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Effect of Exogenous Nucleotides on Ca^{2+} Dependence and V Antigen Synthesis in *Yersinia pestis*

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Cells of *Yersinia pestis* strain EV76 are known to cease growth after a shift from 26 to 37°C in neutral Ca^{2+}-deficient medium; this effect is potentiated by Mg^{2+}. With 2.5 mM Mg^{2+} and no added Ca^{2+}, restriction was relaxed at elevated pH at which maximum cell yields occurred in the presence of 20 mM exogenous ATP. This ATP-dependent growth was inhibited by Ca^{2+} or 20 mM Mg^{2+}; the nucleotide was neither transported into the organism nor hydrolyzed extracellularly. With strain EV76, ATP could be replaced by GTP but not other nucleotides, nucleosides, free bases, or pyrophosphate. CTP and UTP also promoted growth of strain KIM, in which limited division also occurred with nucleoside di- and monophosphates. Intracellular V antigen was detected 1 h after temperature shift in Ca^{2+}-deficient medium containing 20 mM Mg^{2+}, a time corresponding to the earliest known events associated with restriction (shutoff of stable RNA synthesis and reduction of adenylate energy charge). Maximum yield of V was obtained 2 h later when cell division ceased; the titer of the antigen remained constant thereafter. The specific activity of V in cells grown with ATP was significantly reduced, especially at elevated pH. These results would be expected if exogenous nucleotides promote growth by sequestering sufficient Mg^{2+} to prevent restriction of cell division mediated by V antigen.

A major determinant of virulence in *Yersinia pestis* (4), *Yersinia pseudotuberculosis* (2), and *Yersinia enterocolitica* (5) is the ability to produce the plague V and W or virulence antigens (Vwa^+). V antigen from *Y. pestis* is a 75,000-dalton pure protein, whereas coordinately induced W was characterized as a 145,000-dalton lipoprotein (18). The antigens are induced in vitro at 37°C but not at 26°C in aerated enriched medium; synthesis is enhanced by 20 to 40 mM Mg^{2+} (1, 4, 18). Their optimal expression is associated with an ordered stepdown of anabolic functions initiated by a primary block in synthesis of stable RNA (7) and a marked reduction in adenylate energy charge (34) associated with subsequent cessation of cell division without significant loss of viability (33, 34). This form of restriction can be reversed by downshift to 26°C and prevented at 37°C by the presence of at least 2.5 mM Ca^{2+} (11, 34); both of these treatments are known to repress synthesis of virulence antigens (1, 18). In view of the regulatory effects of divalent cations on virulence antigen biosynthesis, it seems unlikely that V and W would be produced in extracellular fluids such as blood or lymph, which are rich in Ca^{2+} (17). Significant synthesis might occur in host intracellular fluids which contain much Mg^{2+} but only trace levels of Ca^{2+} (16).

Although all yersiniae are facultative intracellular parasites (6, 24, 25, 30), it is not yet known whether the bacteria remain within phagosomes during the course of intracellular residence or if they, like rickettsiae (31), gain access to host-cell cytoplasm. An important feature of these obligate intracellular parasites is their ability to exchange nucleotides, including ATP (32), which are normal constituents of host cell cytoplasm. Typical procaryotes, of course, are unable to transport nucleotides although they can often accumulate nucleosides and free bases (20). However, since Vwa^+ *Y. pestis* cultivated under simulated intracellular conditions with respect to Ca^{2+} and Mg^{2+} lost internal adenine nucleotides (34), we considered the possibility that they, like rickettsiae, could exchange internal nucleotides with those of the environment. In this case, addition of ATP to Ca^{2+}-deficient cultures might alleviate restriction. The purpose of this report is to show that exogenous nucleotides do indeed promote Ca^{2+}-independent growth of Vwa^+ organisms and modify their patterns of virulence antigen biosynthesis.

