This is to certify that the

thesis entitled

REPLACEMENT OF Ca^{2-} BY EXOGENOUS NUCLEOTIDES AS A GROWTH FACTOR FOR YERSINIA PESTIS AT 37 C

presented by

Robert John Zahorchak

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

Robert M Brubaker
Major professor

Date March 1, 1979

O-7639

REPLACEMENT OF Ca^{2+} BY EXOGENOUS NUCLEOTIDES AS A GROWTH FACTOR FOR *YERSINIA PESTIS* AT 37 C

By

Robert John Zahorchak

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1978

ABSTRACT

REPLACEMENT OF Ca^{2+} BY EXOGENOUS NUCLEOTIDES
AS A GROWTH FACTOR FOR *YERSINIA PESTIS*
AT 37 C

By

Robert John Zahorchak

Cells of virulent *Y. pestis*, a facultative intracellular parasite, require physiological levels of Ca^{2+} (2.5 mM) for sustained growth *in vitro* at 37 C. This concentration of Ca^{2+} is not found in mammalian intracellular fluid. In the restrictive environment (no Ca^{2+}) the virulence antigens, V and W, which are essential for successful invasion of the host, are expressed. Exogenous ATP allowed for a energy charge in Ca^{2+}-starved and Ca^{2+}-supplemented cells was determined as 0.57-0.67 for the former and 0.75-0.84 for the latter. Exogenous ATP allowed for a growth response equivalent to that of a Ca^{2+}-sufficient control culture. The growth response was dependent upon the concentration of ATP and Mg^{2+} as well as the pH of the culture medium. Other 5'-ribonucleotides that could replace Ca^{2+} were ADP, AMP, GTP, GDP, and CTP. The addition of UMP, CMP, or common free bases or ribonucleosides failed to promote growth. An intermediate response was obtained with UTP, UDP, CDP, and GMP. Cells grown in the presence of [8-^{14}C] ATP did not incorporate the label into TCA-precipitable material. Cells cultivated in Ca^{2+}-supplemented media failed to express the virulence antigen.

However, the specific activity of V antigen measured in extracts of cells grown in the presence of exogenous ATP was similar to that observed in Ca^{2+}-starved non-growing cells. These findings suggest that certain nucleotides may serve a regulatory role, allowing for both the growth of Y. pestis and expression of the virulence antigens, in the intracellular environment of the host.

ACKNOWLEDGMENTS

I am grateful to Dr. R. N. Costilow and Dr. H. L. Sadoff for the use of their laboratory equipment. I wish to express my appreciation for the technical assistance of Janet Fowler. The suggestions openly offered by Dr. R. J. Patterson, Dr. L. R. Snyder, and Dr. N. Band have been very helpful. The advice and guidance of my major professor, Dr. R. R. Brubaker, have been a great asset to my education. For this I wish to extend my sincere appreciation.

ORGANIZATION

This thesis is organized in such a way as to fulfill the departmental requirement for a publishable manuscript as well as to provide the reader with background material helpful in understanding the implications of the results presented. Section I consists of a review of the literature pertinent to the research discussed. Section II is a manuscript prepared to send to internal review and subsequently to Current Microbiology. The third section consists of data obtained that is related to that presented in Section II. A more complete discussion of the results obtained is also presented in Section III. An appendix consisting of a detailed description of the methods used to determine the adenylate energy charge is also included.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SECTION I

LITERATURE REVIEW

Virulence and Yersinia pestis

The plague has been of historical significance to man possibly since as early as 1320 B.C. (61). Epidemics and pandemics of this disease have been responsible for the deaths of large percentages of the human population. Killing 25 to 40 million people, the Black Death of 1347 A.D. - 1350 A.D. was one of the most disastrous of these (65). In modern times the institution of public health practices and the use of insecticides have been largely responsible for the containment of plague. Even though epidemics are rare, the disease is endemic in the rodent population in many areas of the world (4). The fact that the disease is still prominent, although not overtly manifest, maintains the scientific concern to understand plague.

The bacteria that causes the bubonic plague, Yersinia pestis, easily lends itself to scientific investigation of the pathogenic process. The ease with which it is cultivated in the laboratory has helped to establish the organism as a model system to understand infectious disease in general as well as to define specifically the variables of significance in the pathogenesis of plague. The results of investigations into various facets of the plague comprise a considerable amount of material that has been reviewed extensively

(8,17,18,20,61). This literature review is written in order to present those findings of significance in understanding the physiology of Y. pestis and its relationship to the disease caused by the organism.

The etiologic agent of bubonic plague was discovered in 1894 by Alexander Yersin (77). The gram-negative bacillus was subsequently classified as Pasturella pestis (29). Recently, the organism has been reclassified and a new genus, Yersinia, in honor or the discoverer, was defined (62). The genus is composed of three recognized species, Y. pestis, Y. pseudotuberculosis, and Y. entercolitica. A fourth member, Y. ruckeri, has been nominated (31).

These bacteria have been of scientific interest, mainly to medical bacteriologists, because of their pathogenic properties. Organisms of the three accepted species are all facultative intracellular parasites. It is of interest to note that all of these organisms appeared to prefer the fixed macrophages of the mesenteric lymph nodes, liver, and spleen as the host-cell of choice to invade (44,77).

The extreme potential for wild-type Y. pestis to infect and disease laboratory animals has been documented (23). The average lethal dose necessary to kill fifty percent of test animals ($L.D._{50}$) was observed to be less than ten organisms for both guinea pigs and mice when wild-type Y. pestis was injected by the intraperitoneal route.

Three pathologically distinct forms of plague have been defined: pneumonic, bubonic, and septicemic. Each form of the

disease is acute and the mortality is very high in untreated cases (29). Pneumonic plague, the most acute, results from infection of the lungs by Y. pestis. This form of plague is transmitted from person to person by aerosol. The most common form of plague, bubonic, is transmitted from rodents to man via an insect vector, the flea. Septicemic plague may be merely a manifestation of either pneumonic or bubonic plague where the bloodstream is invaded before the classic clinical symptoms have surfaced.

Much research has been done in order to identify the specific properties of Y. pestis that correlate with the disease producing capabilities of the organism. To date, five phenotypic characteristics have been demonstrated to be directly associated with the pathogenicity of Y. pestis; pigmentation (Pgm), fraction I antigen (Fra), purine independence (Pur), pesticin I (Pst), and the V and W antigens (Vwa). These five virulence determinants have been discussed in detail by Burrows (17) and Brubaker (8). A brief description of each follows.

Wild-type Y. pestis (Pgm^+) formed pigmented colonies on the hemin agar of Jackson and Burrows (42) and on the congo red agar of Surgalla and Beesley (69). Mutation resulting in the non-pigmented phenotype (Pgm^-) occurred at a spontaneous rate of about 10^{-5} mutations per bacterium per generation (10). An $L.D._{50}$ of greater than 10^7 was observed when mice were experimentally infected with non-pigmented mutants (43).

Fra^- cells were unable to synthesize the fraction I or capsular antigen and were decreased in virulence for guinea pigs

($L.D._{50}$ of 10^3 to 10^6) but were fully virulent for mice (23). The possibility that the fraction I antigen rendered Y. pestis less susceptible to phagocytosis by polymorphonuclear leukocytes, thereby enhancing the invasiveness of the bacteria, has been investigated by Janssen et al. (45). These investigators showed that there was a marked difference in resistance to phagocytosis among the various strains of Y. pestis. They correlated resistance to phagocytosis with the presence of a visible capsule (fraction I).

It has been demonstrated that purine auxotrophs of Y. pestis (Pur^-) were avirulent (16). Brubaker (11) showed that mutation eliminating guanosine monophosphate synthetase activity increased the intraperitoneal lethal dose in mice to greater than 10^7, whereas mutation affecting the enzymes necessary for the synthesis of inosine monophosphate lowered the virulence of the bacteria only slightly ($L.D._{50}$ of approximately 10^2).

Virulent Y. pestis synthesize a bacteriocin that is active against certain strains of Escherchia coli, Y. pseudotuberculosis, and Y. enterocolitica (8). The role of pesticin in disease production by Y. pestis is obscure but it may be significant that the synthesis of the protein (41) is correlated with that of coagulase and fibrinolysin (7). With respect to the latter point it is important to state that, although non-pesticinogenic mutants (Pst^-) appeared to be avirulent when introduced by the intraperitoneal route, wild-type virulence was observed when the same mutants were injected intravenously into mice (12,14).

Cells of Y. pestis and Y. pseudotuberculosis must possess the ability to express two antigens, V and W, in order to be fully virulent (15,19,22). Mutants that did not possess this characteristic exhibited a mouse intraperitoneal $L.D._{50}$ of greater than 10^7 (23). Vwa^+ cells also displayed a requirement for relatively high concentrations of calcium ions (2.5 mM) as a growth factor at 37 C but not at 26 C (39). The expression of V and W antigens and the Ca^{2+} requirement will be discussed in detail in a later section of this review.

Y. pestis rendered avirulent by the mutational loss of any of the first four determinants defined above, have been shown to exhibit wild-type virulence under certain conditions. Pgm^- and Pur^- mutants exhibited wild-type virulence if the challenge dose was accompanied by an injection of iron or purine, respectively (16, 43). It was suggested that these mutants were avirulent due to their inability to procure sufficient iron or purine for growth inside the host. If, however, the concentrations of these compounds were artificially increased, the invading bacteria would no longer be nutritionally deprived and would cause disease as easily as wild-type Y. pestis. As already mentioned, Fra^- cells were observed to be fully virulent in mice (23) and wild-type virulence was observed with intravenously injected Pst^- Y. pestis (12). To date there is no known manipulation of either the host or the bacteria that results in Vwa^- Y. pestis exhibiting $L.D._{50}$ values similar to those observed with wild-type cells. The expression of the virulence antigens

appears to be essential for a successful invasion of the host by Y. pestis.

Burrows (15) and Burrows and Bacon (22) discovered two antigens, V and W, in cultures of phagocytosis-resistant virulent Y. pestis. The V and W antigens were undetectable in phagocytosis-sensitive avirulent cells. It had already been documented that there was a strong correlation between the disease producing ability of these bacteria and their ability to resist phagocytosis (16,22). It was suggested therefore that the role of the antigens with respect to virulence was to render Y. pestis resistant to phagocytosis.

The results obtained by Janssen et al. (45) did not support this hypothesis. Their observations that fixed macrophages phagocytized virulent cells as readily as avirulent cells led them to suggest that the V and W antigens were important to the survival and multiplication of the bacteria after they had been phagocytized by fixed macrophages. The virulent cells were detected in the bloodstream much earlier than were the avirulent cells (44). Attempts to demonstrate a difference between Vwa^+ and Vwa^- Y. pestis with respect to their ability to survive and multiply within macrophages in cell culture have indicated that, in these systems, there is no significant difference (46).

The expression of the antigens is a temperature dependent phenomenon. V and W were detected in cultures of Y. pestis that were incubated at 37 C but not in those at 26 C (15,22). Lawton et al. (50) noted that 20 mM Mg^{2+} enhanced the expression of the antigens and they were able to purify V antigen 100 fold and W

antigen 1000 fold. Analysis of these preparations resulted in identifying V as a protein of 90,000 daltons and W as a lipoprotein of 145,000 daltons. The mechanism by which the expression of the antigens is regulated is not known. These same authors demonstrated that antibodies to V antigen could protect mice from experimental plague (50). This observation further indicated the importance of the virulence antigens in the pathogenesis of plague.

Vwa^+ Y. pestis displayed a requirement for at least 2.5 mM Ca^{2+} (replaceable by Sr^{2+} or An^{2+}) when cultivated in a defined medium at 37 C (38,39). Vwa^- mutants did not require Ca^{2+}. Cells of neither phenotype require the cation at 26 C.

The mutation from Vwa^+ to Vwa^- occurred at a spontaneous rate of 10^{-4} (39) and appeared to be irreversible (21). These observations implied the presence of a plasmid that carries the genetic information necessary for Y. pestis to express the virulence antigens. No plasmid has been identified by cesium chloride-ethidium bromide density gradient methodology (52). More recently, however, analysis of gently prepared cell extracts by agarose gel electrophoresis revealed the presence of plasmids in Y. pestis (D. Ferber, personal communication). None of these plasmids as yet have been correlated with the Vwa or any other virulence determinant.

In vitro studies performed in order to ascertain the effect of Ca^{2+} on Vwa^+ Y. pestis cultivated at 37 C revealed that Ca^{2+}-starved cells were morphologically and physiologically different from cells cultivated at 26 C or at 37 C in Ca^{2+}-supplemented media (35,51,76). Ca^{2+} starvation resulted in elongated cells with a

diffuse nucleoid (35). DNA synthesis and cell growth ceased after a time that allowed two doublings in mass (76). Significantly, it was only under these "restrictive" conditions that V and W were expressed *in vivo* (13).

Brubaker (9) compared the *in vitro* Ca^{2+}-deficient environment with that of the intracellular environment of the phagocytic cell and suggested that this ion may regulate the expression of V and W *in vivo*. The conflicting observations that Ca^{2+}-starved *Y. pestis* cells cannot multiply *in vitro* at 37 C while they proliferate in the essentially Ca^{2+}-free intracellular environment *in vivo* have not been resolved.

Bacterial Cell Growth and the Adenylate Energy Charge

The ability of a bacterial cell to utilize various compounds as energy sources and/or substrates for the components of the cell is tightly coupled to the growth rate of that cell. Some of the mechanisms by which enzymes involved in macromolecular synthesis are regulated have been elucidated by nutritional shift or growth state transition experiments. When a bacterial cell such as an *E. coli* is starved for an essential nutrient such as a nitrogen or a carbon source, dramatic effects on macromolecular synthesis are observed. Classically, net RNA and protein synthesis cease. This is followed sequentially by the cessation of DNA synthesis and cell division (55).

It has recently been shown that one of the earliest defined events observed in nitrogen-starved *E. coli* was a drastic change in

nucleotide pools (73). The cytoplasmic concentration of all ribonucleoside and deoxyribonucleoside triphosphates dropped before net accumulation of RNA and protein stopped. In addition, the concentration of ppGpp or magic spot I rose drastically and reached maximum levels after cell growth had ceased.

The nucleotides, ppGpp and pppGpp, accumulate in many bacteria as a result of amino-acid or ammonium ion starvation (24). Only the former nucleotide has been observed in cells starved for a carbon source (34,56). The concentration of the "common" nucleoside triphosphates (ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, and TTP) varies directly with the growth rate (58) unlike those of the magic spot compounds which vary inversely with growth rate (24).

