INHIBITION OF THE SYNTHESIS OF DEOXYRIBONUCLEIC ACID IN YERSINIA PESTIS DURING PRODUCTION OF VIRULENCE ANTIGENS

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This is to certify that the

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ABSTRACT

INHIBITION OF THE SYNTHESIS OF DEOXYRIBONUCLEIC ACID IN YERSINIA PESTIS DURING PRODUCTION OF VIRULENCE ANTIGENS

By

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Virulent and potentially virulent cells of Yersinia (Pasteurella) pestis, the causative agent of bubonic plague, were shown by others to produce virulence or V and W antigens (VW^+) but remain static at 37 C during aeration in enriched Ca^{++} -deficient medium containing 0.02 M Mg^{++}. In this environment, which simulates mammalian intracellular fluid VW^+ cells possessed a functional cytoplasmic membrane as judged by concentration of ^{14}C-isoleucine release of ^{32}P, and consumption of oxygen at rates comparable to those of dividing cells cultivated with Ca^{++}. Furthermore, the rates of protein and ribonucleic acid synthesis were essentially identical in both dividing and static VW^+ cells and also in mutant VW^- organisms. However, the synthesis of deoxyribonucleic acid (DNA) ceased in static cells about 4 hr after the removal of Ca^{++}. During this period of time, which corresponded to one generation in the presence of Ca^{++},

the static VW^+ cells completed one chromosomal replication as judged by a two-fold increase in content of DNA and a corresponding degree of resistance to irradiation with ultraviolet light. These findings agree with the results of an independent study which disclosed that the typical static cell possesses at least twice the number of visible nuclei that were observed by direct staining to exist within dividing cells. The static and growing organisms contained essentially identical levels of DNA polymerase.

Accordingly, the static organisms appeared to be unable to initiate septation or to synthesize new DNA following termination of an initial replication that was in progress when Ca^{++} was removed. These metabolic blocks appear to be caused by thermo-inactivation of the membrane-associated proteins that are required for the initiation of DNA replication and the formation of septae.

INHIBITION OF THE SYNTHESIS OF DEOXYRIBONUCLEIC ACID IN *YERSINIA PESTIS* DURING PRODUCTION OF VIRULENCE ANTIGENS

By

Gene Ching-hua Yang

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DEDICATION

This thesis is respectfully dedicated to my mother, Wang Lan, who instilled in me the importance of education, and to my wife, Alice, whose patience and understanding made this achievement possible.

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CHAPTER I

INTRODUCTION

Investigation of the metabolism of obligate intracellular parasites has yielded considerable information regarding the reasons why these organisms are restricted to growth within the host cell. The finding that chlamydiae and viruses evidently lack the ability to produce adenosine triphosphate (ATP) can itself explain why these organisms are restricted to an intracellular existance (97, 116). Plasmodia and Rickettsia, on the other hand, can produce ATP via the tricarboxylic acid (TCA) cycle (95, 117, 119); therefore the restriction of these organisms to an intracellular location is due to other factor(s). It has been suggested that a lack of membrane regulatory capacity is the primary cause of this restriction since the cytoplasmic membranes of rickettsial or plasmodial cells are freely permeable to various phosphorylated organic compounds (6, 91). However, the recent finding of Myers, Provost and Wissman (92) demonstrates that rickettsial cell membranes are easily damaged by the standard procedure for purification of these parasites from tissue culture cells. This observation may indicate that leakage of metabolites observed by previous workers is the result of unavoidable

damage associated with the process of preparation. The search for a suitable explanation for restriction to living mammalian cells thus continues.

Moulder (91) suggested that the dependence of the obligate intracellular parasites on the host cell and the highly restricted ability of these organisms to parasitize such cells are presumably closely related phenomena. Therefore, gaining an understanding of one of these mechanisms should greatly facilitate the elucidation of the other. In order to minimize the difficulties associated with the cultivation and purification of obligate intracellular parasites, one line of investigation has focused on the metabolic study of facultative intracellular parasites of the genus Yersinia (Pasteurella). These organisms exhibit distinct responses in synthetic media which simulate the intra- and extracellular environments of mammalian tissue.

When cells of wild type Y. pestis or Y. pseudotuberculosis are incubated at 37 C in an enriched chemically defined medium containing 0.02 M Mg^{++} but no added Ca^{++}, they produce the V and W antigens (VW^{+}) of Burrows and Bacon (22), but remain static. This medium simulates mammalian intracellular fluid with respect to the ion concentrations of magnesium and calcium (70). However, the addition of Ca^{++} (0.0025 M) to such a medium promotes cellular division but suppresses the production of V and W antigens (16, 53). Mutants which lack the ability to

produce V and W antigens are avirulent and do not require Ca^{++} for growth at 37 C (21).

Brubaker (8) suggested that bacterial-stasis with the expression of the V and W antigens, and cell division with apparent repression of these antigens may reflect metabolic patterns which are essential for intra- and extracellular growth, respectively. Therefore, identification of the nature of the metabolic block in static VW^{+} cells, which occurs upon cultivation in the simulated intracellular environment, should not only lead to a greater understanding of virulence in Yersinia but should also be valuable in elucidating the metabolic blocks of some obligate intracellular parasites during in vitro cultivation.