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TABLE 1. Composition of chemically defined medium[a]

Component	Final concentration (mM)
Salt solution[b]	
K_2HPO_4	2.5
Citric acid	10.0
NH_4Cl	10.0
$FeCl_2$	0.1
$MnCl_2$	0.01
Vitamin solution[c]	
Thiamine	0.003
Calcium pantothenate	0.004
Biotin	0.002
Amino acid solution[d]	
DL-alanine	4.5
L-Isoleucine	3.8
L-Leucine	2.0
L-Methionine	1.6
L-Phenylalanine	2.4
L-Threonine	1.4
L-Valine	6.8
L-Arginine	1.0
L-Proline	7.0
L-Glutamic acid	81.1
L-Lysine	1.1
Glycine	2.6
L-Tyrosine	1.1
Miscellaneous solutions	
HEPES, neutralized with 5.5 N NaOH[c]	25.0
Potassium gluconate[e]	10.0
$MgCl_2$[f]	1.25 to 20
$CaCl_2$[f]	0 to 4.0
L-Cysteine[e]	1.0
L-Tryptophan[e]	0.1

[a] Salt solution, amino acid solution, and an appropriate volume of distilled water were autoclaved, cooled, and neutralized with 5.5 M NaOH; remaining solutions were then added aseptically.
[b] Prepared as 10× stock.
[c] Prepared as 10× stock; sterilized by filtration.
[d] Prepared as 2× stock.
[e] Prepared as 100× stock; sterilized by filtration.
[f] Prepared as 100× stock; sterilized by autoclaving.

MATERIALS AND METHODS

Bacteria and cultivation. Vwa^+ and isogenic Vwa^- derivatives of nonpigmented (13) but potentially virulent (14) *Y. pestis* strains EV76, KIM, G32, MP6, and 2C were maintained and cultivated as previously described (34). Unless stated otherwise, cells of EV76 were used in the experiments described.

Media. Organisms removed from slopes of blood agar base (Difco Laboratories, Detroit, Mich.) in 0.033 M potassium phosphate, pH 7.0 (phosphate buffer), were used to inoculate initial cultures of a further modification (Table 1) of the defined liquid medium of Higuchi et al. (11). The major change from a version of this medium used previously (34) was reduction of the K_2HPO_4 concentration and replacement of the resulting lost buffer capacity with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES), thereby permitting addition of both 4.0 mM Ca^{2+} and 20 mM Mg^{2+} without precipitation. D-xylose was also omitted since this carbohydrate was not significantly catabolized until D-gluconate was exhausted.

Sufficient volume of an initial culture was used to directly inoculate experimental cultures at an optical density (O.D.) of 0.1 (620 nm); this concentration, measured with a model 2000 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio), corresponded to about 10^8 cells per ml. Growth was similarly monitored by using samples of culture diluted appropriately in phosphate buffer. Experimental cultures varied with respect to initial pH (which, by using D-gluconate as a source of energy, remained constant for 12 to 16 h), concentration of Mg^{2+} (1.25 to 20 mM), addition (2.5 to 4.0 mM) or absence of Ca^{2+}, and presence at various concentrations of exogenous nucleotides, nucleosides, free bases, or chelators.

Purification of [8-^{14}C]ATP. Before the incorporation studies described herein, [8-^{14}C]ATP purchased from Amersham/Searle Corp. (Arlington Heights, Ill.) was purified by paper electrophoresis at 1,500 V for 1.5 h in 0.5 M sodium acetate buffer, pH 3.5. This step was necessary to remove a minor radioactive contaminant that underwent incorporation into cellular trichloroacetic acid-insoluble material.

Utilization of exogenous ATP. Samples of cultures (0.5 ml) containing 20 mM [8-^{14}C]ATP (2.5 μCi/mmol) were removed at intervals and immediately added to 0.5 ml of cold 10% trichloroacetic acid. Precipitated material was collected by passage through 0.22-μm pore size membrane filters (Millipore Corp., Bedford, Mass.) after storage in an ice bath for 30 min. After drying, the filters were placed in vials containing 10 ml of ACS (Amersham). Radioactivity was measured with a model LS7500 Beckman liquid scintillation spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

In separate experiments, cells were cultivated in medium supplemented with 20 mM ATP. At intervals, samples were removed and filtered (Millipore; 0.22-μm pore size) and diluted appropriately before determination of ATP as previously described (34).