Based upon experiments with various enzymes, Atkinson (2) proposed that the activities of energy-producing and energy-consuming enzymes in vitro are more influenced by the ratio of adenine nucleotides in the cell than by the absolute concentrations of these compounds. The development and definition of the adenylate energy charge theory has been presented (1,3). Briefly, the metabolic energy immediately available to a cell is reflected in the degree of phosphorylation of the adenylate pool. A linear measure of this parameter can be achieved by applying the equation:

$$\frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]} \quad (2).$$

The importance of this parameter in the whole cell has been investigated by Chapman et al. (27). For instance, it has been

shown that the value of the energy charge (EC) is generally above 0.8 for most growing organisms that have been studied (see compiled data in ref. 27). In dividing E. coli the EC appears to be stabilized around a value of 0.8 or higher regardless of the growth rate (70). The EC value observed for stationary phase cells slowly decreased from 0.8 to 0.5 over a period of about 60 h (27). During this time the viability of the cells remained constant. However, a decrease in viability was observed when the EC dropped below 0.5.

Variations in energy charge have been observed after nutrient deprivation of glucose-limited E. coli (27). Adenine limitation of an adenine auxotroph of E. coli resulted in a drop in EC value from 0.8-0.9 to 0.6-0.7 after growth had ceased (70). In the case of adenine limitation, the total adenylate pool fell to a relatively low level before any significant change in the EC value was observed. Investigations with other organisms, in general, support the concept that Atkinson originally presented.

The parameter is generally accepted as a measure of the metabolic state of a cell. Caution should be exercised, however, before applying hard-fast rules with respect to energy charge and bacterial cell growth. For instance, it has been reported that iron limitation of Candida utilis resulted in a low EC value (0.6). These cells, however, continued to grow (71). Furthermore, although it might be inferred from the above discussion that an extremely low EC value corresponds to cell death, it had been shown that freshly isolated Rickettsia typhi exhibit EC values of about 0.2-0.5 (74).

These cells were presumably viable based on results of metabolic activity and infectivity (74).

SECTION II

ARTICLE

REPLACEMENT OF Ca^{2+} BY EXOGENOUS NUCLEOTIDES AS A GROWTH FACTOR FOR *YERSINIA PESTIS* AT 37 C

By

R. J. Zahorchak and R. R. Brubaker

(Manuscript to be published)

ABSTRACT

REPLACEMENT OF Ca^{2+} BY EXOGENOUS NUCLEOTIDES AS A GROWTH FACTOR FOR *YERSINIA PESTIS* AT 37 C

By

Robert John Zahorchak

Cells of virulent *Y. pestis*, a facultative intracellular parasite, require physiological levels of Ca^{2+} (2.5 mM) for sustained growth *in vitro* at 37 C. This concentration of Ca^{2+} is not found in mammalian intracellular fluid. In this restrictive environment the virulence antigens, V and W, which are essential for successful invasion of the host, are expressed. Exogenous ATP allowed for a growth response equivalent to that of a Ca^{2+}-sufficient control culture. The growth response was dependent upon the concentration of ATP and Mg^{2+} as well as the pH of the culture medium. Other 5'-ribonucleotides that could replace Ca^{2+} were ADP, AMP, GTP, and CTP. The addition of UMP, CMP, or common free bases or ribonucleosides failed to promote growth. An intermediate response was obtained with UTP, UDP, CDP, and GMP. Cells grown in the presence of [8-^{14}C] ATP did not incorporate the label into TCA-precipitable material. Cells cultivated in Ca^{2+}-supplemented media failed to express the virulence antigen. However, the specific activity of V antigen measured in extracts of cells grown in the presence of

exogenous ATP was similar to that observed in Ca^{2+}-starved non-growing cells. These findings suggest that certain nucleotides may serve a regulatory role, allowing for both the growth of Y. pestis and expression of the virulence antigens, in the intracellular environment of the host.

INTRODUCTION

Yersinia pestis, the causative agent of bubonic plague, is a facultative intracellular parasite. Wild-type cells (Vwa^+) express two antigens, V and W, at 37 C [4,5]. These antigens were not detected in Vwa^+ cell cultures incubated at 26 C or in avirulent Vwa^- cell cultures. It has been suggested that these antigens are important to the survival and multiplication of Y. pestis within fixed macrophages [16].

Vwa^+ cells required at least 2.5 mM Ca^{2+} for sustained growth in a defined medium at 37 C whereas Vwa^- mutants did not require the cation [13]. Furthermore, Ca^{2+} inhibited the expression of the V and W antigens [3].

The response of growth and virulence antigen expression in vitro, with respect to the presence or absence of Ca^{2+}, led Brubaker [2] to postulate that the ion may serve as a signal indicating to the yersinial cell that it is in either an extracellular or an intracellular environment. The observation that Y. pestis did divide in the Ca^{2+}-limited intracellular environment of the phagocytic cell [16] indicates that some other intracellular molecule(s) may replace Ca^{2+} in allowing the sustained growth of these bacteria after phagocytosis.

Investigations defining the morphological and physiological consequences of Ca^{2+} starvation on Vwa^+ Y. pestis in vitro have

provided information helpful in understanding this phenomenon. Cells cultivated in a Ca^{2+}-free defined medium are elongated with a diffuse nucleoid [10]. Ca^{2+}-starved cells were unable to synthesize significant amounts of DNA [28]. Stable RNA and protein synthesis were also depressed (submitted for publication). Analysis of nucleotide pools in these cells revealed that the ribonucleoside but not the deoxyribonucleoside triphosphate pools were significantly lower than those observed in Vwa^{+} Y. pestis growing cells (submitted for publication). The adenylate energy charge in Ca^{2+}-starved Vwa^{+} Y. pestis cells was significantly lower than in growing cells (submitted for publication).

These latter results suggested that Ca^{2+}-starved Y. pestis may be unable to generate sufficient energy to maintain cell growth. The possibility that exogenous ATP may promote growth in a Ca^{2+}-deficient environment was therefore investigated. In this communication we present results indicating that exogenous ATP and other ribonucleotides promote both cell growth and the expression of V and W antigens in Ca^{2+}-deficient medium at 37 C. The significance of these results with respect to the survival and multiplication of Y. pestis in the intracellular environment of the fixed macrophage is discussed.

MATERIALS AND METHODS

Bacteria. The laboratory strains of Y. pestis, EV76 and KIM, were employed in the experiments presented. The organisms are both non-pigmented mutants and, therefore, are avirulent but still retain the potential to express the V and W antigens [15]. Stock suspensions of the bacteria were maintained at -20 C in glycerol-phosphate buffer as previously described [1].

Media. Bacteria were cultivated in the liquid synthetic medium described by Higuchi et al. [13] with the following modifications. It was observed that ammonium acetate and xylose were neither stimulatory nor necessary for growth at 37 C. Therefore, these compounds were not included. The L-isomers of the following amino acids were included: phenylalanine, 4.8 mM; tyrosine, 2.2 mM; threonine, 2.8 mM; methionine, 3.2 mM; isoleucine, 7.6 mM; valine, 13.6 mM; leucine, 4.0 mM; glutamic acid, 162.0 mM; proline, 14.0 mM; arginine, 2.0 mM; glycine 5.2 mM. D-L alanine was included at the final concentration of 9.0 mM. The final K_2HPO_4 concentration was 2.5 mM and buffering capacity was provided by the inclusion of N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) at a final concentration of 25 mM. The medium was adjusted to the desired pH with 5.5 N NaOH. The desired $MgCl_2$ and $CaCl_2$ concentrations were achieved by adding the appropriate amount of a sterile 100X stock solution of either of these salts. Nucleotides were prepared as 10X stock solutions in

25 mM HEPES buffer, adjusted to pH 7.8, and filter sterilized before addition to the growth medium.

Cultivation of bacteria. Cells were preliminarily cultivated on slopes of blood agar base (BBL, Cockeyville, MD) at 26 C for 48 h. The growth was then washed from the slope with freshly prepared sterile medium. This cell suspension was used directly to inoculate the liquid growth medium. Prior to all experiments, bacteria were cultivated in the growth medium for at least 12 h in order to eliminate possible nutritional shift complications. This primary culture was used directly as an inoculum for experimental cultures. Experimental cultures, consisting of medium in an Erlenmeyer flask (10% vol/vol), were inoculated at a cell density of approximately 1×10^8 cells per ml ($O.D._{620} = 0.1$). The cultures were aerated at the appropriate temperature by shaking at 200 rpm on a model G76 gyrotory water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ). Bacterial growth in the cultures was monitored by assessing the optical density of culture aliquots, appropriately diluted, with a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) at a wavelength of 620 nm.

Utilization of exogenous ATP. Cells were cultivated in the defined liquid medium supplemented with 20 mM ATP. At designated times aliquots were removed and filtered through a millipore filter (0.22 μm pore size). The filtrate was diluted appropriately and ATP was determined spectrophotometrically with hexokinase and glucose-6-phosphate dehydrogenase as previously described [21].

Incorporation of radioactive ATP. The rationale behind these experiments is based on the fact that if [8-^{14}C] ATP was transported into the cytoplasm of the bacterial cells, it would become available for RNA synthesis. Therefore, an increase in radioactivity in trichloroacetic acid (TCA) precipitable material over time would indicate uptake. [8-^{14}C] ATP (0.1 μCi/mmol) was added to a growing culture of Y. pestis EV76. Aliquots of 0.1 ml were removed at the designated times and immediately added to 10 ml of cold 5% TCA. The TCA precipitable material was collected on millipore filters (0.22 μm pore size) after incubation on ice for 30 minutes. After drying, the filters were placed in scintillation vials containing 10 ml of 0.4% 2,5-diphenyloxazole (PPO) and 0.005% 1,4-di-2(5-phenyloxazolyl)-benzene (dimethyl POPOP) in toluene. Radioactivity was measured on a Model 3320 Packard Tri-Carb Liquid Scintillation Spectrophotometer.

Immunological Methods. Anti-V serum was obtained essentially as previously described [22]. The quantitation of V antigen has also been described [22].

Protein determination. Protein was determined by the method of Lowry et al. [23] with bovine serum albumin as a standard.

Electrophoresis of [8-^{14}C] ATP. [8-^{14}C] ATP purchased from Amersham Corporation was further purified by paper electrophoresis at 1500 volts for 1.5 h in 0.5 M acetate buffer, pH 3.5, prior to the incorporation studies presented herein. This procedure was necessary in order to remove a minor unidentified contaminant that resulted in artifactual results.

Chemicals. All chemicals were of the highest purity available from commercial sources. Enzymes were purchased from Sigma Chemical Company, St. Louis, MO.

RESULTS

Replacement of Ca^{2+} by ATP as a growth factor at 37 C.

Because Ca^{2+}-starved cells had decreased ATP pools and energy charge value, the possibility that exogenous ATP could sustain cell growth at 37 C in the absence of Ca^{2+} was investigated. Figure 1 shows that the addition of 20 mM ATP to Ca^{2+}-deficient medium resulted in cell growth equivalent to that observed for a Ca^{2+}-sufficient control culture.

pH dependence. Preliminary experiments indicated that the growth of EV76 in the presence of exogenous ATP was strongly dependent on the pH of the medium at the time of inoculation. The effect of this variable was therefore investigated in order to define conditions for further experimentation.

The growth curves presented in Figure 2 indicate that slightly alkaline conditions favored growth in the presence of the nucleotide. The medium pH did not significantly affect the growth response in medium containing Ca^{2+}. At pH 8.0 the growth yield in Ca^{2+}-deficient medium was increased but was not equal to that observed in either Ca^{2+}- or ATP-supplemented cultures. Cultivation at a pH higher than 8.0 resulted in reduced growth yields with exogenous ATP compared to those obtained at pH 7.8 (data not shown). In all future experiments the medium was adjusted to pH 7.8. This allowed for a

FIGURE 1.--ATP effect.

Growth of Y. pestis strain EV76 at 37 C in the defined medium (see Materials and Methods), pH 7.8, containing 2.5 mM $MgCl_2$ and either 2.5 mM $CaCl_2$ (□), 20 mM ATP (●), or no supplement (○).

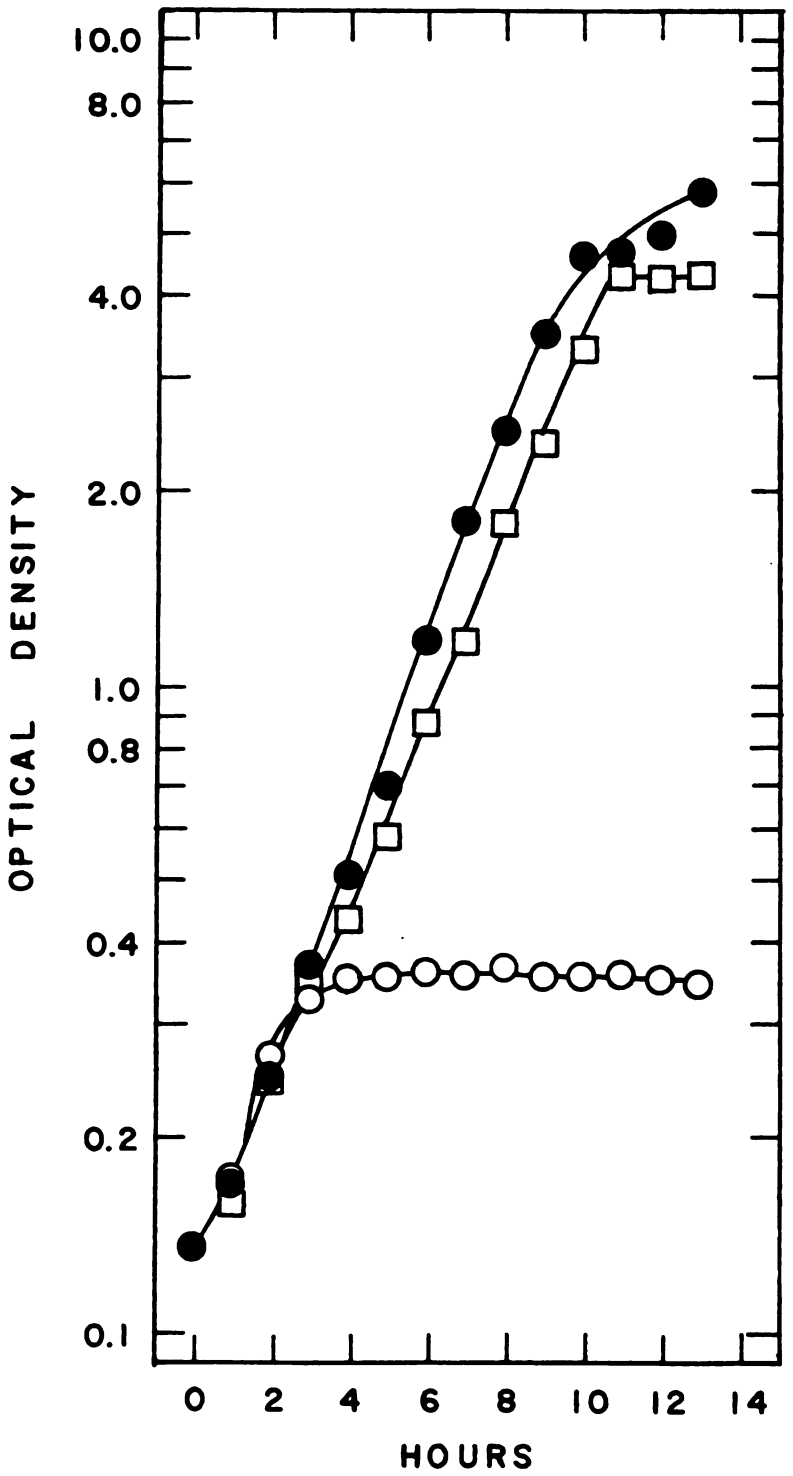

Figure 1.--ATP Effect.