A definition of the physiological block in static VW^{+} cells is presented in this dissertation.

CHAPTER II

LITERATURE REVIEW

Taxonomy and Morphology

The genus Pasteurella comprises the etiological agents responsible for pasteurellosis (hemorrhagic septicemia), plague, pseudotuberculosis, and tularemia. Although these agents share superficial morphological, staining, and biochemical properties, there are several fundamental distinctions between them. These include differences in nutritional requirements (88), antigenic properties (118), and lack of deoxyribonucleic acid (DNA) homology except between organisms of plague and pseudotuberculosis (97). Due to these differences, it has been suggested by several workers (79, 87) that a separate genus, Yersinia, be established for the organisms of plague and pseudotuberculosis. Stocker (108) pointed out that this new genus Yersinia is taxonomically related to the Enterobacteriaceae. His conclusion was based on the findings that Yersinia and Salmonella share antigenic properties (67) and that bacteriophages of Yersinia also lyse species of Escherichia, Shigella and Salmonella (52, 77, 104, 108). Martin and Jacob (84) and Lawton, Morris and Burrows (76) demonstrated the transfer

of the F-lac episome from E. coli to Y. pestis and Y. pseudotuberculosis, respectively.

Y. pestis was isolated by Yersin in 1894. The organism is a gram-negative ovoid bacillus, o.5 to 0.8 micron wide and 1.5 to 2.0 microns long (118). Unlike Y. pseudotuberculosis, this organism lacks flagella and is not motile (88). Y. pestis is divided into three physiological varieties: orientalis, antiqua and mediaevalis, based on the organism's ability to ferment glycerol and to reduce nitrate to nitrite (118).

Carbohydrate Metabolism

Due to a deficiency of glucose-6-phosphate dehydrogenase (7, 89 90), Y. pestis utilizes the Enbden-Meyerhof pathway almost exclusively to ferment hexoses (101). Gluconate is readily metabolized by a combination of Weimberg and Wolochow (78) reported that Y. pestis decarboxylates pyruvate to acetate, which is then completely oxidized to CO_2 via the TCA cycle (34, 35, 36). Anaerobically grown cells are unable to oxidize acetate; however, upon aeration for a brief period of time, anaerobically grown cells become adapted to aerobiosis and then oxidize acetate readily.

Growth and Virulence

Virulent strains of Y. pestis are usually defined as those having average lethal doses for mice and guinea pigs

of less than 10 cells following subcutaneous or intraperitoneal injection (20). Due to its high degree of pathogenicity, Y. pestis has been used by many workers as a model for the study of virulence.

The optimum temperature for in vitro cultivation of Y. pestis is 27 C for the majority of pathogens. At room temperature, these organisms have less complex nutritional requirements than at 37 C (23, 30, 50, 55).

The influence of culture conditions on the loss of virulence in Y. pestis has frequently been noted. Fukui et al. (40) found that cultivation in aerated broth medium at 37 C consistently resulted in a loss of virulence. This process of attenuation was attributed to selective conditions favoring growth of avirulent mutants which normally persist in the virulent inoculum (40, 94). Several effective treatments, at least for the initial 24 hr incubation period, have been shown to prevent this population shift. These processes include lowering the incubation temperature to 26 C (39), addition of spent culture filtrate of avirulent mutants, adjustment of the initial culture medium to pH 7.8 (94), or the addition of compounds such as $NaHCO_3$, pyrimidine (3), 2.4 dinitro phenol, potassium iodide, salicylate ions and biliverdine which all act to selectively retard the growth of VW^- organisms (28, 110). The shift to avirulence may also be prevented by addition of Ca^{++}, Sr^{++}, Zn^{++} (53), KCN, potassium oleate and sodium

deoxycholate which act by selectively stimulating the growth of VW^+ cells (111). As noted by Surgalla, Andrews and Cavanaugh (110), all of these substances with the exception of $NaHCO_3$ and pyrimidine, could directly interact with cell membrane. The CO_2 requirement for the *in vitro* cultivation of virulent, but not avirulent, strains was also confirmed by Burrows and Gillett (23) in nutritional experiments utilizing chemically defined solid media.

Fukui *et al.* (38) showed that the phenotypic expression of virulence was repressed when virulent cells were grown at temperatures below 26 C. This repression, however, was reversed by raising the temperature to 37 C for 6 hr. Further investigation revealed that restoration of virulence required vigorous aeration and a high concentration of an adequate nitrogen and energy source (93). Other findings presented by Fukui, Lawton and Mortlock (39) indicated that the expression of maximal virulence required *de novo* synthesis of ribonucleic acid (RNA) and protein, but not DNA. These results appear to be consistent with the finding of Baugh, Andrews and Surgalla (3), that pyrimidines are required for retention of virulence during aeration at 37 C.

