0	0	0	15	0
+ K_2HPO_4	10 s	0	1.5	53	12	0	0	7	0	17.5	9
	1 min	0	1.7	38	8.6	0	0	27	6	15	10
	8 min	10	1	7	3	4	14	23	8	15	14
+ Na_2HAsO_4	10 s	0	3	31	66	0	0	0	0	0	0
Adenosine, 0.4 μM											
+ K_2HPO_4	1 min	0	0	0	0	14	17	18	13	23	15
	8 min	0	0	0	0	13	6.5	44	6.5	9	21

Cell suspensions were preincubated with 1 mM of either phosphate or arsenate; thin-layer chromatography of cell extracts was performed as in section 2

not restored by addition of PMS (not shown). Inhibitory effect of KCN on respiration was judged by measurement of the oxygen uptake, of membrane potential with 8-anilino-1-naphthalene sulfonic acid, of the proton gradient with 9-aminoacridine and of the changes in the redox state of NAD and of *b*-type cytochrome. However, PMS did oxidize NADH in cyanide-treated cells as illustrated in fig.4. Aerobic–anaerobic transitions in the NADH/NAD^+ redox state were recorded at 360 nm because of the interference of PMS absorption at 340 nm [22] where a broad absorption peak appeared when PMS was reduced upon the onset of anaerobiosis. The wavelength of 360 nm was found to be isoasbestic point for reduced *vs* oxidised PMS. Lines I and III in fig.4 reflect aerobic redox state of NAD in the absence and presence of PMS, respectively. Lines II and IV reflect anaerobic NAD^+ reduction under the above conditions. Cyanide poisoning of the cell suspension in the presence of PMS brought about reoxidation of reduced NAD to a new redox steady state (line V of fig.4), while in the absence of PMS addition of cyanide left NAD in the anaerobic reduced state. These data indicated that inhibition of adenosine uptake by KCN was not due directly to block of respiration. This inhibition was probably exerted through alteration in the redox state of NAD or increase in cellular nucleoside and base levels via enhanced degradation of nucleotides as was found in animal cells [23,24].

A currently accepted view of nucleoside uptake as an energy-dependent transport process was based on the use of uncouplers of oxidative phosphorylation and respiration inhibitors [10,12,14] and on transport assays in mutant strains lacking one or more enzymes of nucleoside metabolism [9,10,13,14]. However, as exemplified here, inhibition of nucleoside uptake by uncouplers and respiration inhibitors may result from depletion of cellular energy sources rather than from direct effect on respiration and proton gradient. On the other hand, interpretation of results obtained with mutant strains may also be erroneous, since in bacteria [3], as in animal cells [15], there exist several alternative salvage pathways. The analysis

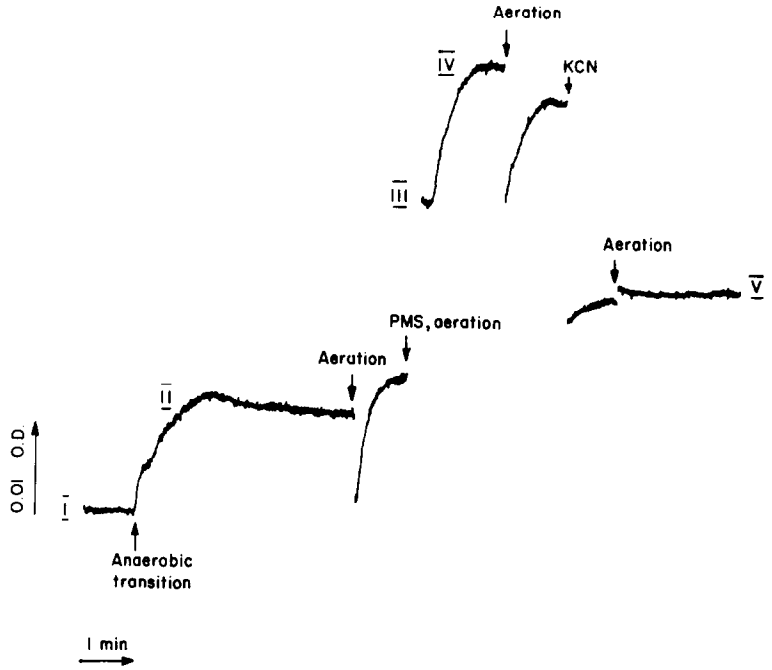

Fig.4. Effects of PMS and KCN on the oxidation–reduction state of NAD in intact cells of *Vibrio harveyi*. Washed cells equivalent to 0.6 mg protein were suspended in 3 ml of the buffer containing final concentrations of 20 mM HEPES (pH 7.8), 2 mM $MgCl_2$ and 0.5 M NaCl; final concentrations of PMS and KCN were 5 μM and 4 mM, respectively. Anaerobiosis was complete in 90 s after aeration. All other conditions were as described in the text.

of intracellular labeled products of nucleoside uptake was only performd in one of the works employing mutant strains, and an unaltered purine nucleoside was shown to have accumulated intracellularly [13].

These results can serve as an example of similarity in the mechanisms of transport of biological substrates between prokaryotic and eukaryotic cells. Another such example is the transport of amino acids which is believed to proceed at the expense of electrochemical proton gradient, in symport with Na^+, in both bacteria and animal cells [25].

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