FIGURE 2.--pH effect.

The effect of pH on the growth of Y. pestis strain EV76 in an unsupplemented medium (A) or medium containing either 20 mM ATP (B) or 2.5 mM $CaCl_2$ (C) as a supplement. Bacterial cells, previously cultured at pH 7.0, were used to inoculate media adjusted to either pH 7.0 (●), 7.3 (○), 7.5 (■), 7.8 (□), or 8.0 (◐). The cultures were then incubated with aeration at 37 C and the optical density was monitored. Cultures contained 2.5 mM $MgCl_2$.

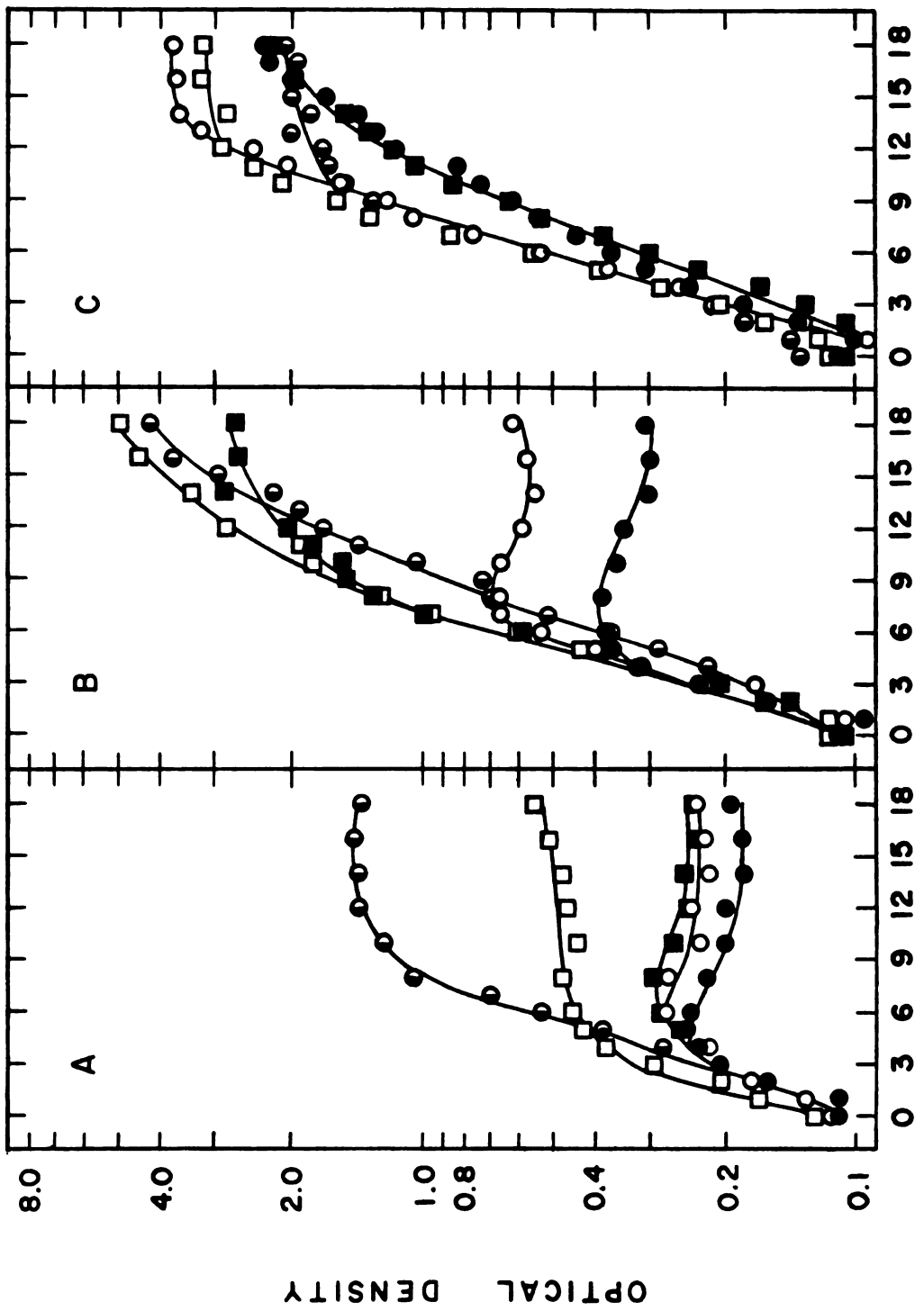

Figure 2.--pH Effect.

reproducible system in which to study the effect of ATP on Ca^{2+}-starved cells.

Concentration dependence. The growth response of Y. pestis was dependent upon the concentration of ATP present in the medium (Figure 3). At 10 and 20 mM ATP the cell growth was equal to or better than that observed in a Ca^{2+}-supplemented culture.

Utilization of exogenous ATP. As a direct method to determine whether the ATP added to the medium was utilized by the cells, the extracellular concentration of the nucleotide was enzymatically determined in a growing culture (see Materials and Methods). The results indicated that there was no measurable decrease in the extracellular concentration of ATP over a period of 10 h (Figure 4). During this time the cell mass in culture, determined as a function of optical density, doubled four times.

Uptake and incorporation of ATP. Additional evidence supporting the conclusion that exogenous ATP was not utilized by these cells was obtained by investigating the ability of Y. pestis to incorporate exogenous [8-^{14}C] ATP into TCA-precipitable material. The results of this experiment are presented in Table 1. No increase in the radioactivity in the TCA-precipitable material was observed over an 8 h period. During this time, cell mass increased almost ten-fold.

Specificity. It is known that Ca^{2+} could be replaced by Sr^{2+} or Zn^{2+} as a growth factor for Vwa^{+} Y. pestis [13]. In order to ascertain how specific the phenomenon was with respect to

FIGURE 3.--Dependence on ATP concentration.

Growth of Y. pestis strain EV76 at 37 C in a defined medium (see Materials and Methods), pH 7.8, containing exogenous ATP at a concentration of 20 mM (○), 10 mM (●), 5 mM (□), 2.5 mM (■), 1.25 mM (△), or 0.0 mM (▲). Cultures contained 2.5 mM $MgCl_2$.

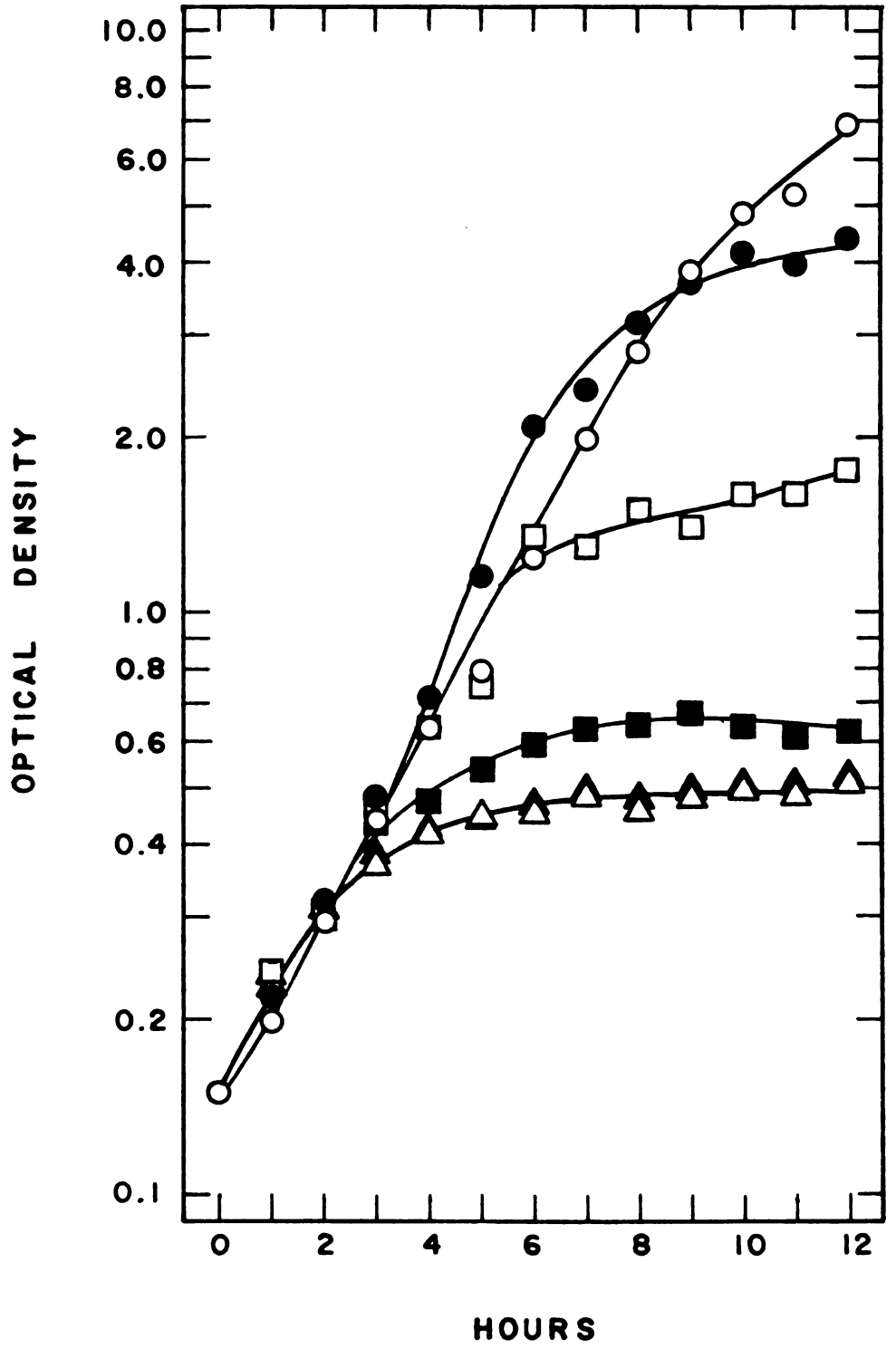

Figure 3.--Dependence on ATP Concentration.

FIGURE 4.--Inability of Y. pestis strain EV76 to utilize exogenous ATP.

Bacterial cells previously cultivated at 26 C in medium, pH 7.8, were used to inoculate the experimental cultures consisting of the same medium supplemented with 20 mM ATP. At the times indicated, samples were removed, filtered, and assayed for ATP as described in Materials and Methods (●). The optical density of the culture was monitored at 620 nm (○). Cultures contained 2.5 mM $MgCl_2$.

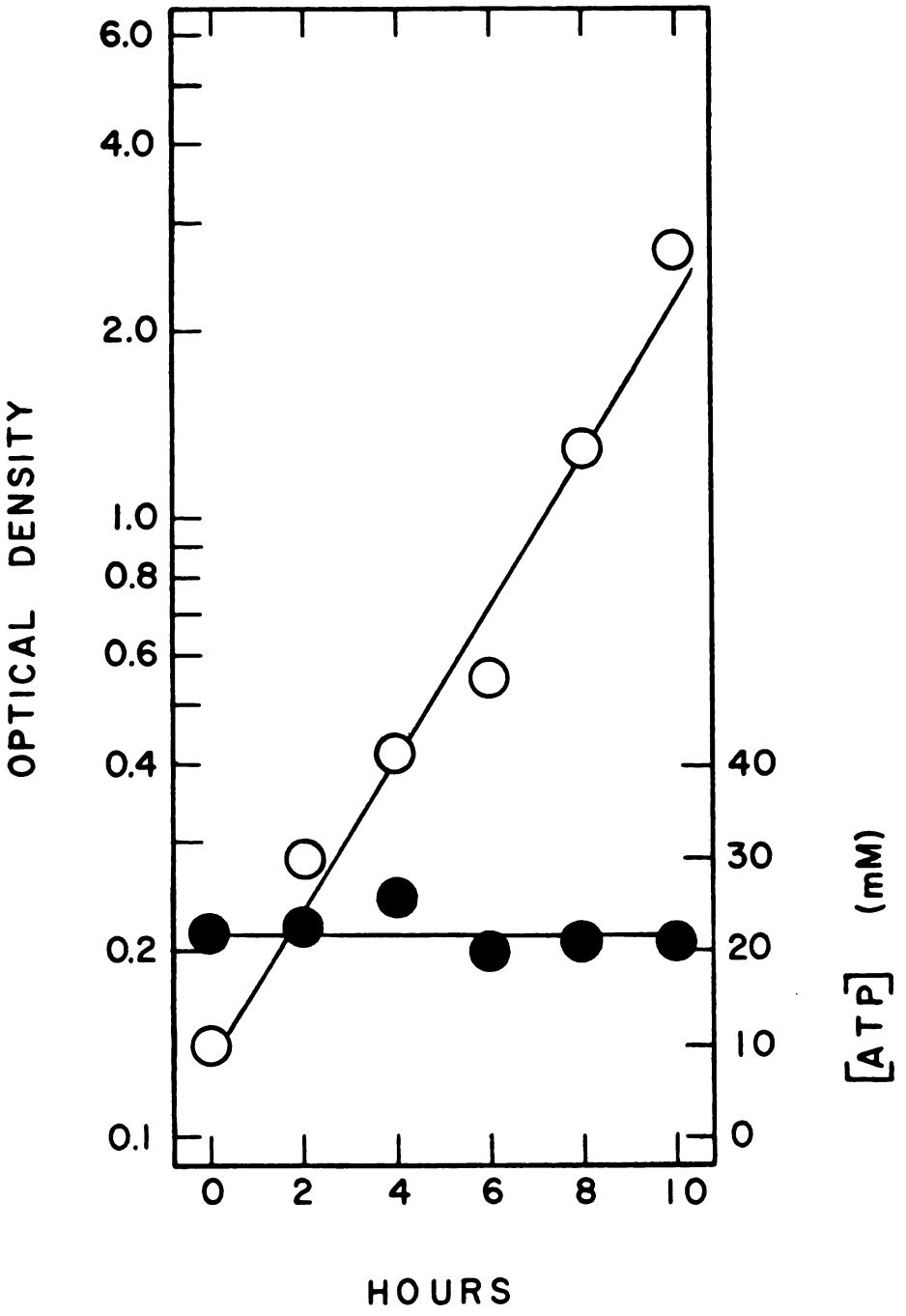

Figure 4.--Inability of Y. pestis strain EV76 to utilize exogenous ATP.