Virulence Determinants

Compared to the intensive and fruitful studies on general bacterial genetics, the effort devoted to the genetic study of virulence determinants of pathogens has been minimal. The major difficulty has been in finding an adequate host

and parasite system which would permit the use of the mutant approach to study virulence properties. The problem, in part, has been caused by lack of laboratory facilities suitable for this type of research. In spite of such limitations, a number of virulence determinants have been discovered in Y. pestis in the last 20 years.

Fl^+ determinant

Baker et al. (2) isolated several protein antigens from the surface envelope of virulent Y. pestis. Strains that are genetically capable of synthesizing fraction one (Fl) of these capsular antigens are designated Fl^+. Mutants which lack the ability to produce this antigen (Fl^-) are of reduced virulence in guinea pigs but not mice (109). The Fl antigen is produced at 27 C and 38 C (21), but its accumulation on the cell surface only occurs at 37 C. This seems to indicate that the Fl^+ state is determined by two gene loci--one determining Fl antigen production, the other its expression as the surface envelope. Spontaneous mutation from Fl^+ to Fl^- occurs readily, but no case of back mutation has yet been reported. The role of the Fl antigen is unknown. However, Burrows (21), and Englesberg et al. (33) found that Fl^+ strains were more resistant to phagocytosis than Fl^- strains. This statement is challenged by the recent finding of Janssan and Surgalla (66) who showed that both virulent and avirulent cells were phagocytized at equal rates in mice.

VW$^+$ determinant

The search for a second virulence determinant stemmed from the finding that Fl$^-$ strains remain virulent in mice. Burrows and Bacon (22) demonstrated that all virulent strains produce V and W antigens, and may thus be termed VW$^+$. Mutants which are unable to produce these gene products (VW$^-$) are avirulent for mice and for guinea pigs. The V and W antigens were partially purified by Lawton, Erdman and Surgalla (74) who reported that the former was a protein and the latter was a lipoprotein; both were easily extractable from the culture medium. Virulent strains always produce these two antigens together and mutants producing one without the other have not been isolated. Most of the nutritional requirements and culture conditions previously described under Growth and Virulence are found to be associated with production of V and W antigens (28, 38, 39, 94). Janssen and his coworkers showed that the VW$^+$ determinant is associated with survival and multiplication of virulent cells within fixed macrophages of the host reticuloendothelial system (65, 66). The mutation rate from VW$^+$ to VW$^-$ is 10^{-4} per bacterium per generation (54).

The inhibitory effect of Ca^{++} on the production of V and W antigens in vitro was noted by Brubaker and Surgalla (16). Lawton (73) had earlier demonstrated that maximal production of V and W antigens occurred at 37 C in aerated enriched medium containing 0.02 M Mg^{++}, but no added Ca^{++}.

When VW^+ cells were grown under these conditions, they became elongated (16) but remained static (53). These virulent static cells could initiate growth in the absence of Ca^{++} if the culture medium was adjusted in such a way that its ionic strength was increased and the osmolarity was decreased (8). Brubaker and Surgalla (15) isolated an avirulent mutant which retained all other virulence characters but did not require Ca^{++} for growth at 37 C. This result clearly established the fact that the Ca^{++} requirement is an essential virulence property.

P^+ determinant

Virulent strains grown on solid synthetic media containing hemin form dark pigmented colonies (P^+). Mutants which form light, non-pigmented colonies (P^-) on the same medium are avirulent (63). Colony-pigmentation readily occurs at 26 C but not at 37 C. Like the Fl^+ and VW^+ determinants, no back mutation from P^- to P^+ has yet been observed. However, the full virulence of P^- cells can be restored if Fe^{++}, Fe^{+++}, or hemin is simultaneously injected with cells into the mice (64). Therefore P^- strain is usually described as potentially virulent cells (16). The role of iron is unknown, but it has been suggested that the ability to acquire iron *in vivo* is associated with the expression of virulence. To date, it is still unknown whether the P^+ determinant is directly concerned with virulence or indirectly related to some other virulence

determinant. Recently, Surgalla and Beesley (112) have developed a new technique for detection of colony pigmentation by substituting Congo red for Hemin. They suggested that basic residues of protein or mucopolysaccharide of P^+ cells may serve as the binding sites for chromogens. The mutation rate from P^+ to P^- is 10^{-5} per bacterium per generation (11).

Pu^+ determinant

Purine auxotrophs (Pu^-) of Y. pestis are of reduced virulence since the availability of purines in host cells is limited (19). This nutritional requirement for purines as a virulent determinant has also been characterized in Salmonella typhi (19), Klebsiella pneumoniae (41), and Bacillus anthracis (62). Concomitant injection of purines, but not iron, restored full virulence of Pu^- strains in mice (21). Mutants which were incapable of synthesizing inosine monophosphate by the de novo pathway retained a considerable degree of virulence in mice, whereas strains which could convert inosine monophosphate to guanosine monophosphate were completely avirulent (10).