Determination of V antigen. At appropriate intervals, cells from parallel cultures (25 ml of medium per 250-ml flask) were centrifuged (10,000 × *g* for 30 min), washed once in phosphate buffer, and then suspended in 1.0 ml of 0.05 M Tris buffer (pH 7.8). Bacteria in these samples were then disrupted for 1 min with a sonic probe (MSE Instruments, Ltd., London, England), and cellular debris was removed by centrifugation (10,000 × *g* for 30 min). The resulting cell-free extracts (containing at least 1.5 mg of protein per ml) were diluted linearly in phosphate buffer; these preparations were assayed by gel diffusion as described by Lawton et al. (18) except that wells contained about 10 μl of reagent. Antiserum against V antigen was prepared as described by these workers. Protein was determined by the method of Lowry et al. (19) with bovine serum albumin as a standard.

RESULTS

The primary observation leading to this investigation was the finding that exogenous ATP

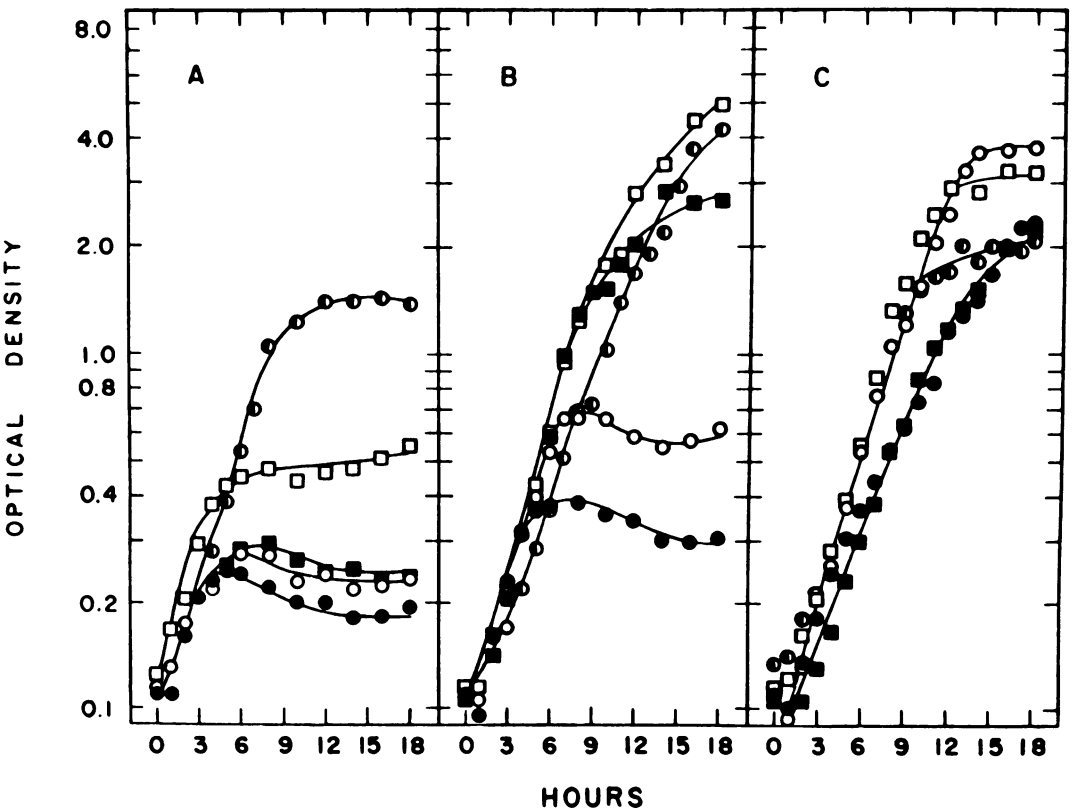

FIG. 1. Effect of pH on growth of *Y. pestis* strain EV76 at 37°C in medium containing 2.5 mM Mg^{2+} without supplement (A), with 20 mM ATP (B), or with 2.5 mM Ca^{2+} (C). Cells previously cultured at pH 7.0 (26°C) served to inoculate media adjusted to pH 7.0 (●), 7.3 (○), 7.5 (■), 7.8 (□), or 8.0 (◐).

could replace Ca^{2+} as a temperature-dependent growth factor of Vwa^+ *Y. pestis* strain EV76. This effect was strongly influenced by pH and concentration of added Mg^{2+}.