TABLE 1.--Inability of Y. pestis to incorporate exogenous [8-^{14}C] ATP.

Time (h)	Cultural Optical Density	TCA Precipitable Radioactivity[a] (cpm)
1	0.23	73
2	0.32	79
3	0.44	70
4	0.57	62
5	0.75	49
6	1.12	79
7	1.37	65
8	1.69	65

[a]Determined as described in Materials and Methods.

ribonucleotides, the ability of other ribonucleotides to replace Ca^{2+} was assessed. The results of these experiments are presented in Table 2. It was obvious that a number of ribonucleotides could replace Ca^{2+} as a growth factor for Y. pestis EV76 at 37 C. There appeared to be no preference for purine or pyrimidine nucleotides nor was a "high-energy" phosphate a necessary moiety. Pyrophosphate was also observed to enhance the growth yield over that of a Ca^{2+}-deficient control culture. However, this effect was not observed consistently and the growth rate observed in pyrophosphate-supplemented cultures was much slower than that in other growing cultures. Ethylenediaminetetraacetic acid did not support the growth of Y. pestis EV76 at the concentrations used. None of the respective ribosides or free bases supported growth in Ca^{2+}-deficient medium at 37 C.

Expression of V antigen by ATP-supplemented Y. pestis.

The expression of the V antigen appears to be essential for the successful invasion of the mammalian host by Y. pestis [4,5]. It was, therefore, of interest to determine whether this antigen was expressed by cells cultivated at 37 C in Ca^{2+}-deficient media that was supplemented with ATP. For these experiments the KIM strain of Y. pestis was chosen because it exhibited an enhanced growth response in the presence of 20 mM Mg^{2+}, previously shown to potentiate the expression of the V and W antigens [22]. The growth of these cells under various conditions is shown in Figure 5. The cells were harvested at the end of the experiment, disrupted by sonication,

TABLE 2.--Effect of various ribonucleotides on the growth of Vwa^+ *Y. pestis* EV76 at 37 C.

Supplement[a]	Final[b] $O.D._{620}$
None	0.37
Ca^{2+}	4.36
ATP	5.87
GTP	3.81
CTP	3.51
UTP	0.94
ADP	4.83
GDP	4.01
CDP	1.16
UDP	1.00
AMP	4.39
GMP	0.75
CMP	0.40
UMP	0.40

[a] 2.5 mM Ca^{2+}, all nucleotides were 20 mM.

[b] The optical densities presented are those measured after 13 h of incubation (ATP, ADP, AMP) or when growth ceased (all other cultures).

FIGURE 5.--ATP-stimulated growth of Y. pestis strain KIM.

Growth of Y. pestis strain KIM at 37 C in the defined medium (see Materials and Methods), pH 7.8, containing 2.5 mM $MgCl_2$ (A) or 20 mM $MgCl_2$ (B). The medium was either supplemented with 3.0 mM $CaCl_2$ (●), 20 mM ATP (□), or remained unsupplemented (○).

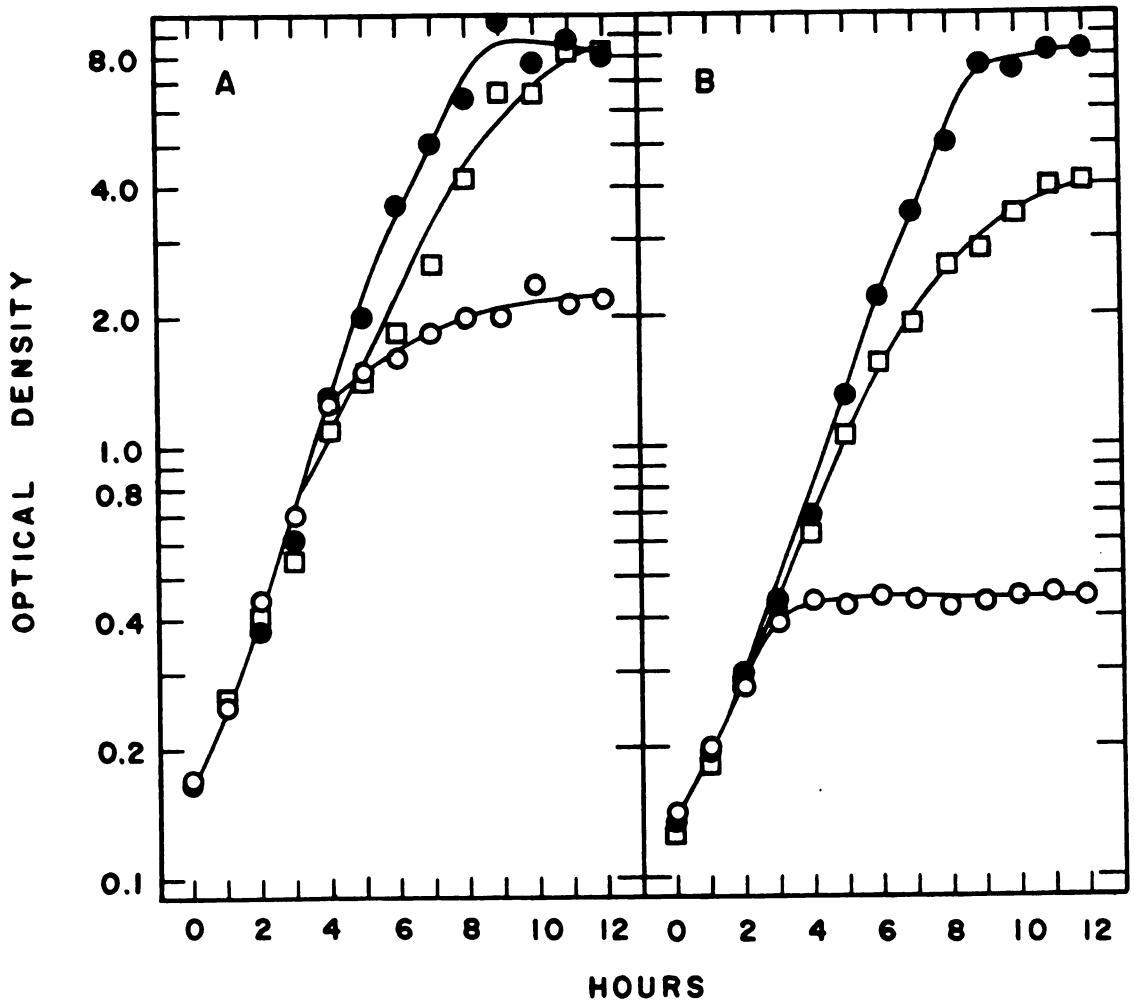

Figure 5.--ATP-stimulated growth of Y. pestis strain KIM.

and the resultant extracts were assayed for V antigen. It was observed that the cells cultivated either in the presence or the absence of the nucleotide expressed the antigen as long as Ca^{2+} was absent and sufficient Mg^{2+} was available (Table 3).

TABLE 3.--Expression of V antigen.

Addition	V antigen (sp. act.)[a]	
	2.5 mM Mg^{2+}	20 mM Mg^{2+}
3.0 mM Ca^{2+}	< 0.05	< 0.04
20 mM ATP	< 0.08	1.30
None	1.00	1.30

[a]After cultivation for 12 h at 37 C in medium containing the designated supplements, the cells were harvested by centrifugation at 27,000 X G for 15 min, washed in 0.033 M potassium phosphate buffer (pH 7.0), resuspended in buffer and disrupted by sonication. The resultant extract was assayed for V antigen and protein as described in Materials and Methods. The results are expressed as units of V antigen per mg of protein.

DISCUSSION

The expression of the V and W antigens appears to be essential for a successful invasion of the host by Y. pestis [4,5]. Vwa^+ cells cultivated in vitro in a simulated intracellular environment with respect to Ca^{2+} and Mg^{2+} concentration (10^{-7} M Ca^{2+} and 20 mM Mg^{2+} [18]) express V and W [3]. These same cells fail to multiply at 37 C unless 2.5 mM Ca^{2+} is present [13]. This latter environment mimics the high extracellular Ca^{2+} concentration in the mammalian host [19]. However, under these permissive conditions, V and W are not expressed [3].

It has been suggested that the inability of Vwa^+ Y. pestis to grow in vitro in the absence of Ca^{2+} at 37 C reflects a physiological change that makes the bacterium dependent upon the host cell [2]. The observations that both the ATP pool and the energy charge (submitted for publication) were decreased in Ca^{2+}-starved Vwa^+ Y. pestis prompted investigations to see if exogenous ATP would stimulate the growth of these cells.

Two obligate intracellular parasites, the rickettsiae and the chlamydiae, have been shown to transport host-cell derived nucleotides. Rickettsia prowazeki possesses an ADP-ATP exchange system and thus has access to the host ATP pool [27]. Chlamydia psittaci incorporated host-cell derived UTP and CTP into 16S RNA [11].

Although the nucleotide sustained the growth of Vwa^+ Y. pestis under normally restrictive conditions, it does not seem likely that Y. pestis depends upon host cell ATP as a substrate for growth as in the previously mentioned systems. The effect is not specific to ATP or even to triphosphorylated ribonucleosides. Furthermore, ATP was not utilized by Y. pestis even though growth proceeded normally.

The observation that ATP was not transported into Y. pestis cells was not totally surprising. Bacterial cell membranes are usually impermeable to these highly charged compounds and their transport has been demonstrated in very few cases [11,27]. However, exogenous nucleotides have been observed to affect other bacterial systems [8,9,17,26,29]. Transport of the nucleotides in these systems was not evaluated.

The mechanism by which the various ribonucleotides promote the growth of Vwa^+ Y. pestis remains as obscure as does that for the effect of Ca^{2+} [3,13]. Any model presented to explain the observed results must account for the observations that either specific cations (Ca^{2+}, Sr^{2+}, or Zn^{2+}) or specific anions (see Table 4) produce similar effects.

It may be that Ca^{2+} competes with another cation in the medium that inhibits the growth of Vwa^+ Y. pestis. The effect observed with the various ribonucleotides may then be due to the chelation of the same cation, thereby preventing growth inhibition.

It has been demonstrated that increasing concentrations of Mg^{2+} hastened the onset of restriction of Vwa^+ Y. pestis in Ca^{2+}-deficient medium at 37 C (submitted for publication). ATP and other

nucleotides form complexes with Mg^{2+} although the association constants vary over a large range [25]. The observations that ATP, ADP, and AMP were able to equally substitute for Ca^{2+}, even though their affinities for Mg^{2+} are enormously different, suggests that Mg^{2+} complexing is probably not of sole importance to the effect observed.

Another possibility surfaces upon examination of the literature with respect to the effects of Ca^{2+} and ATP on biological membranes. It has been shown that Ca^{2+} can affect the activity of membrane-bound enzymes by its interaction with the membrane [14]. The fluidity of artificial membranes is affected by the cation [24]. Of particular interest to this study was the observation that ATP, ADP, or AMP could substitute for Ca^{2+} in the stabilization of neural membranes [20]. It is possible that the specific interactions of either Ca^{2+} or a ribonucleotide with the yersinial membrane results in the stabilization of some membrane state that is necessary for growth. Obviously, there is at this time not enough data to support directly either the above or any alternative possibility.

The most significant observation presented here is that ATP-supplemented Y. pestis expressed the V antigen. The ability to express this antigen significantly affects the virulence of Y. pestis [4,5,6]. If it is true that the virulence antigens are necessary to the survival and multiplication of the bacterial cells after phagocytosis by fixed macrophages, as previously suggested [16], then they should be expressed in a simulated intracellular environment. Previous studies indicated that the virulence antigens were expressed in vitro only by restricted cells [3]. However, the results

obtained here indicate that growing Y. pestis cells do express V antigen in nucleotide-supplemented media.

The observation presented in this study may mean that certain ribonucleotides replace Ca^{2+} as a growth factor intracellularly. In the essentially Ca^{2+}-free environment of the cytoplasm, the expression of the V and W antigens would not be inhibited and the cells would still be able to multiply due to the presence of the effective ribonucleotide. Intracellular concentrations of adenine nucleotides have been measured [12] and they approach the concentrations that were found to be effective in vitro. Since the most abundant nucleotide in a growing cell is ATP [7,12] it is quite possible that this nucleotide is the biological effector.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grants AI 13590 from the National Institute of Allergy and Infectious Diseases and GM 01911 from the Department of Health, Education, and Welfare. The excellent technical assistance of Janet Fowler is gratefully appreciated.

LITERATURE CITED

1. Beesley, E. D., Brubaker, R. R., Janssen, W. A., Surgalla, M. J. 1967. Pesticins. III. Expression of coagulase and the mechanism of fibrinolysin. Journal of Bacteriology. 94: 19-26.

2. Brubaker, R. R. 1967. Growth of Pasteurella pseudotuberculosis in simulated intracellular and extracellular environments. The Journal of Infectious Diseases. 117:403-417.

3. Brubaker, R. R., Surgalla, M. J. 1963. The effect of Ca^{2+} and Mg^{2+} on lysis, growth, and production of virulence antigens by Pasteurella pestis. The Journal of Infectious Diseases. 114:13-25.

4. Burrows, T. W. 1956. An antigen determining virulence in Pasteurella pestis. Nature. 177:426-427.

5. Burrows, T. W., Bacon, G. A. 1956. The basis of virulence in Pasteurella pestis: an antigen determining virulence. British Journal of Experimental Pathology. 37:481-493.

6. Burrows, T. W., Bacon, G. A. 1958. The effect of the loss of different virulence determinants on the virulence and immunogenicity of strains of Pasteurella pestis. British Journal of Experimental Pathology. 39:278-291.

7. Chapman, A. G., Fall, L., Atkinson, D. E. 1971. Adenylate energy charge in Escherichia coli during growth and starvation. Journal of Bacteriology. 108:1072-1086.