PI^+ determinant

At least two types of bacteriocin-like substances are produced by wild type Y. pestis. The first, designated pesticin I, inhibites the growth of certain strains of Y. pseudotuberculosis (5, 51), E. coli, and pesticin-deficient

(PI^-) mutants of Y. pestis (13). Brubaker and Surgalla (13, 14) demonstrated the presence of a second type of pesticin, pesticin II, which is also produced by Y. pseudotuberculosis. The activity of pesticin I, but not pesticin II, can be supressed by Fe^{+++}, Mg^{++}, inorganic phosphate, hemin and an acid soluble metabolite (termed pesticin I inhibitor) produced by the pesticin I forming (PI^+) cells. Whether these cations and hemin affect pesticin I directly or indirectly by either stimulating the production or activation of pesticin I inhibitor is still unclear. Suppression of the activity of pesticin I by Fe^{+++} or pesticin I inhibitor can be reversed by the addition of either Ca^{++} or chelating agents (14). Brubaker and his coworkers (4, 17) found that all PI^+ strains are capable of synthesizing coagulase and fibrinolysin, whereas PI^- strains are devoid of these two biological activities. Since fibrinolysin and coagulase are important invasive factors of pathogens, they speculated that the PI^+ determinant is another bona fide virulence determinant. This suggestion was confirmed in a later report (12) by the isolation of an avirulent mutant, strain G 32, which was positive for all known virulence determinants except PI^-. Simultaneous injection of iron also restored the full virulence of the PI^- avirulent mutant in mice (21).

The optimum temperature for pesticin I production is 26 C (5). Brubaker (11) found that most pesticin I-sensitive

strains are $PI^{-}P^{+}$. When the organisms mutate to PI^{-} P^{-}, they concomitantly lose sensitivity to pesticin I. As a result of this correlation, pesticin I was used as the selective agent to estimate the mutation rate of P^{+} to P^{-}.

Control of Replication and Cell Division

Although the biochemical nature of the control of cell division is not well understood, the sequential events of this process have been characterized in E. coli as follows: 1) the cell replicates DNA and forms two sets of nuclei; 2) the cell elongates and the newly formed chromosomes are progressively segregated; 3) a "septum" is formed near the equatorial plane; 4) two daughter cells, each containing a complete genome, physically separate from each other, and the new cycle is re-initiated. For organisms which have short generation times, new rounds of replication are initiated before the occurrence of division (24).

Helmstetter (48) divided the entire cycle into a three step process, I+C+D, representing the periods required for synthesis of an "initiator complex" (I), completion of a round of replication (C), and that occurring between the end of a round of replication and the following cell division (D). He showed experimentally that the length of the C+D period for E. coli growing exponentially at a defined temperature is constant. Accordingly the generation time of this organism in a particular medium is determined by the period I.

The "initiator complex" appears to comprise at least two pieces of heat labile membrane-associated protein, which also function in the regulation of nuclear segregation (32, 72, 101). Initiation of a new round of replication in cells with a short generation time is not affected by the completion of the previous round (49, 81), but is regulated by a crucial value of the cellular mass/DNA ratio (29, 82), or the availability of chromosome attachment sites (72).

Evidently, the process of cellular division in gram-positive and gram-negative organisms is different. In the former, cell wall synthesis is initiated equatorially and peripherally at the same time. Thus the new portions of the daughter cells develop adjacently, and the old ends of parental cells remain intact but are gradually pushed apart (83). The formation of cross wall appears to be associated with mesosome (98). In gram-negative cells, on the other hand, the insertion of new wall material occurs along the entire cell surface. The peripheral wall and cytoplasmic membrane grow inwards, resulting in a pinching-off effect. However, under special conditions of growth and fixation, the formation of septae have been demonstrated in cells of E. coli (107). The role of mesosomes in the cell division of gram-negative organisms has not been characterized (98).

The initiation of septum formation, under normal conditions, is obviously coordinated with DNA replication. If DNA synthesis is arrested during the process of replication, the viability of the cells declines rapidly. On the other

hand, if the inhibition occurs after completion of a round of replication, the cells divide once and then die (24, 49, 85). These observations suggest a very appealing mode of regulation; namely, that septation does not occur in cells which cannot complete replication of their chromosomes, whereas cells which have completed this replication can divide without further DNA synthesis. In this manner each daughter cell is assured of a complete genome.

Under abnormal growth conditions, however, some mutants of E. coli and B. subtilis deviate from this regulatory pattern (44, 57, 86). For example, when thermosensitive mutants of E. coli strains CRT 115 and PAT 84 are incubated at 40 C, they continue to synthesize DNA and other macromolecules but do not divide. As a result, multinuclear filaments are produced (57). On the other hand, strain PAT 421, while unable to synthesize DNA at the impermissive temperature, can still form a septum to one side of the chromosome. Consequently some anucleated daughter cells are produced (56). It would appear from these observations that cellular division and DNA replication are not coordinated.

In addition to completion of chromosome replication, septation also requires an accumulation of a heat sensitive membrane-associated protein which may be different from the components of "initiator complex" (61, 105). So far, this hypothetical protein can only be demonstrated indirectly.