Effect of pH. In Ca^{2+}-deficient medium containing 2.5 mM Mg^{2+}, alkaline conditions (pH 7.5 to 8.0) favored growth with 20 mM ATP (Fig. 1B); elevated pH was not necessary to stimulate multiplication by addition of 2.5 mM Ca^{2+} (Fig. 1C). Significant cell division in the absence of added ATP or Ca^{2+} only occurred at pH 8.0 (Fig. 1A) to 8.5 (not illustrated).

Concentration of Mg^{2+} and ATP. In Ca^{2+}-deficient medium at alkaline pH, Vwa^+ cells exhibited full scale growth in the presence of 20 mM ATP with low levels (1.25 to 5.0 mM) of Mg^{2+} (Fig. 2). Either a further increase in molarity of Mg^{2+} or a decrease in concentration of ATP resulted in premature cessation of growth.

Utilization of exogenous ATP. Organisms previously cultivated with radioactive ATP were grown with the compound for about four generations at alkaline pH in Ca^{2+}-deficient medium containing 2.5 mM Mg^{2+}. Initial trichloroacetic acid-precipitable radioactivity was not significant (79 cpm/ml) and did not vary significantly in samples removed thereafter at hourly intervals. This finding indicates that the intact molecule was not transported, in which case the rate of adenine incorporation into nucleic acids would have paralleled or at least approached that observed for generation of cell mass.

To determine whether exogenous ATP was hydrolyzed by the organisms, its extracellular concentration was assayed enzymatically. No significant decrease was detected over a period of time sufficient to permit four doublings. These results indicate that exogenous ATP, and therefore probably other stimulatory nucleotides (see below), were neither transported nor degraded.

Nucleotide specificity. The ability of various ribonucleotides to promote growth of Vwa^+ cells at 37°C with 2.5 mM Mg^{2+} in Ca^{2+}-deficient medium is shown in Table 2. Only ATP and GTP significantly stimulated cells of strain EV76 under these otherwise restrictive conditions. In contrast, all tested nucleotides, especially nucleoside triphosphates, enhanced multiplication of strain KIM. Sodium pyrophosphate slightly increased the yield of cells over those of control