8. Fanica-Gaignier, M., Clement-Metral, J., Kamen, M. D. 1971. Adenine nucleotide levels and photopigment synthesis in a growing photosynthetic bacterium. Biochimica et Biophysica Acta. 226:135-143.

9. Gajdos, A., Gajdos-Torok. 1969. The quantitative regulation of the biosynthesis of porphyrins by intracellular ATP concentration. Biochemical Medicine. 2:372-388.

10. Hall, P. J., Yang, G. C., Little, R. V., Brubaker, R. R. 1974. Effect of Ca^{2+} on morphology and division of Yersinia pestis. Infection and Immunity. 9:1105-1113.

11. Hatch, T. P. 1975. Utilization of L-cell nucleoside triphosphates by Chlamidia psittaci for ribonucleic acid synthesis. Journal of Bacteriology. 122:393-400.

12. Hauschka, P. V. 1973. Analysis of nucleotide pools in animal cells, pp. 361-462. In: Prescott, D. M. (ed.), Methods in cell biology, vol. 7. New York: Academic Press.

13. Higuchi, K., Kupferberg, L. L., Smith, J. L. 1959. Studies on the nutrition and physiology of Pasteurella pestis. III. Effects of calcium ions on the growth of virulent and avirulent strains of Pasteurella pestis. Journal of Bacteriology. 77:317-321.

14. Horvath, C., Sovak, M. 1973. Membrane coarctation by calcium as a regulator for bound enzymes. Biochimica et Biophysica Acta. 298:850-860.

15. Jackson, S., Burrows, T. W. 1956. The virulence enhancing effect of iron on non-pigmented mutants of virulent strains of Pasteurella pestis. British Journal of Experimental Pathology. 37:577-583.

16. Janssen, W. A., Surgalla, M. J. 1969. Plague bacillus: survival within host phagocytes. Science. 163:950-952.

17. Kight-Olliff, L. C., Fitzgerald, J. W. 1978. Inhibition of induction if Pseudomonas aeruginosa by exogenous nucleotides. Canadian Journal of Microbiology. 24:811-817.

18. Kretsinger, R. H. 1977. Evolution of the informational role of calcium in eukarotes, pp. 63-72. Inc: Wasserman, R. H., et al. (eds.), Calcium binding proteins and calcium function. New York: North Holland.

19. Kugelmass, N. I. 1959. Biochemistry of blood in health and disease. Springfield, Illinois: Ch. C. Thomas, Inc.

20. Kuperman, A. S., Okamoto, M., Gallin, E. 1967. Nucleotide action on spontaneous electrical activity of calcium deficient nerve. Journal of Cellular Physiology. 70:257-264.

21. Lamprecht, W., Trautschold, I. 1963. Adenosine-5'-triphosphate: determination with hexokinase and glucose-6-phosphate dehydrogenase, pp. 543- . In: Bergmeyer, H. U. (ed.), Methods of enzymatic analysis. New York: Academic Press.

22. Lawton, W. D., Erdman, R. L., Surgalla, M. J. 1963. Biosynthesis and purification of V and W antigen in Pasteurella pestis. Journal of Immunology. 91:179-184.

23. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry. 193:265-275.

24. Papahadjopoulos, D., Portis, A. 1978. Calcium-induced lipid phase transitions and membrane fusion. Annals of the New York Academy of Science. 308:50-66.

25. Rose, I. A. 1968. The state of magnesium in cells as estimated from the adenylate kinase equilibrium. Proceedings of the National Academy of Sciences. 61:1079-1086.

26. Schmidt, G. L., Kamen, M. D. 1971. Control of chlorophyll synthesis in Chromatium vinosum. Archives of Microbiology. 76:61-64.

27. Winkler, H. H. 1976. Rickettsial permeability: an ADP-ATP transport system. Journal of Biological Chemistry. 251:389-396.

28. Yang, G. C. H., Brubaker, R. R. 1971. Effect of Ca^{2+} on the synthesis of deoxyribonucleic acid in virulent and avirulent Yersinia. Infection and Immunity. 3:59-65.

29. Zilinsky, J. W., Sojka, G. A., Gest, H. 1971. Energy charge regulation in photosynthetic bacteria. Biochemical and Biophysical Research Communications. 42:955-961.

SECTION III

INTRODUCTION

The data presented in Section II of this thesis indicate that exogenous ATP present *in vitro* can replace Ca^{2+} in sustaining growth of Vwa^{+} *Y. pestis* at 37 C. The growth response observed was dependent upon the pH of the medium as well as the concentration of ATP present. Growing cells did not appear to either hydrolyze significant amounts of exogenous ATP nor did they incorporate the nucleotide into TCA precipitable material. Various other ribonucleotides could serve equally well to sustain the growth of these bacteria. Unlike cells cultivated in the presence of 2.5 mM Ca^{2+}, those grown in 20 mM ATP expressed the V antigen. These results are consistent with the hypothesis that a ribonucleotide, possibly ATP, may serve a regulatory role in sustaining the intracellular growth of *Y. pestis*.

This section is included in this thesis in order to present the data relating to energy charge in *Y. pestis* which will appear in a separate communication. In addition, experiments that further investigated the phenomenon of nucleotide-stimulated growth of *Y. pestis* at 37 C are presented.

MATERIALS AND METHODS

Bacteria. The following strains of Y. pestis were employed in these studies: EV76, KIM, MP6, G25, G32. The phenotypes of these strains with respect to the virulence determinants are presented in Table 4.

Adenylate energy charge. Adenine nucleotides were extracted by the addition of a 2.0 ml aliquot of the bacterial culture to 0.4 ml of 35% $HClO_4$ (70) containing 67 mM EDTA (54). The resulting precipitate was removed by centrifugation and 2.0 ml of the supernatant was neutralized by the addition of 0.7 ml of 2.5 M KOH, 0.58 M $KHCO_3$ (70). This extract was either assayed directly for ATP or was assayed after enzymatic conversion of AMP and ADP to ATP (27). ATP was determined by the luciferase assay in a scintillation vial containing 1.8 ml of the assay buffer (40 mM glycylglycine, 3 mM $MgSO_4$, pH 7.4) and 0.2 ml of the extract. A 75 µl volume of partially purified luciferase and luciferin (57) was added and after 15 sec of incubation, the light emitted over a period of 6 sec was measured with a Model 3320 Packard Tri-Carb scintillation spectrophotometer as previously described (66). Quintuplicate assays were performed on each sample and the mean value was used to determine the adenylate energy charge. All values fell in the linear portion of an ATP standard curve. Analysis of external standards which were treated

identically to culture aliquots resulted in recoveries of 101, 107, and 91% for ATP, ADP, and AMP, respectively. Less than 2% cross-reactivity was observed with GTP and ADP.

Other methods. In the presentation of the energy charge data, optical density was converted to dry weight with a standard curve comparing the two parameters in the appropriate environment. All other methods used have been described in Section II of this thesis.

TABLE 4.--Phenotypes of *Y. pestis*.

Strain	Phenotypes[a]			
	Vwa	Pgm	Pst	Fra
EV76	+	0	+	+
KIM	+	0	+	+
MP6	+	0	+	+
G32	+	0	0	+
G25	+	0	0	+
M23	+	0	+	0

[a]Vwa, virulence antigen production; Pgm, pigmentation, Pst, pesticin production; Fra, Fraction I antigen; +, expressed; -, not expressed.

RESULTS

Adenylate energy charge. The adenylate energy charge was determined for cells of Y. pestis cultivated under various conditions. The results of these experiments are presented in Table 5. The energy charge in cells cultivated at 37 C in Ca^{2+}-deficient media was significantly lower than that observed in cells cultivated at 37 C in Ca^{2+}-supplemented media or at 26 C. The decrease in energy charge observed appeared to be due to a decrease in intracellular ATP and a concommitant increase in intracellular AMP.

ATP antagonism of Ca^{2+}-sustained growth. The effect of ATP on the growth of EV76 cells at 37 C in medium containing 2.5 mM Ca^{2+} was investigated. It was observed that exogenous ATP resulted in antagonizing the effect of Ca^{2+} on these cells (Figure 6). The degree of the antagonism was dependent upon the concentration of ATP added to the medium.

Effect of ATP on other strains of Y. pestis. In order to assure the fact that the phenomenon observed with exogenous ribonucleotides was not unique to EV76, other strains were tested for their response to exogenous ATP. Various strains of Y. pestis were not able to grow in Ca^{2+}-deficient medium at 37 C in the presence of 20 mM Mg^{2+}. However, the addition of either 2.5 mM Ca^{2+} or 20 mM

TABLE 5.--Adenine nucleotide pools in Vwa$^+$ Y. pestis strain EV76 cultivated under various conditions.

°C	Ca^{2+} (mM)	Mg^{2+} (mM)	ATP (μmoles/g dry wt)	ADP (μmoles/g dry wt)	AMP (μmoles/g dry wt)	Total (μmoles/g dry wt)	E.C.[a]
26	0	2.5	3.2 (0.4)[b]	1.7 (0.3)	N.D.[c]	4.6 (0.2)	0.82 (0.04)
26	0	20.0	3.1 (0.3)	1.4 (0.4)	N.D.	4.5 (0.2)	0.84 (0.04)
37	2.5	2.5	2.4 (0.3)	2.3 (0.1)	N.D.	4.7 (0.2)	0.75 (0.06)
37	2.5	20.0	2.3 (0.2)	1.5 (0.2)	N.D.	3.9 (0.2)	0.80 (0.01)
37	0	2.5	1.6 (0.2)	2.0 (0.2	0.3 (0.2)	3.9 (0.2)	0.67 (0.04)
37	0	20.0	1.3 (0.2)	2.8 (0.2)	0.5 (0.2)	4.6 (0.2)	0.57 (0.02)

[a]Adenylate energy charge = $\frac{ATP + \frac{1}{2} ADP}{\text{total adenylates}}$

[b]Values presented are the means of three separate determinations with the standard deviations enclosed in parentheses.

[c]None detectable.

FIGURE 6.--ATP antagonism of Ca^{2+} effect.

Growth of Vwa^{+} Y. pestis strain EV76 at 37 C in medium, pH 7.8, containing 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, and either 0 mM (○), 1.25 mM (●), 2.5 mM (□), 5.0 mM (■), or 10 mM ATP (△).

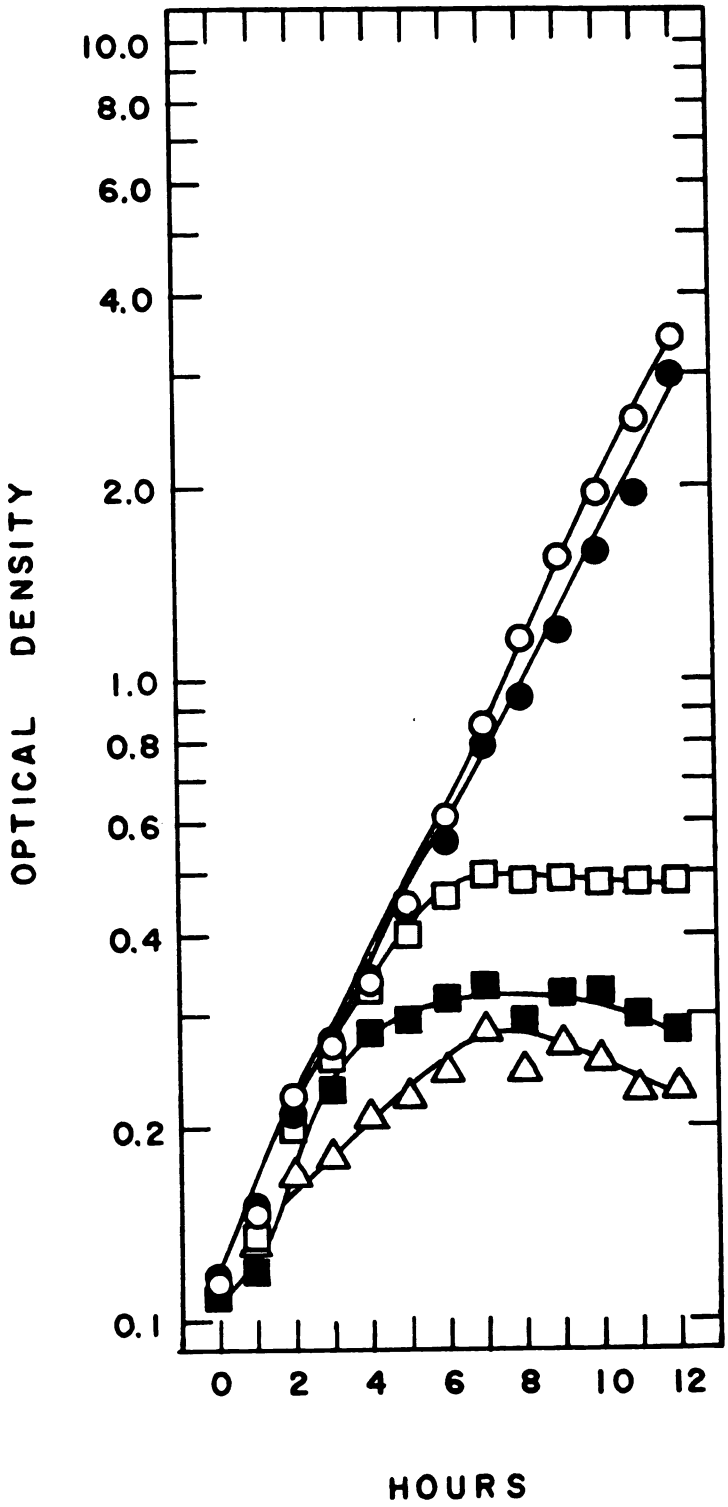

Figure 6.--ATP Antagonism of Ca^{2+} Effect.

ATP to the medium resulted in sustained growth of all strains of Y. pestis investigated except EV76 (Table 6).

The influence of Mg^{2+} and ATP concentrations on the growth of EV76. An antagonism was also observed between Mg^{2+} and ATP when EV76 was cultivated in the presence of varying concentrations of either Mg^{2+} or ATP. Figures 7 and 8 show that increasing the Mg^{2+} concentration required an increasing ATP concentration in order to sustain the growth of this strain. Other strains did not appear to be as sensitive to the Mg^{2+} concentration when cultivated in the presence of 20 mM ATP (see Figure 5).

Effect of pH on the growth of Y. pestis. It was observed that an alkaline pH favored the growth of both the EV76 (Figure 2) and KIM strains (Figure 5) of Y. pestis even in Ca^{2+}-deficient media in the presence of 2.5 mM Mg^{2+}. Other strains of Y. pestis also exhibited this property (Table 7). However, as observed with EV76, increasing the Mg^{2+} concentration to 20 mM hastened the onset of restriction.