Septum formation is also regulated by the biosynthesis of cell walls. Inhibition of peptidoglycan formation by cycloserine results in the inhibition of cell division of Erwinia (45). This inhibition, however, can be overcome by the addition of Ca^{++}, Mn^{++}, D-alanine and pantoyl lactone. The mechanisms whereby these compounds promote growth are not understood with certainty.

CHAPTER III

METHODS

Bacterial Strain

Unless otherwise stated, *Y. pestis* strain EV76 was used throughout the course of this investigation. Strain EV76 possesses all of the virulence determinants of the species except P^+. A VW^- mutant was selected on the magnesium oxalate (MGOX) agar of Higuchi and Smith (54). Stock cultures were preserved in buffered glycerol at -20 C as described by Brubaker (8).

Cultivation and Culture Media

Following incubation for 2 days at 26 C on slopes of blood agar base (BAB), the organisms were centrifuged at 4 C for 10 min in a Sorvall RC2-B refrigerated centrifuge at 27,000 x *g*, washed once with 0.033 M potassium phosphate buffer, pH 7.0 (phosphate buffer), and inoculated at a concentration of about 3 to 5 x 10^8 cells per ml into a 250 or 500 ml Erlenmyer flask containing 20 or 50 ml of a modification of the chemically defined medium of Higuchi, Kupferberg, and Smith (53). The composition of this synthetic medium is described in Appendix A of this dissertation. Cultures were aerated at 37 C on a model BB wrist

action shaker (Burrell Corp.) for specific intervals. To permit the growth of VW^+ cells, 0.0025 M $CaCl_2$ was included in the medium. Appropriate steps, including routine plating on BAB and MGOX agar, were taken to assure that cultures were not contaminated or overgrown with VW^- cells.

Measurement of Growth

Optical absorbance was determined at 620 nm with a Beckman DU spectrophotometer using appropriate uninoculated medium as blank. Assays of viable cells were performed by spreading 0.1 ml of culture, appropriately diluted in the phosphate buffer, on BAB plates; the latter were incubated for 2 days at 26 C.

Characterization of Virulence Determinants

V and W Antigen Production

The immunodiffusion technique of Lawton, Fukui and Surgalla (75) was used to estimate these antigens qualitatively. The overnight culture of strain EV76 aerated at 37 C in an enriched medium containing 0.02 M Mg^{++} but no added Ca^{++} with a concentration of 10^9 cells per ml, was used as a source of antigens. The monospecific antisera against V or W antigen were kindly provided by Dr. M. J. Surgalla.

Pesticin I Production

Cells of Y. pestis strains EV76 and Y. pseudotuberculosis strain PB1/+ (a PI^- strain) were spotted on a BAB plate and incubated at 26 C for 2 days. In order to kill the producer cells, the plate was exposed to chloroform vapors for about 10 min, and then overlaid with 5 ml of sterile 4% BAB containing 0.1 M $CaCl_2$, 0.5% Ca-ethylenediaminetetraacetic acid (Ca-EDTA) seeded with 10^5 cells of Y. pseudotuberculosis strain PB1/0 (a pesticin-sensitive strain). The zones of inhibition were observed after incubating the plate at 37 C for 24 hr.

Pigmentation

The technique of Surgalla and Beesley (112) was used. The agar medium contained 1% heart infusion broth, 2% agar (BBL), 0.01% Congo red and 0.2% D-galactose. Colony pigmentation was observed after 2 days of incubation at 26 C.

Measurement of Amino Acid Uptake

Cells of strain EV76 (VW^+), incubated in synthetic medium (with or without Ca^{++}) at 37 C for 12 hr, were washed, resuspended in phosphate buffer containing 0.025 M potassium gluconate and 10 uM ^{14}C-L-isoleucine (2 uC per umole), and continuously incubated at 37 C in a model G76 gyrotory water bath shaker (New Brunswick Scientific). Samples of 1 ml were withdrawn at brief intervals, immediately

filtered through a 0.45 u pore millipore filter membrane, and the residue was washed twice with 3 ml of prewarmed phosphate buffer. After the filters had been dried at 80 C for at least 60 min, the radioactivity was measured in a Mark I liquid scintillation spectrometer (Nuclear Chicago Corp.). The counting fluid consisted of 10 ml of a mixture of 0.4% 2,5-diphenyloxazole (PPO) and 00.5% 1,4-di-[2-(5-phenloxazolyl)]-benzene (POPOP) in toluene. The control experiments indicated that there was no significant quenching and the counting efficiency was constant in all of the samples.

Measurement of Oxygen Uptake

The standard manometric technique of Umbreit, Burris, and Stauffer (113) was employed. Cell suspensions were prepared with a procedure similar to that just described except that the cells were suspended in distilled water. The reaction mixture consisted of 0.1 ml of D-gluconate (o.1M), 0.3 ml of potassium phosphate buffer (o.5 M, pH 7.0), and 2.5 ml of cell suspension. The center well contained 0.1 ml of 10 N KOH. Prior to introduction of substrate, organisms were shaken for 30 min in the buffer in order to reduce the endogenous metabolism.