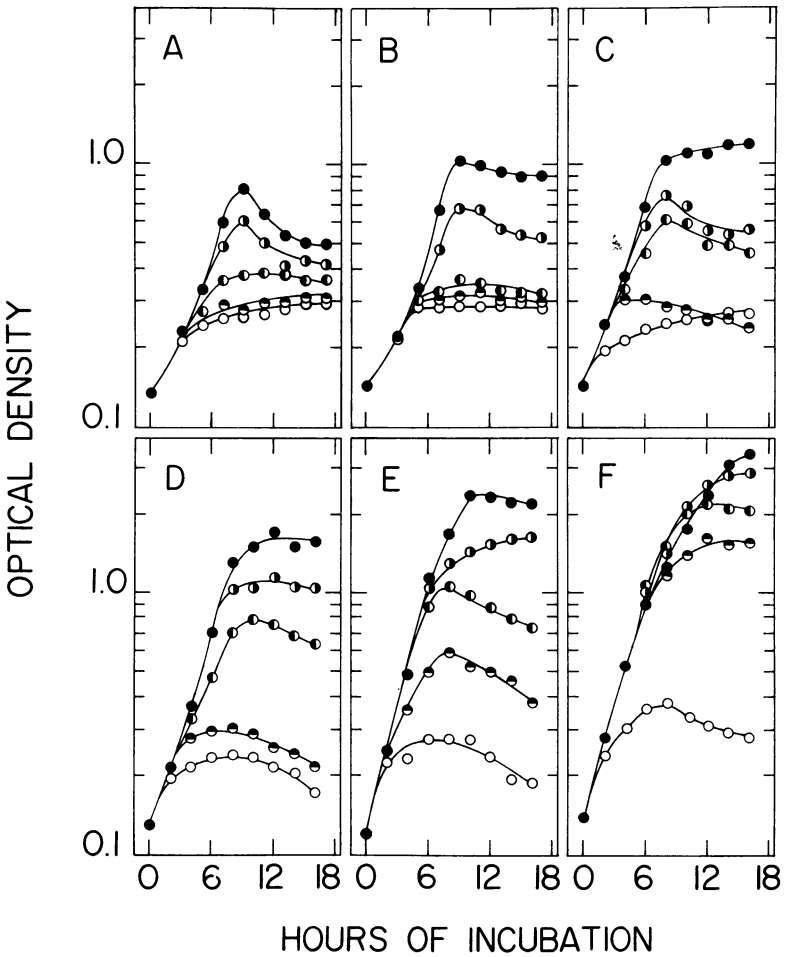

FIG. 2. Growth of Vwa$^+$ *Y. pestis* strain EV76 at 37°C in Ca^{2+}-deficient medium, pH 7.8, containing none (A), 1.25 mM (B), 2.5 mM (C), 5.0 mM (D), 10 mM (E), or 20 mM (F) exogenous ATP. Concentrations of Mg^{2+} were 1.25 mM (●), 2.5 mM (◑), 5.0 mM (◐), 10 mM (◓), or 20 mM (○).

cultures lacking the compound. However, this effect was not observed consistently, and the rate of growth obtained in pyrophosphate-supplemented cultures was always slower than that observed in those containing ATP. Equimolar or saturating concentrations of ribosides or free bases failed to support significant growth, and the chelating agents tested, including 0.001 to 20 mM levels of EDTA, were either ineffective or toxic.

To determine whether ATP-dependent growth in Ca^{2+}-deficient medium is a general phenomenon, cells of additional strains of *Y. pestis* were incubated with and without the nucleotide at neutral and alkaline pH in the presence of 2.5 or 20 mM Mg^{2+}. The response of these isolates was more similar to that of strain KIM than EV76 in that growth with 20 mM Mg^{2+} and equimolar ATP at alkaline pH was generally sustained. In fact, after 16 h of cultivation under these conditions at neutral pH, the O.D. of cultures inoculated with cells of strain KIM, G32, MP6, and 2C often exceeded 2 or 3.

Virulence antigen biosynthesis. The kinetics of V antigen biosynthesis were determined in cells of strain KIM shifted from 26 to 37°C at neutral pH with 20 mM Mg^{2+} and no added Ca^{2+} (28, 29). The antigen was first observed 1 h after shift (Fig. 3), and its titer rose sharply for another 2 h, at which time cell division ceased. The titer remained constant thereafter. V was not observed in cells growing at 37°C with added 2.5 mM Ca^{2+}, but low levels were synthesized after such organisms entered the stationary phase. Maximum specific activity in restricted cells was about 10 U per mg of protein, a value that was nearly 25 times greater than that observed after full-scale growth with 2.5 mM Ca^{2+}. Extracellu-

TABLE 2. Effect of various ribonucleotides on the growth of Vwa$^+$ isolates of *Y. pestis* at 37°C[a]

Supplement[b]	Strain EV76		Strain KIM	
	Maximum O.D.	R[c]	Maximum O.D.	R[c]
None	0.300		0.375	
ATP	5.800	19.3	4.400	11.7
GTP	6.600	22.0	3.600	9.6
CTP	0.920	3.1	4.700	12.5
UTP	0.460	1.5	4.800	12.8
ADP	0.900	3.0	3.600	9.6
GDP	1.100	3.7	4.500	12.0
CDP	0.460	1.5	2.000	5.3
UDP	0.840	2.8	4.000	10.7
AMP	0.290	1.0	2.700	7.2
GMP	1.100	3.7	4.200	11.7
CMP	0.760	2.5	2.600	6.9
UMP	0.500	1.7	3.000	8.0
PP[d]	0.900	3.0	1.400	3.7
Ca^{2+}	4.600	15.3	4.900	13.1

[a] Organisms were inoculated at an O.D. (620 nm) of 0.1; maximum O.D. recorded between 12 and 24 h of subsequent incubation.