TABLE 6.--Growth response of various strains of Y. pestis in the presence and absence of ATP in media supplemented with 20 mM $MgCl_2$.

Strain	Growth Response at 37 C[a]	
	20 mM ATP	No Addition
M23	+	-
MP6	+	-
G-25	+	-
G-32	+	-
KIM	+	-
EV76	-	-

[a]symbols: +, growth similar to Ca^{2+}-sufficient control culture; -, growth ceased after no more than two doublings.

FIGURE 7.--Effect of $MgCl_2$ and ATP on the growth of *Y. pestis*.

Growth of Vwa^+ *Y. pestis* strain EV76 in medium, pH 7.8, containing no added supplement (A), 1.25 mM ATP (B), or 2.5 mM ATP (C). $MgCl_2$ was incorporated into the medium at 1.25 mM (●), 2.5 mM (◑), 5.0 mM (◐), 10 mM (◒), or 20 mM (○).

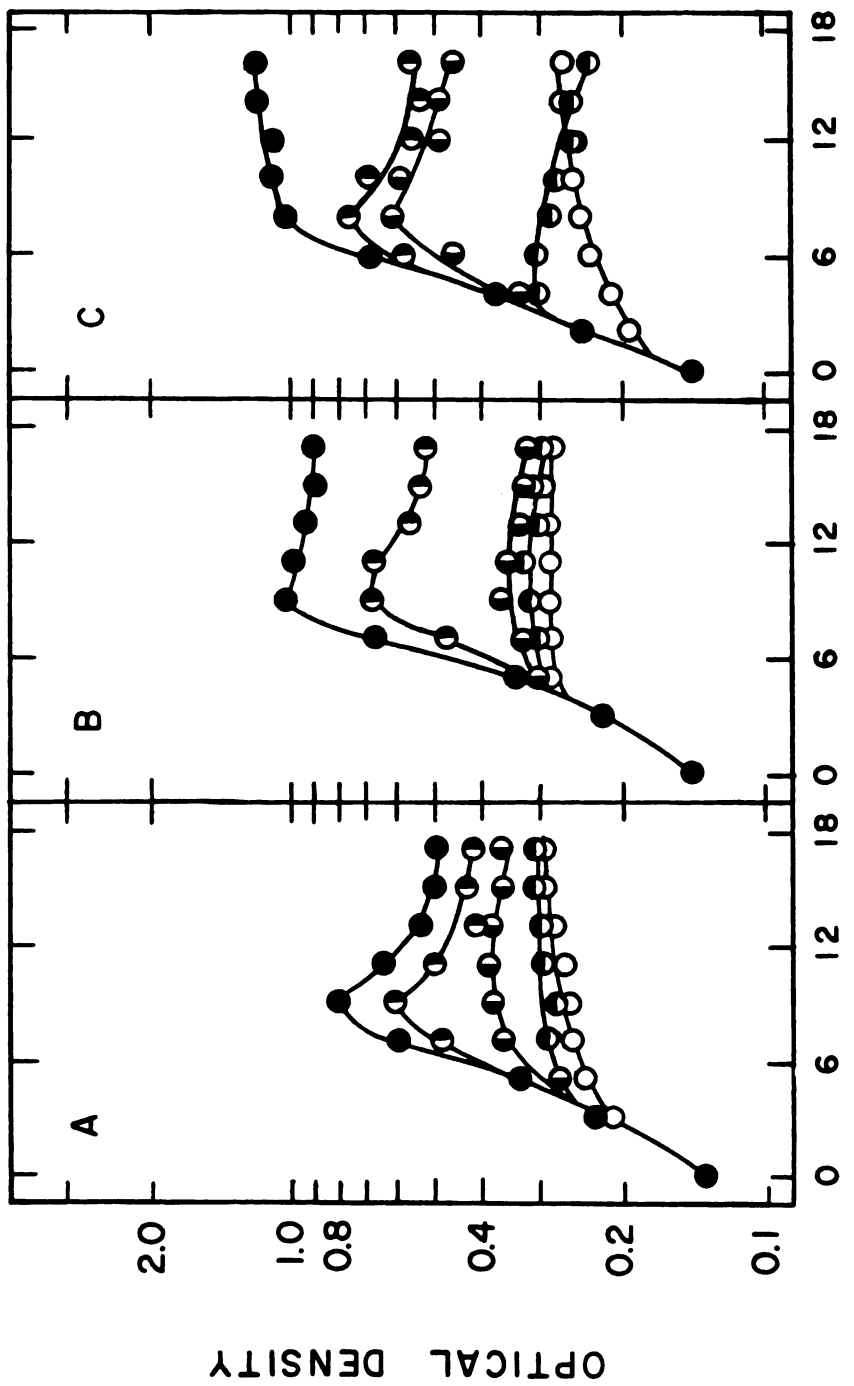

Figure 7.--Effect of $MgCl_2$ on the Growth of Y. pestis.

FIGURE 8.--Effect of $MgCl_2$ and ATP on the growth of *Y. pestis*.

Growth of Vwa^+ *Y. pestis* strain EV76 in medium, pH 7.8, containing 5.0 mM (A), 10 mM (B), or 20 mM ATP (C). $MgCl_2$ was incorporated into the medium at 1.25 mM (●), 2.5 mM (◑), 5.0 mM (◐), 10 mM (◒), or 20 mM (○).

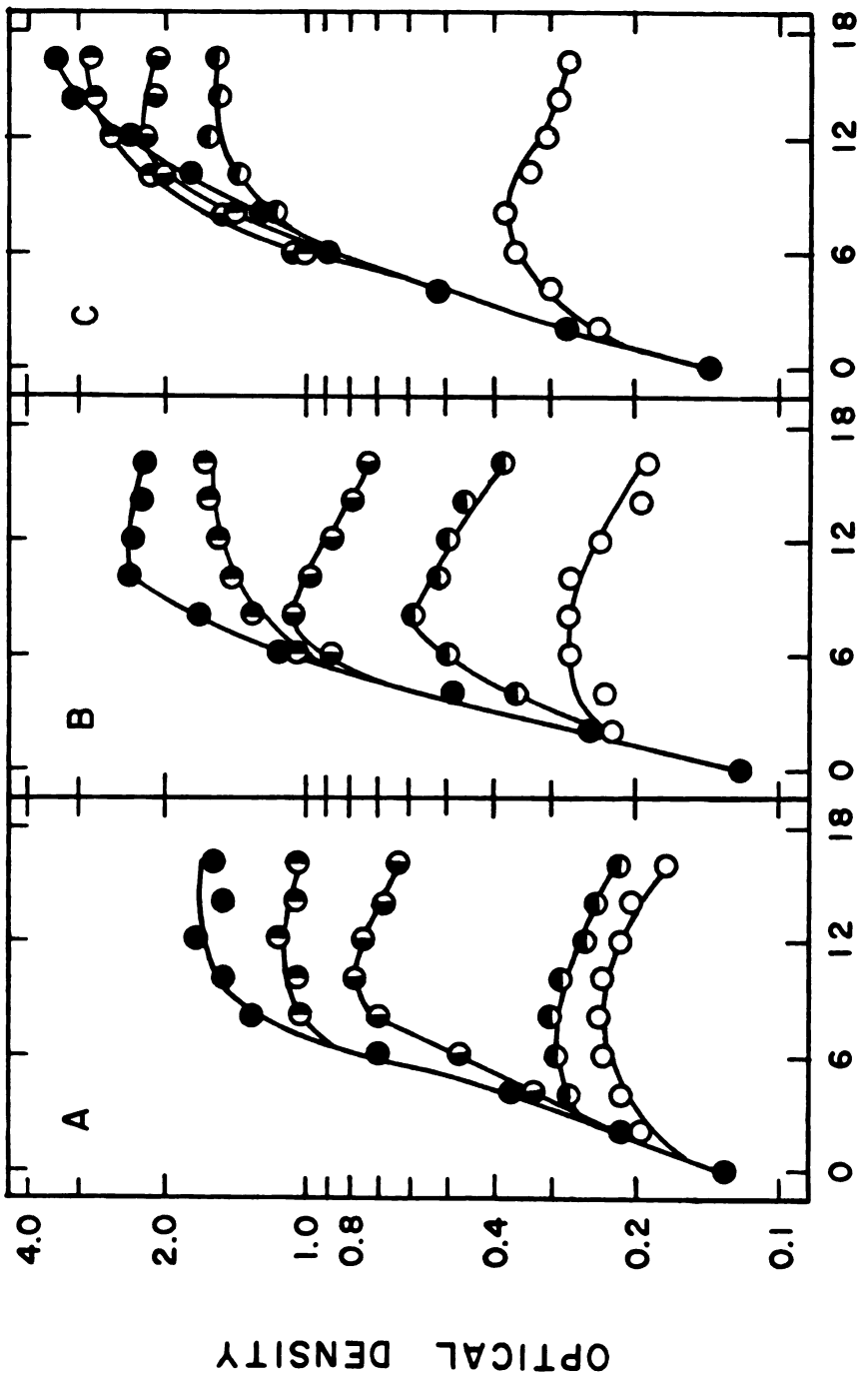

Figure 8.--Effect of $MgCl_2$ and ATP on the Growth of Y. pestis.

TABLE 7.--Effect of pH on growth of various strains of *Y. pestis* at 37 C.

Strain	Optical Density[a]	
	pH 7.0	pH 7.8
G25	0.51	5.0
MP6	0.44	3.5
G32	1.1	7.7

[a]Values represent either the maximum optical density or that observed after 16 h of incubation in medium containing 2.5 mM $MgCl_2$.

DISCUSSION

Atkinson (1) has postulated that a cell must maintain an energy charge of 0.8 or higher in order to sustain growth. The energy charge values observed for a number of organisms support this postulate (27). This parameter has been measured for Y. pestis cultivated under various conditions in order to gain some insight into the metabolic alterations accompanying Ca^{2+} starvation in these cells.

The adenylate energy charge observed for Vwa^{+} Y. pestis cultivated at 26 C or at 37 C in Ca^{2+}-supplemented media was 0.75 to 0.84. There was a significantly lower energy charge in Ca^{2+}-starved cells (0.57-0.67).

Decreases in the energy charge value have been observed in other systems following nutrient deprivation. In E. coli, for example, the energy charge dropped from about 0.8 to 0.6 after the depletion of glucose (27). Likewise, the starvation of an adenine auxotroph for adenine resulted in a similar decrease in energy charge (70). The decreases in energy charge values due to nutrient limitation often occur within a short period of time, whereas the decrease observed with stationary cells of E. coli was very slow (27).

The cells used to evaluate the energy charge in Ca^{2+}-starved Y. pestis were analyzed within 4 to 5 h after the shift to the restrictive temperature. It seems likely that the decrease in energy charge in these cells is rather abrupt. Preliminary experiments, not presented here, indicate that the energy charge in Ca^{2+}-starved Y. pestis drops simultaneously with or very shortly after the cessation of growth. These results suggest that the cells may be unable to obtain an energy source from the medium in the absence of Ca^{2+}. Alternatively, Ca^{2+}-starved cells may be unable to couple the catabolism of various energy sources to ATP synthesis.

The results presented earlier in this thesis indicated that exogenous ATP or a variety of other ribonucleotides could replace Ca^{2+} as a growth factor for Vwa^{+} Y. pestis at 37 C. The observations that the ATP was not utilized by the bacteria suggest that the nucleotide may play a regulatory role in the growth of Y. pestis at 37 C. Such a regulatory role has been postulated for Ca^{2+} (9).

Although ATP can replace Ca^{2+} in sustaining the growth of Vwa^{+} Y. pestis, the overall effect of the exogenous nucleotide does not appear to be identical to that of the cation. It has been demonstrated that Ca^{2+} inhibits the expression of the virulence or V and W antigens in vitro (13). Exogenous ATP, however, did not affect the expression of V antigen as long as sufficient Mg^{2+} was present (Figure 3). Furthermore, simultaneous addition of both ATP and Ca^{2+} to the medium resulted in an antagonistic rather than an additive effect (Figure 6). This result can be due to the complexing ability of ATP for Ca^{2+} (48). Presumably, neither the nucleotide nor the

cation are active in sustaining the growth of Vwa$^+$ Y. pestis when they are present as a Ca-ATP complex.

All the Vwa$^+$ strains of Y. pestis tested, except EV76, displayed an enhanced growth response at 37 C in medium containing 20 mM Mg^{2+} if 20 mM ATP was added. The increased sensitivity of EV76 was taken advantage of to investigate the relationship between Mg^{2+} and ATP and the growth of Y. pestis.

The interaction was observed to be complex (Figures 7 and 8). In the absence of ATP, decreasing the Mg^{2+} concentration resulted in an enhanced growth response. Similar results have been reported elsewhere (submitted for publication).

Increasing the ATP concentration in the medium resulted in counteracting the effects of Mg^{2+}. This result suggests that the nucleotide may merely be chelating the Mg^{2+} in the medium. The corollary to this hypothesis states that as the Mg^{2+} concentration approaches zero, the maximal cell density would approach that observed in a culture supplemented with 20 mM ATP. The maximal optical densities from panel A of Figure 7 are replotted in Figure 9. Extrapolation indicates that an optical density of less than 1.0 would theoretically be obtained in Vwa$^+$ Y. pestis were cultivated at 37 C in the absence of both Mg^{2+} and Ca^{2+}. Obviously, this experiment cannot be performed because Mg^{2+} is an essential ion. However, the value obtained by this manipulation of the data is far less than that observed in ATP-supplemented cultures. In the latter environment, optical densities as high as 7.0 or more have been observed.

FIGURE 9.--Effect of Mg^{2+} on the growth of Y. pestis.

Effect of Mg^{2+} concentration on growth of Vwa^{+} Y. pestis EV76. ●, data points; ○, value at 0 mM Mg^{2+} obtained by extrapolation.