Leakage of Radioactive Compounds

The ability of dividing and static cells to retain internal compounds containing ^{32}P was determined by

cultivating the VW^+ organisms for 12 hr at 26 C in the synthetic medium containing $KH_2^{32}PO_4$ (0.4 uC per mole). Following this process of preloading, the organisms were washed 3 times in non-radioactive phosphate buffer containing 0.07 M NaCl in order to remove unchangeable isotope and then incubated in the synthetic medium, in the presence or absence of Ca^{++}, at 37 C. Samples of 1 ml were then removed at 90 min intervals, filtered through 0.45 u pore membrane filters, and, after drying, the radioactivity in the filtrate was estimated on the aluminum planchets with a model 447 gas flow counter (Nuclear Chicago Corp.).

Protein Synthesis

The synthesis of protein was estimated by measuring the incorporation of ^{14}C-L-isoleucine into cold trichloroacetic acid precipitable fraction. In order to increase the specific activity of radioactive isoleucine, cells that had been cultivated in the synthetic medium for 12 hr at 37 C were resuspended in the prewarmed fresh medium containing 0.15 mM ^{14}C-isoleucine (1.3 uC per umole) instead of the normal concentration. Samples of 1 ml were removed at 10 min intervals from the cell culture, mixed with 1 ml of cold 10% trichloroacetic acid containing 50 ug per ml of non-radioactive isoleucine and incubated at 4C for at least 30 min. The contents were filtered through 0.45 u millipore membrane filters and washed with 5 ml of cold 5% trichloroacetic acid. The membranes were mounted on aluminum

planchets, dried, and the radioactivity was estimated in the gas flow counter.

RNA Synthesis

Essentially a procedure similar to the estimation of protein was used to determine the synthesis of RNA. In this case, 0.44 uM ^{14}C-uracil (1.0 uC per umole) was added to 12 hr cultures without changing the medium. In order to minimize the incorporation of radioactivity into DNA, non-radioactive cytosine was added to the culture at a concentration of 20 ug per ml (71). Samples were collected and the radioactivity was determined by the procedures used for estimation of protein synthesis.

DNA Synthesis

Due to the inability of wild type Yersinia to accumulate thymine or thymidine, the synthesis of DNA was determined with ^{14}C-uracil by a pulse labeling procedure similar to those of Goss, Deitz and Cook (43). Radioactive uracil (1 uC per umole) at a concentration of 0.05 mM was added at various intervals to the cultures undergoing incubation at 37 C. Samples of 1 ml, withdrawn at 10 min intervals, were added to the tubes containing 0.1 ml of 5.5 N NaOH; the latter were sealed with parafilm (Marathon) and incubated for 18 hr at 37 C. The contents were neutralized and precipitated by adding 0.1 ml 6 N HCl and 1.2 ml cold 10% trichloroacetic acid containing non-radioactive uracil

(50 ug per ml) and incubated at 4 C for at least 30 min. The samples were filtered, washed with 5% trichloroacetic acid and the radioactivity was determined either by a gas flow or liquid scintillation counter as described in the preceding experimental methods. RNA but not DNA is hydrolyzed by this procedure (102), thus the radioactive uracil of RNA appeared in the filtrate and the radioactive thymine of DNA remained on the membrane filter.

Sensitivity to Ultraviolet Irradiation

The procedures of ultraviolet (UV) sensitivity tests were similar to those of Howard-Flanders, Simson and Theriot (58). Cells that were incubated in the synthetic medium at 37 C for 12 hr were washed and re-inoculated into phosphate buffer at a concentration of 1 to 5 x 10^7 cells per ml. UV irradiation was performed in a box fitted with a shutter with a standard glass petri dish containing 6 ml of cell suspension (2mm in depth) with light from a 30 watt General Electric germicidal lamp at a distance of 50 cm from the surface of platform. The cell suspension was constantly swirled at 26 C during the irradiation period. Dilutions for plating were made in the buffer under a dark environment and the inoculated plates were incubated at 26 C for 2 days. Intensity of the light, as estimated by comparing data from E. coli strain K12 with those of Howard-Flanders, Simson and Theriot (58) was about 13 ergs per mm^2 per sec.

Quantitative DNA Determination

A modified procedure of Copeland (25) was used to extract DNA from the cell suspension. The nucleic acids of the cell suspension were precipitated by adding 1 volume of cold 10% trichloroacetic acid and incubated at 4 C for 30 min. After the acid was decanted following centrifugation, the precipitates were resuspended in 1 volume of 1 N KOH and incubated at 37 C for 15 to 18 hr. The hydrolysates were neutralized and precipitated by adding 4/10 volume of a cold acid mixture (0.28 ml 1 N HCl, 0.62 ml 10% trichloroacetic acid and 0.1 ml H_2O) and incubated in an ice bath for 1 hr. Pellet and supernatant fluid were separated by centrifugation at 27,000 x g. DNA content of the pellet was determined by the method of Giles and Myers (42) using highly polymerized calf thymus (Calbiochem) as standard. Total cell counts were determined by use of a Petroff-Hausser counting chamber.