[b] Ca^{2+} was 2.5 mM, and remaining compounds were 20 mM.

[c] R, Ratio of maximum O.D. plus nucleotide to that of control.

[d] PP, Sodium pyrophosphate.

lar V antigen was not detected in these experiments.

Cells of strains KIM and EV76 were grown in Ca^{2+}-deficient medium at neutral and alkaline pH with 20 mM Mg^{2+} in the absence and presence of exogenous ATP (20 mM). After 6 h of growth at 37°C, extracts of the cultures were prepared and assayed for V as performed previously. In both organisms, addition of ATP or increase in pH reduced the specific activity of the antigen (Table 3); the inhibitory effect of both ATP and alkaline pH on V synthesis by strain KIM was dramatic. Similar experiments performed with cells grown with 2.5 mM Mg^{2+}, either with or without added ATP, revealed only trace levels of V antigen (0.2 to 0.5 U per mg of protein) during logarithmic growth.

Ca^{2+} and ATP antagonism. Since added ATP was not metabolized but rather, like Ca^{2+}, appeared to reduce production of V and presumably W, we considered the possibility that exogenous nucleotides simply removed sufficient Mg^{2+} from solution to prevent full induction of these antigens, thereby indirectly preventing restriction. To determine the effect of similar chelation of Ca^{2+}, cells of strain EV76 were grown with 2.5 mM Ca^{2+} and Mg^{2+} in the presence of increasing concentrations of exogenous ATP. Mixtures of Ca^{2+} and ATP at levels sufficient to individually promote growth were restrictive (not illustrated). This result probably reflects preferential formation of the Ca-ATP chelate without removal of sufficient Mg^{2+} to prevent induction of sufficient concentrations of virulence antigens to mediate restriction.

DISCUSSION

Although transport of nucleoside triphosphates has only been demonstrated for a few obligate intracellular parasites (10, 32), exogenous nucleotides are known to influence the metabolism of a variety of bacteria (8, 15, 27, 35). Results of the present study showed that added ATP favored growth of Vwa$^+$ *Y. pestis* at 37°C by removing its temperature-dependent requirement for Ca^{2+}. The nature of this requirement is unknown although the consequences of Ca^{2+} deficiency, especially in the presence of excess Mg^{2+}, involve profound changes of normal metabolic patterns (7, 34). *Y. pestis* did not transport or otherwise catabolize significant ATP in Ca^{2+}-deficient medium. Some other tested nucleotides, including low energy AMP, could also stimulate Ca^{2+}-independent growth of strain KIM but not EV76. Accordingly, it is

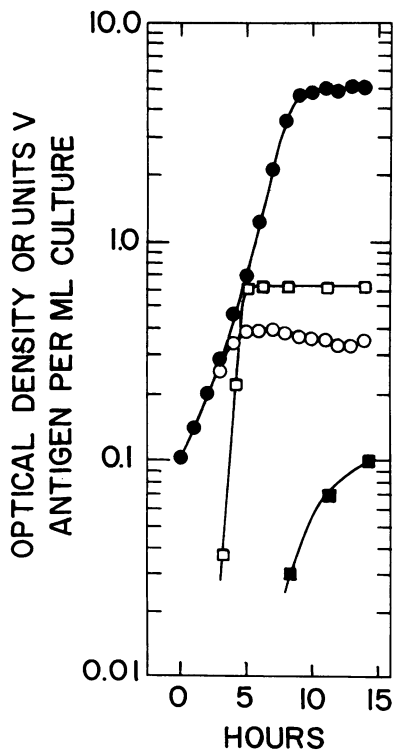

FIG. 3. O.D. (●) and titer of V antigen (■) during growth of *Y. pestis* strain KIM with 4.0 mM Ca^{2+} and O.D. (○) and titer of V antigen (□) during growth without added Ca^{2+}; cultures contained 20 mM Mg^{2+} and were shifted from 26 to 37°C at an O.D. of 0.2.