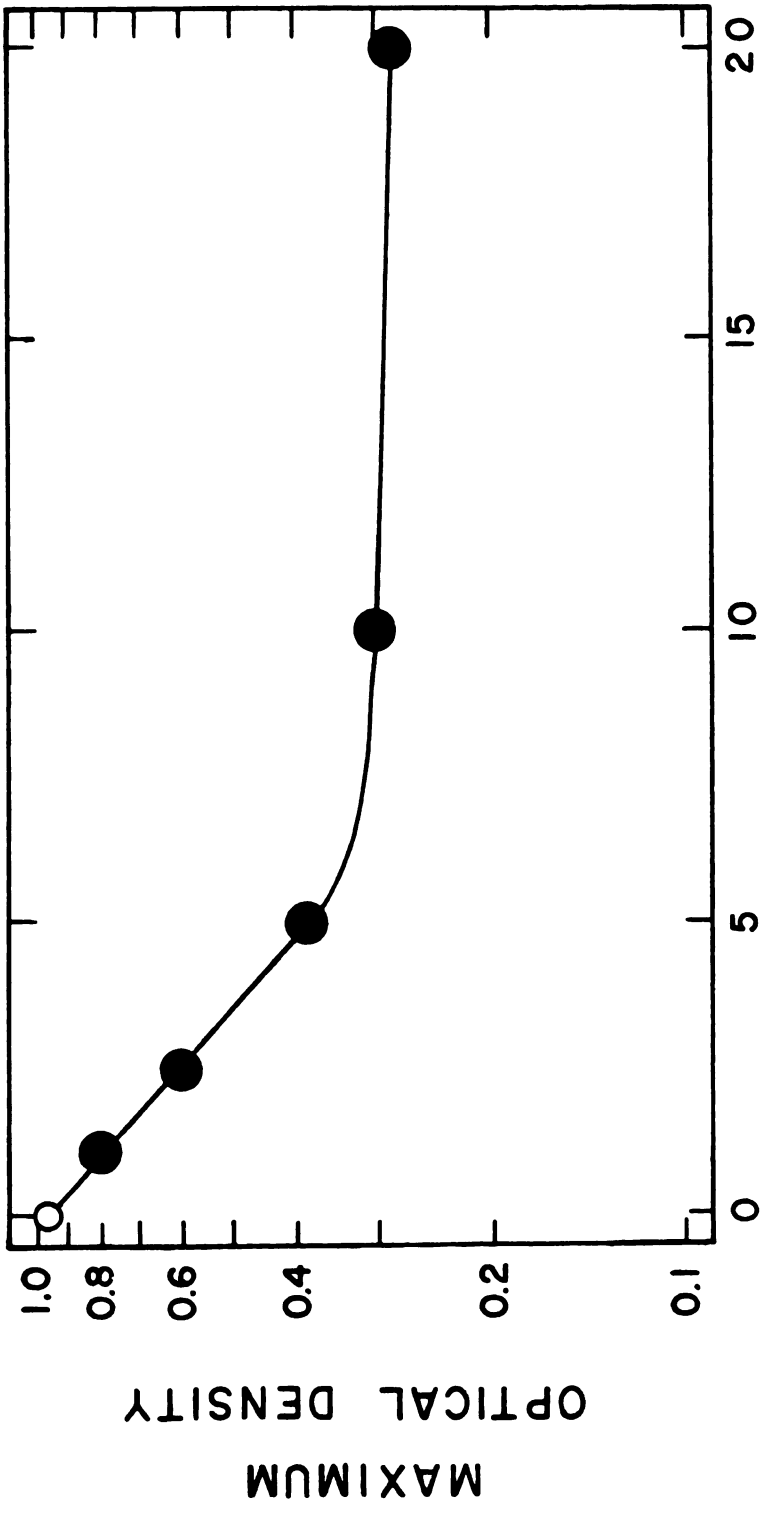

Figure 9.--Effect of Mg^{2+} on the Growth of Y. pestis.

It was noted that sharp decreases in optical density occurred at low Mg^{2+} concentrations in the absence of ATP after about 8 h of incubation at 37 C. In these experiments it was not determined whether this result was due to cell lysis or some other phenomenon. This type of growth response was not observed in ATP-supplemented cultures. In fact, increasing ATP concentrations appeared to protect against a decline in the optical density of the culture during the last 8 hours of incubation (see Figures 7 and 8). These results further suggest that the enhanced growth response observed with exogenous nucleotides is not solely due to a chelation of Mg^{2+}. However, the lowering of the free Mg^{2+} concentration due to chelation by ATP or other nucleotides must affect the growth of the bacteria. The effect of ATP on the growth of Vwa^+ Y. pestis cells at 37 C may reflect a combination of Mg^{2+}-chelation and a more direct, and as yet undefined, interaction of the nucleotide with the bacterial cell.

The strain EV76 was also affected differently than other strains by variations of the pH of the medium. Although four other strains displayed an increased growth response at 37 C if media containing 2.5 mM Mg^{2+} was adjusted to pH 7.8 prior to seeding the culture, EV76 was only moderately affected, if at all, by this manipulation (see Figure 2 and Table 7). It appears as if the EV76 strain observed in a slightly alkaline environment is particularly sensitive to the cultural conditions and therefore is a desirable strain to use in defining various parameters that affect the growth of Vwa^+ Y. pestis.

The enhanced growth response of Y. pestis observed in a slightly alkaline environment at low Mg^{2+} concentrations (2.5 mM) may explain the early observations of Ogg et al. (59) that the attenuation of virulent cultures could be prevented by adjusting the medium to pH 7.8. Other investigators suggested that the pH effect may be due to the expected increase of the CO_2 in solution at the higher pH (30). Although they demonstrated that the addition of bicarbonate to the culture medium resulted in the maintenance of a virulent culture, the effect was found to be attributed to the inhibition of Vwa^- mutants which spontaneously arise (68). Further work showed that orotic acid and other products of CO_2 fixation also were effective in maintaining virulent cultures by enhancing the growth of Vwa^+ cells (5). The addition of bicrabonate did not suffice in promoting the growth of Vwa^+ Y. pestis in the studies presented in this thesis. Although orotic acid was not tested, neither cytosine nor uracil could substitute for Ca^{2+} in sustaining growth under the conditions presented in this thesis. It does not seem likely, therefore, that the pH effect observed in these studies reflects a problem in CO_2 fixation as postulated by Baugh et al. (6).

These studies on the effects of various nucleotides on the growth of Vwa^+ Y. pestis in a chemically defined medium have resulted in the definition of various parameters of importance in vitro. These parameters may play a crucial role in the intracellular growth of Y. pestis. The observation that growing Y. pestis cells incubated in the presence of ATP at 37 C express the virulence antigens suggests that this or some other ribonucleotide may regulate the

growth of Y. pestis in the host-cell. Whether ATP or other ribonucleotides are the actual regulators of yersinial cell growth in vivi remains to be elucidated.

APPENDIX

APPENDIX

Determination of the Adenylate Energy Charge in Y. pestis

Since the methods used to determine adenine nucleotide pools in bacterial cells can influence the values obtained, a brief review of the methodology is presented. Some aspects of matters discussed here are more completely covered by other authors (26,67). I also include a detailed description of the methods used to obtain the energy charge values presented in this thesis.

Among the factors known to most critically affect the accurate measurement of adenine nucleotides are the length of time between sampling and extraction of nucleotides (26), the extraction procedure used (54), the presence of ATPase or other interfering enzymes in the sample (28,70), the presence of inhibitors of luciferase (67), and the presence of nucleoside diphosphokinase and/or adenylate kinase in the luciferase preparation (57).

Since the turnover rate of ATP is very fast (in the range of 1 sec), it is of prime importance that samples be extracted as quickly as possible after removal from the culture being studied. The exact effect that this parameter has on the measurement of the adenylate energy charge (EC) appears to be variable with respect to the organism being studied. The oxygen-dependent ATP-producing reactions in an aerobe may cease due to the anaerobic environment

quickly produced in the pipet with which the sample is taken (26). A prolonged exposure to the anaerobic environment may result in artifactually low EC values. This parameter may not be very critical in the study of anaerobes (26). In general, an interval of 5 sec or less between the time a sample is taken and that at which the nucleotides are extracted is recommended if possible. With bacterial cultures in liquid media this is easily done by merely pipetting a sample of the culture directly into the extraction reagent.

The interference of any possible extracellular nucleotides can be assessed by treating culture filtrates identically to whole cultures (27). Intracellular pools of nucleotides can then be calculated from the difference of the two values.

Lundin and Thore (54) have evaluated various procedures for the extraction of nucleotides and the reader should refer to this article before embarking on one of these adventures. For obvious reasons, it is important that the extraction procedure used does not destroy the nucleotide(s) of interest. Also, the extracting reagent should inactivate any enzymes involved in ATP consuming or producing reactions.

The most popular method found in the literature consists of an acid extraction with either perchloric (PCA) or trichloracetic acid (TCA) (54,70). Both PCA and TCA inhibit firefly luciferase. Therefore, the acids must be removed before analysis of ATP with luciferase. Trichloroacetic acid can be removed by ether extraction (54) and perchlorate can be precipitated with KOH (27). Alternatively, ethanol extraction has been shown to produce results similar to those

obtained with acid extraction (27,54). As an additional measure to prevent the alteration of the ATP, ADP, or AMP pools, EDTA may be included in the extraction reagent (54). This compound chelates Mg^{2+} which is often required in reactions involving ATP.

The samples must be neutralized before analysis. It is desirable to remove precipitated protein from the sample prior to neutralization. Some enzymes are reactivated upon neutralization and may alter the ratios of nucleotides. This has been found to be the case in E. coli (70). The inclusion of an internal standard may expose problems in this area.

A number of compounds have been observed to inhibit the activity of firefly luciferase. These include PCA, TCA, pyrophosphate, and Cl^- ions. Other influential parameters include temperature, oxygen concentration, Mg^{2+} concentration, and pH. The review presented by Strehler (67) describes the effects of these parameters.

Crude extracts of firefly tails contain, in addition to luciferase and luciferin, adenylate kinase and nucleoside diphosphate kinase (57). These latter two enzymes catalyze the formation of ATP from other nucleotides. The presence of these enzymes can result in artifactually high ATP values. Luciferin and luciferase can be separated from the two kinases by gel filtration (57).

The method that follows resulted in reproduceable measurements of adenine nucleotides.

Extraction of adenine nucleotides. A 2.0 ml aliquot of the bacterial culture was pipetted into 0.4 ml cold 35% $HClO_4$ (70)

containing 67 mM EDTA (54) within 5 sec after the sample was removed. The solution was briefly mixed with a vortex and incubated on ice for 30 min. The precipitated protein was removed by centrifugation. Neutralization of the supernatant was accomplished by adding 0.7 ml of 2.6 M KOH, 0.58 M $KHCO_3$ to 2.0 ml of the supernatant (70). After incubation on ice for 1 h the white precipitate was removed by centrifugation.

Conversions of AMP and ADP to ATP. The extracted neutralized culture was divided into three 0.7 ml portions. For ATP determinations, 0.7 ml of 10 mM morpholinopropane sulfonic acid (MOPS), pH 7.4, containing 10 mM $MgSO_4$ was added to one portion. For ADP plus ATP determinations, 0.7 ml of 10 mM MOPS buffer, pH 7.4, containing 10 mM $MgSO_4$, 20 µg pyruvate kinase (Sigma), and 1.5 mM phosphoenolpyruvate was added to the second portion. For ATP, ADP plus AMP determinations, 0.7 ml of 10 mM MOPS, pH 7.4, containing 10 mM $MgSO_4$, 20 µg pyruvate kinase, 1.5 mM phosphoenolpyruvate, and 25 µg myokinase (Sigma) was added to the third portion.

All three mixtures were incubated at 37 C for 30 min. The enzymes were then denatured by placing the mixtures in a boiling water bath for 20 min. Myokinase was desalted by passage through a short Sephadex G-25 column (1 X 10 cm) which was pre-equilibrated with the MOPS buffer used in the conversions. The preparations were assayed either immediately for adenine nucleotides or they were stored at -20 C for up to 7 days.

Luciferase assay. Firefly lantern extracts (FLE-50) were obtained from Sigma Chemical Company, St. Louis, MO. Luciferase and luciferin were partially purified from these extracts by a minor modification of the method described by Neilson and Rasmussen (57).

The extracts from 5 vials of FLE-50 were resuspended in 5 ml of distilled water. After incubation on ice for 1 h the solution was clarified by centrifugation at 12,000 X g for 20 min at 4 C. Luciferin was separated from luciferase on a Sephadex G-25 column (Pharmacia Fine Chemicals, Piscastaway, NJ) as previously described (57). The fractions containing luciferase activity were pooled, concentrated to 1 ml, and applied to a Sephadex G-100 (superfine grade) column (2.5 X 90 cm) which was equilibrated with glycine-arsenate buffer (50 mM glycine, 10 mM K_2HAsO_4, 1 mM EDTA, pH 7.7). The flow rate was 0.1 ml/min at an operating pressure of 80 cm. Fractions of 3 ml were collected and assayed for luciferase (57). Luciferase positive fractions were then assayed for nucleoside diphosphokinase activity (57). Those fractions containing little or no nucleoside diphosphokinase activity were pooled and used in determining ATP. Luciferase prepared in this manner displayed less than 2% cross-reactivity with GTP and ADP. This luciferase preparation was combined with the luciferin-containing fractions from the G-25 column.

ATP was assayed in a scintillation vial containing 2 ml of glycylglycine buffer (40 mM glycylglycine, 3 mM $MgSO_4$, pH 7.4) and 0.2 ml of the extract. Partially purified luciferase-luciferin (50 µl) was added. After 15 sec of incubation at room temperature, the light emitted over a period of 6 sec was measured in a Model 3320

Tri-Carb Liquid Scintillation Spectrophotometer. The lower and upper discriminators were set at 60 and 65 divisions, respectively. The gain was set at 100% and the coincidence circuit was off (66). The counts accumulated over the 6 sec period were converted to picomoles of ATP with a standard curve generated by assaying known amounts of ATP.

ADP was calculated by the difference between the untreated extract and that converted with pyruvate kinase. Similarly, AMP was calculated by the difference between the latter value and that obtained by conversion with myokinase and pyruvate kinase. Intracellular nucleotides were determined by subtracting values obtained by assaying culture filtrates. At the time of culture sampling, an aliquot was removed and filtered through a 0.22 μm pore size Millipore filter contained in a Swinnex filter apparatus (Millipore Corp.). Acid extractions and nucleotide determinations were performed as was described for whole cultures.

The treatment of known amounts of ATP, ADP, and AMP by the above methods resulted in recovery of 101, 107, and 91%, respectively. The ATP solution for generating the standard curve consisted of 0.5 mM ATP in 20 mM Tri-SO_4, 2 mM EDTA, pH 7.8. The ATP concentration in this solution was confirmed by enzymatic analysis with hexokinase and glucose-6-phosphate dehydrogenase essentially by the method of Lamprecht (49) by spectrophotometrically monitoring the generation of NADPH at a wavelength of 340 nm. Appropriate dilutions were then made in MOPS buffer, pH 7.4, so that amounts of 0-100 picomoles of ATP were assayed by the luciferase method.