DNA Polymerase Assay

The modified procedures of Richardson (96) were employed. The reaction mixture contained 60 ul of 0.35 M glycine buffer (pH 9.2). 3.5×10^{-2} M $MgCl_2$, 5 mM 2-mercaptoethanol; 30 ul of 2-deoxythymidine-5-triphosphate (dTTP), 2 deoxycytidine-5-triphosphate (dCTP), 2-deoxyguanosine-5-triphosphate (dGTP), 0.1 umole each, ^{3}H-2-deoxyadenosine-5-triphosphate (37 uC per umole; ^{3}H-dATP), 0.1 umole; 30 ul of calf thymus "activated"

DNA; and the appropriate volume of extract and H_2O to make a total volume of 300 ul. The reaction mixture was incubated at 37 C for 30 min and the reaction was terminated by adding 5 ml of cold 10% trichloroacetic acid containing 1% sodium pyrophosphate. After incubating in an ice bath for 10 min, the mixture received 2 drops of carrier DNA (salmon sperm) with agitation and was then filtered through a 0.45 u pore Scheicher and Schuell nitrocellulose filter membrane. The membrane was dried and the radioactivity was determined with a liquid scintillation counter. The control determinations which consisted of a series of reaction mixtures lacking enzyme extract, dTTP, and "activated" DNA, respectively, were treated similarly. The calf thymus "activated" DNA was prepared by partial enzymatic degradation of DNA (96). The reaction mixture contained 0.2 ml calf thymus DNA (2.3 mg per ml); 0.1 ml pancreatic deoxyribonuclease (5×10^{-3} ug per ml; DNAse); 0.1 ml tris buffer, pH 8.0 (0.5 M); and 0.4 ml H_2O. The mixture was incubated at 37 C for 15 min and was then heated at 80 C for 15 min to terminate the DNAse activity. The degraded DNA was kept frozen until use. Protein was estimated by the Folin-phenol method of Lowry et al. (80) using bovine serum albumin as standard.

Effect of Ca^{++} on Initiating Growth of Static Cells

VW^{+} cells were preincubated at 26 C for 12 hr in synthetic medium on a model R25 gyrotory shaker (New Brunswick Scientific), resuspended in prewarmed fresh medium and then incubation was continued at 37 C. At time intervals, 0.0025 M $CaCl_2$ was added to the individual cell cultures and 1 ml samples were withdrawn for the estimation of viable cells.

CHAPTER IV

RESULTS

Characterization of Virulence Determinants

The results of Table 1 confirmed that Y. pestis strain EV76 produced V and W antigens and pesticin I but was non-pigmented, whereas Y. pseudotuberculosis strain PB1/+ produced V and W antigen but not pesticin I. Since the temperature-dependent nutritional Ca^{++} requirement is only associated with VW^{+} character (8), the results indicated that both organisms were suitable for use in the subsequent study. The results also indicated that Ca^{++} -independent mutants of strains EV76 and PB1/+ were VW^{-}.

Growth Pattern

A lag period of about 6 hr occurred before growth of VW^{+} cells of Y. pestis commenced in cultures containing Ca^{++} (Fig. 1). Following further incubation for 6 hr, such organisms had entered the exponential growth phase whereas stasis was completed by this time in parallel cultures lacking added Ca^{++}. Accordingly, cells used in subsequent experiments were preincubated for 12 hr in order to permit the expression of maximum phenotypic differences related to growth and stasis. The generation times of growing VW^{+}