TABLE 3. Specific activity of V antigen in yersiniae 6 h after shift from 26 to 37°C in Ca^{2+}-deficient medium containing 20 mM Mg^{2+}

Strain	Added ATP (20 mM)[a]	pH	V antigen (U/mg protein)
EV 76	0	7.0	9.4
	+	7.0	4.4
	0	7.8	8.4
	+	7.8	4.0
KIM	0	7.0	11.7
	+	7.0	5.5
	0	7.8	4.3
	+	7.8	0.6

[a] 0, No ATP added; +, ATP added.

unlikely that exogenous ATP served any unique bioenergetic function by directly elevating the adenylate energy charge or by energizing the cytoplasmic membrane from without. A search for an alternative mode of action was therefore initiated. In addition to the presence of Ca^{2+} and nucleotides, other variables considered were pH, concentration of Mg^{2+}, and expression of V antigen.

Effects of pH were determined because Ogg et al. (23) showed that virulent cells grow normally in undefined medium at alkaline pH but were overgrown at neutral pH by avirulent mutants, now known to be Vwa^-. These workers also noted that at neutral pH an unidentified product excreted into spent medium could prevent the population shift to avirulence. By using chemically defined Ca^{2+}-deficient medium, we observed that significant but not full-scale growth of Vwa^+ cells of strain EV76 incubated at 37°C occurred at alkaline pH with added 2.5 mM Mg^{2+}, a concentration insufficient for maximum induction of V and W. However, nearly full-scale growth without Ca^{2+} did occur at alkaline pH with added 20 mM ATP. Significant multiplication of other isolates, typified by strain KIM, was observed in Ca^{2+}-deficient medium at neutral pH in the presence of 20 mM Mg^{2+} plus equimolar ATP.

This finding would be expected if exogenous nucleotides promoted growth by removing sufficient Mg^{2+} from solution to prevent full induction of V and W with attendant restriction. In favor of this notion is the fact that ATP is an efficient chelator of Mg^{2+}, Ca^{2+}, and other metallic cations. Indeed, the majority of ATP in intact mammalian cells is complexed with Mg^{2+}, and formation of both natural and artificial Mg complexes is favored by alkaline pH (9, 12, 21, 22, 26).

To test the possibility that exogenous ATP might promote growth by removing inducing levels of Mg^{2+}, we determined the specific activity of V antigen in cells grown at neutral and alkaline pH, with and without added nucleotide. With strain KIM, cultivation with ATP, especially at alkaline pH, resulted in marked reduction of biosynthesis. Findings with strain EV76 were less dramatic but in accord with the concept that ATP serves to promote growth by sequestering excess Mg^{2+}. Inconsistent with this hypothesis was the finding that certain nucleotides incapable of binding Mg^{2+} as effectively as ATP (9, 12, 26) also supported Ca^{2+}-independent growth. This observation suggests that these compounds may serve in some other capacity, possibly as biological effectors as does Ca^{2+}. Alternatively, inefficient chelators such as AMP might increase the rate of excretion of effective natural ligands. In any event, the phenomenon described herein provides a possible explanation for the role of alkaline pH on growth of Vwa^+ cells and suggests that nucleotides, either excreted or exogenous, may account for the dialyzable heat-labile stimulatory nutritional activity described by Ogg et al. (23). Not yet resolved is the atypical response of strain EV76 to the presence of exogenous UTP and CTP. Many substrains of this isolate, but not that used here, possess a nutritional requirement for pyrimidines (3). However, Vwa^+ cells of our strain exhibit other unique temperature-dependent properties including a nutritional requirement for fermentable carbohydrate (unpublished observations). Results of further studies may equate this curious lesion with evident toxicity of exogenous UTP and CTP.

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