LIST OF REFERENCES

LIST OF REFERENCES

1. Atkinson, D. E. 1968. The energy charge of the adenylate pool as a regulatory parameter, interactions with feedback modifiers. Biochemistry. 7:4030-4034.

2. Atkinson, D. E. 1966. Regulation of enzyme activity. Ann. Rev. Biochem. 35:85-124.

3. Atkinson, D. E. 1969. Regulation of enzyme function. Ann. Rev. Microbiol. 23:47-68.

4. Baltazard, M. 1960. Declin et destn d'une maladie infectiense: la peste. Bull. Wld. Hlth. Org. 23:247-262.

5. Baugh, C. L., A. W. Andrews, and M. J. Surgalla. 1964. Effects of bicarbonate on growth of Pasteurella pestis. III. Replacement of bicarbonate by pyrimidines. J. Bacteriol. 88:1394-1398.

6. Baugh, C. L., J. W. Lanham, and M. J. Surgalla. 1964. Effects of bicarbonate on the growth of Pasteurella pestis. II. Carbon dioxide fixation into oxaloacetate by cell-free extracts. J. Bacteriol. 88:553-558.

7. Beesley, E. D., R. R. Brubaker, W. A. Janssen and M. J. Surgalla. 1967. Pesticins. III. Expression of coagulase and the mechanism of fibrinolysin. J. Bacteriol. 94:19-26.

8. Brubaker, R. R. 1972. The genus Yersinia: biochemistry and genetics of virulence. Curr. Top. Microbiol. 57:111-158.

9. Brubaker, R. R. 1967. Growth of Pasteurella pseudotuberculosis in simulated intracellular and extracellular environments. J. Inf. Dis. 117:403-417.

10. Brubaker, R. R. 1969. Mutation rate to non-pigmentation in Pasteurella pestis. J. Bacteriol. 98:1404-1406.

11. Brubaker, R. R. 1970. Interconversion of purine mononucleotides in Pasteurella pestis. Infect. Immunity. 1:446-654.

12. Brubaker, R. R., E. D. Beesly, and M. J. Surgalla. 1965. Pasteurella pestis: role of pesticin I and iron in experimental plague. Science. 149:422-424.

13. Brubaker, R. R., and M. J. Surgalla. 1963. The effect of Ca^{2+} and Mg^{2+} on lysis, growth, and production of virulence antigens by Pasteurella pestis. J. Infect. Dis. 114:13-25.

14. Brubaker, R. R., M. J. Surgalla, and E. D. Beesley. 1965. Pesticinogeny and bacterial virulence. Zentr. Bakteriol. Parasitenk, Abt. Orig. 196:302-312.

15. Burrows, T. W. 1956. An antigen determining virulence in Pasteurella pestis. Nature. 177:426-427.

16. Burrows, T. W. 1955. The basis of virulence for mice of Pasteurella pestis, pp. 152-175. In: J. W. Howie and A. J. O'Hea (eds.), Mechanisms of microbial pathogenicity. Fifth symposium of the Society for General Microbiology. Cambridge, England: Cambridge University Press.

17. Burrows, T. W. 1962. Genetics of virulence in bacteria. Brit. Med. Bull. 18:69-73.

18. Burrows, T. W. 1960. Biochemical properties of virulent and avirulent strains of bacteria: Salmonella typhosa and Pasteurella pestis. Ann. N.Y. Acad. Sci. 88:1125-1135.

19. Burrows, T. W. 1960. V and W antigens in strains of Pasteurella pseudotuberculosis. Brit. J. Exptl. Path. 41:38-44.

20. Burrows, T. W. 1963. Virulence of Pasteurella pestis and immunity to plague. Ergbn. Mikrobiol. 37:59-113.

21. Burrows, T. W., and G. A. Bacon. 1954. The basis of virulence in Pasteurella pestis: attempts to induce mutation from avirulence. Brit. J. Exptl. Path. 35:129-133.

22. Burrows, T. W., and G. A. Bacon. 1956. The basis of virulence in Pasteurella pestis: an antigen determining virulence. Brit. J. Exptl. Path. 37:481-493.

23. Burrows, T. W. and G. A. Bacon. 1958. The effect of the loss of different virulence determinants on the virulence and immunogenicity of strains of Pasteurella pestis. Brit. J. Exptl. Path. 39:278-291.

24. Cashel, M. 1975. Regulation of bacterial ppGpp and pppGpp. Ann. Rev. Microbiol. 29:301-318.

25. Cavanaugh, D. C., and R. Randall. 1959. The role of multiplication of Pasteurella pestis in mononuclear phagocytes in the pathogenesis of flea-borne plague. J. Immunol. 83: 348-363.

26. Chapman, A. G., and D. E. Atkinson. 1977. Adenine nucleotide concentrations and turnover rates. Their correlation with biological activity in bacteria and yeast. Adv. Microbiol. Physiol. 15:253-306.

27. Chapman, A. G., L. Fall, and D. E. Atkinson. 1971. Adenylate energy charge in Escherichia coli during growth and starvation. J. Bacteriol. 108:1072-1086.

28. Davidson, J. A., and G. H. Flynn. 1974. The assay of ATP by the luciferase method: interference by a bacterial phosphatase enzyme stable to perchlorate treatment. Anal. Biochem. 58:632-637.

29. Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood. 1970. Microbiology. pp. 802-807. Harper and Row, New York, Evanston and London.

30. Delwiche, E. A., G. M. Fukui, A. W. Andrews, and M. J. Surgalla. 1959. Environmental conditions affecting the population dynamics and the retention of virulence of Pasteurella pestis: the role of carbon dioxide. J. Bacteriol. 77:355-360.

31. Ewing, W. H., A. J. Ross, D. J. Brenner, G. R. Fanning. 1978. Yersinia ruckeri sp. nov., the redmouth (RM) bacterium. Int. J. Sys. Bacteriol. 28:37-44.

32. Fanica-Gaignier, M., J. Clement-Metral, and M. D. Kamen. 1971. Adenine nucleotide levels and photopigment synthesis in a growing photosynthetic bacterium. Biochim. Biophys. Acta. 226:135-143.

33. Gajdos, A., and M. Gajdos-Torok. 1969. The quantitative regulation of the biosynthesis of porphyrins by intracellular ATP concentration. Biochem. Med. 2:372-388.

34. Gallant, J., and R. A. Lazzarini. 1976. The regulation of ribosomal RNA synthesis and degradation in bacteria, pp. 309-359. In: E. H. McConkey (ed.), Protein synthesis: a series of advances. Dekker, New York.

35. Hall, P. J., G. C. Yang, R. V. Little, and R. R. Brubaker. 1974. Effect of Ca^{2+} on morphology and division of Yersinia pestis. Infect. Immunity. 9:1105-1113.

36. Hatch, T. P. 1975. Utilization of L-cell nucleoside triphosphates by Chlamydia psittaci for ribonucleic acid synthesis. J. Bacteriol. 122:393-400.

37. Hauschka, P. V. 1973. Analysis of nucleotide pools in animal cells, pp. 361-467. In: D. M. Prescott (ed.), Methods in cell biology, vol. 7. Academic Press, New York.

38. Higuchi, K., L. L. Kupferberg, and J. L. Smith. 1959. Studies on the nutrition and physiology of Pasteurella pestis. III. Effects of calcium ions on the growth of virulent and avirulent strains of Pasteurella pestis. J. Bacteriol. 77:317-321.

39. Higuchi, K., and J. L. Smith. 1961. Studies on the nutrition and physiology of Pasteurella pestis. VI. A differential plating medium for the estimation of mutation rate to avirulence. J. Bacteriol. 81:605-608.

40. Horvath, C., and M. Sovak. 1973. Membrane coarctation by calcium as a regulator for bound enzymes. Biochim. Biophys. Acta. 298:850-860.

41. Hu, P. C., and R. R. Brubaker. 1974. Characterization of pesticin: separation of antibacterial activities. J. Biol. Chem. 249:4749-4753.

42. Jackson, S., and T. W. Burrows. 1956. The pigmentation of Pasteurella pestis on a defined medium containing hemin. Brit. J. Exptl. Path. 37:570-576.

43. Jackson, S., and T. W. Burrows. 1956. The virulence enhancing effect of iron on non-pigmented mutants of virulent strains of Pasteurella pestis. Brit. J. Exptl. Path. 37:577-583.

44. Janssen, W. A., G. M. Fukui, and M. J. Surgalla. 1958. A study of the fate of Pasteurella pestis following intracardial injection into guinea pigs. J. Inf. Dis. 103:183-187.

45. Janssen, W. A., W. D. Lawton, G. M. Fukui, and M. J. Surgalla. 1963. The pathogenesis of plague. I. A study of the correlation between virulence and relative phagocytosis of some strains of Pasteurella pestis. J. Inf. Dis. 113:139-143.

46. Janssen, W. A., and M. J. Surgalla. 1969. Plague bacillus: survival within host phagocytes. Science. 163:950-952.

47. Kight-Olliff, L. C., and J. W. Fitzgerald. 1978. Inhibition of enzyme induction in Pseudomonas aeruginosa by exogenous nucleotides. Can. J. Microbiol. 24:811-817.

48. Kuperman, A. S., M. Okamoto, and E. Gallin. 1967. Nucleotide action on spontaneous electrical activity of calcium deficient nerve. J. Cell. Physiol. 70:257-264.

49. Lamprecht, W., and I. Trautschold. 1963. Adenosine-5'-triphosphate: determination with hexokinase and glucose-6-phosphate dehydrogenase, pp. 543- . In: H. U. Bergmeyer (ed.), Methods of enzymatic analysis. Academic Press, New York.

50. Lawton, W. D., R. L. Erdman, and M. J. Surgalla. 1963. Biosynthesis and purification of V and W antigen in Pasteurella pestis. J. Immunol. 91:179-184.

51. Little, R. V. 1974. Temperature-dependent regulation by Ca^{2+} of macromolecular syntehsis in Yersinia pestis. Doctoral Dissertation. Michigan State University, East Lansing, Michigan.

52. Little, R. V., and R. R. Brubaker. 1972. Characterization of deoxyribonucleic acid from Y. pestis by ethidium bromide-cesium chloride density gradient centrifugation. Infect. Immunity. 5:630-631.

53. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

54. Lundin, A., and Thore. 1975. Comparison of methods for extraction of bacterial adenine nucleotides determined by firefly assay. Appl. Microbiol. 30:713-721.

55. Maaløe, O., and N. O. Kjeldgaard. 1966. Control of macromolecular synthesis. Benjamin, New York.

56. Neidhardt, F. C. 1964. The regulation of RNA synthesis in bacteria. Prog. Nucleic Acid Res. Mol. Biol. 3:145-181.

57. Neilson, R., and H. Rasmussen. 1968. Fractionation of extracts of firefly tails by gel filtration. Acta. Chem. Scand. 22:1757-1762.

58. Nierlich, D. P. 1978. Regulation of bacterial growth, RNA, and protein synthesis. Ann. Rev. Microbiol. 32:393-432.

59. Ogg, J. E., S. B. Friedman, A. W. Andrews, and M. J. Surgalla. 1958. Factors influencing the loss of virulence in Pasteurella pestis. J. Bacteriol. 76:185-191.

60. Papahadjopoulos, D., and A. Portis. 1978. Calcium-induced lipid phase transitions and membrane fusion. Ann. N.Y. Acad. Sci. 308:50-66.

61. Pollitzer, R. 1954. Plague. W.H.O. Monograph series No. 22. World Health Organization, Palais des Nations, Geneva.

62. Report (1966-1970) of the subcommittee of Pasteurella, Yersinia and Francisella to the International Committee of Nomenclature of Bacteria. 1970. Int. J. Sys. Bacteriol. 21:157.

63. Rose, I. A. 1968. The state of magnesium in cells as estimated from the adenylate kinase equilibrium. Proc. Nat. Acad. Sci. 61:1079-1086.

64. Schmidt, G. L., and M. D. Kamen. 1971. Control of chlorophyll synthesis in Chromatium vinosum. Arch. Mikrobiol. 76:51-64.

65. Smith, A. L. 1972. Microbiology and Pathology. C. V. Mosby Co., St. Louis.

66. Stanley, P. E., and S. G. Williams. 1969. Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. Anal. Biochem. 29:381-392.

67. Strehler, B. L. 1968. Bioluminescence assay: principles and practice, pp. 99-181. In: D. Glick (ed.), Methods of biochemical analysis, vol. 16. Wiley, New York.

68. Surgalla, M. J., A. W. Andrews, and C. L. Baugh. 1964. Effects of bicarbonate on the growth of Pasteurella pestis. I. Differential response of virulent and avirulent cells. J. Bacteriol. 88:269-272.

69. Surgalla, M. J., and E. D. Beesley. 1964. Congo red agar plating medium for detecting pigmentation in Pasteurella pestis. Appl. Microbiol. 18:834-837.

70. Swedes, J. S., R. J. Sedo, and D. E. Atkinson. 1975. Relation of growth and protein synthesis to the adenylate energy charge in an adenine-requiring mutant of Escherichia coli. J. Biol. Chem. 250:6930-6938.

71. Thomas, K. C., and P. S. S. Dawson. 1977. Variations in the adenylate energy charge during phased growth (cell cycle) of Candida utilis under energy excess and energy-limiting growth conditions. J. Bacteriol. 132:36-43.

72. Une, Tsutomi. 1977. Studies on the pathogenicity of Yersinia enterocolitica. III. Comparative studies between Y. enterocolitica and Y. pseudotuberculosis. Microbiol. Immunol. 21:505-516.

73. Villadsen, I. S., and O. Michelsen. 1977. Regulation of PRPP and nucleoside tri- and tetra-phosphate pools in Escherichia coli under conditions of nitrogen starvation. J. Bacteriol. 130:136-143.

74. Williams, J. C., and E. Weiss. 1978. Energy metabolism of Rickettsia typhi pools of adenine nucleotides and energy in the presence and absence of glutamate. J. Bacteriol. 134:884-892.

75. Winkler, H. H. 1976. Rickettsial permeability: an ADP-ATP transport system. J. Biol. Chem. 251:389-396.

76. Yang, G. C. H., and R. R. Brubaker. 1971. Effect of Ca^{2+} on the synthesis of deoxyribonucleic acid in virulent and avirulent Yersinia. Infect. Immunity. 3:59-65.

77. Yersin, A. 1894. La peste bubonque a Hong-Kong. Ann. Inst. Pasteur. 8:662-667.

78. Zilinsky, J. W., G. A. Sojka, and H. Gest. 1971. Energy charge regulation in photosynthetic bacteria. Biochim. Biophys. Acta. 42:955-961.