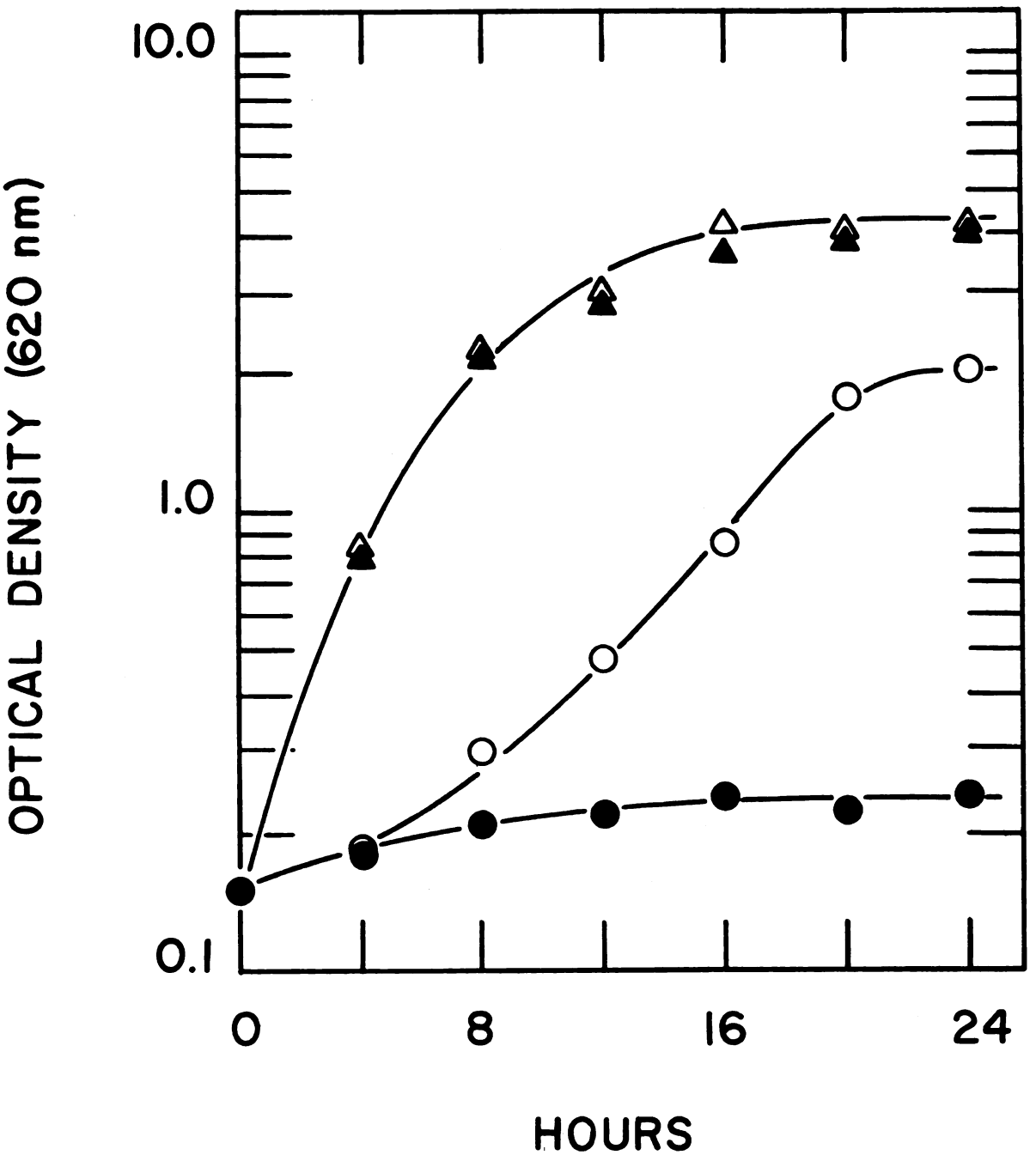
TABLE 1.--Characterization of virulent determinants in strains of Y. pestis and Y. pseudotuberculosis.

	VW$^+$	PI$^+$	P$^+$
Y. pestis			
EV76 (prototroph)	+[a]	+	0
EV76 (selected on MGOX[b])	0	+	0
Y. pseudotuberculosis			
PB1/+ (prototroph)	+	0	-
PB1/0 (selected on MGOX)	0	0	-

[a]Symbols +, 0, and - represent positive, negative, and not tested, respectively.

[b]Magnesium oxalate agar (MGOX).

Fig. 1. Growth of Y. pestis strain EV76 in the modified synthetic medium of Higuchi, Kupferberg and Smith following inoculation at a level of 3×10^8 cells per ml; (●) VW^+ cells without added Ca^{++}, (○) VW^+ cells plus 0.0025 M Ca^{++}, (▲) VW^- cells without added Ca^{++}, and (△) VW^- cells plus 0.0025 M Ca^{++}.


10.0
1.0
0.1
OPTICAL DENSITY (620 nm)
0
8
16
24
HOURS

and VW^{-} cells, as estimated from Figure 1, are 4 and 3 hr respectively.

The finding of Higuchi, Kupferberg and Smith (53) was verified that the synthetic medium was toxic to Y. pestis at inocula of less than 10^8 cells per ml (not illustrated). This toxicity was not observed in the case of Y. pseudotuberculosis, which could initiate growth without lag from inocula of 10^7 VW^{+} cells per ml (Fig. 2). Unlike VW^{+} cells of Y. pestis, those of Y. pseudotuberculosis were able to undergo about 3 divisions in Ca^{++}-deficient medium before growth ceased. This phenomenon was even more pronounced when a high inoculum was used (not illustrated). The VW^{-} cells of both species did not show the Ca^{++}-requirement for growth. This observation is consistent with the results shown in Table 1.

Effect of Antibiotics on the Growth of VW^{+} Cells

Penicilin was introduced into the cultures in order to show with certainty that stasis was a property of all VW^{+} organisms within the population rather than a function of growth with concomitant lysis. As shown in Figure 3, static VW^{+} cells were not significantly influenced by as much as 250 units of penicillin per ml of medium, whereas organisms growing in the presence of Ca^{++} and penicillin were rapidly killed. These results demonstrate that there was no significant turnover within the population of static VW^{+} cells.

Fig. 2. This figure represents the repetition of the experiment of Figure 1 with Y. pseudotuberculosis strain PB1; the inocula were 2×10^7 cell per ml.

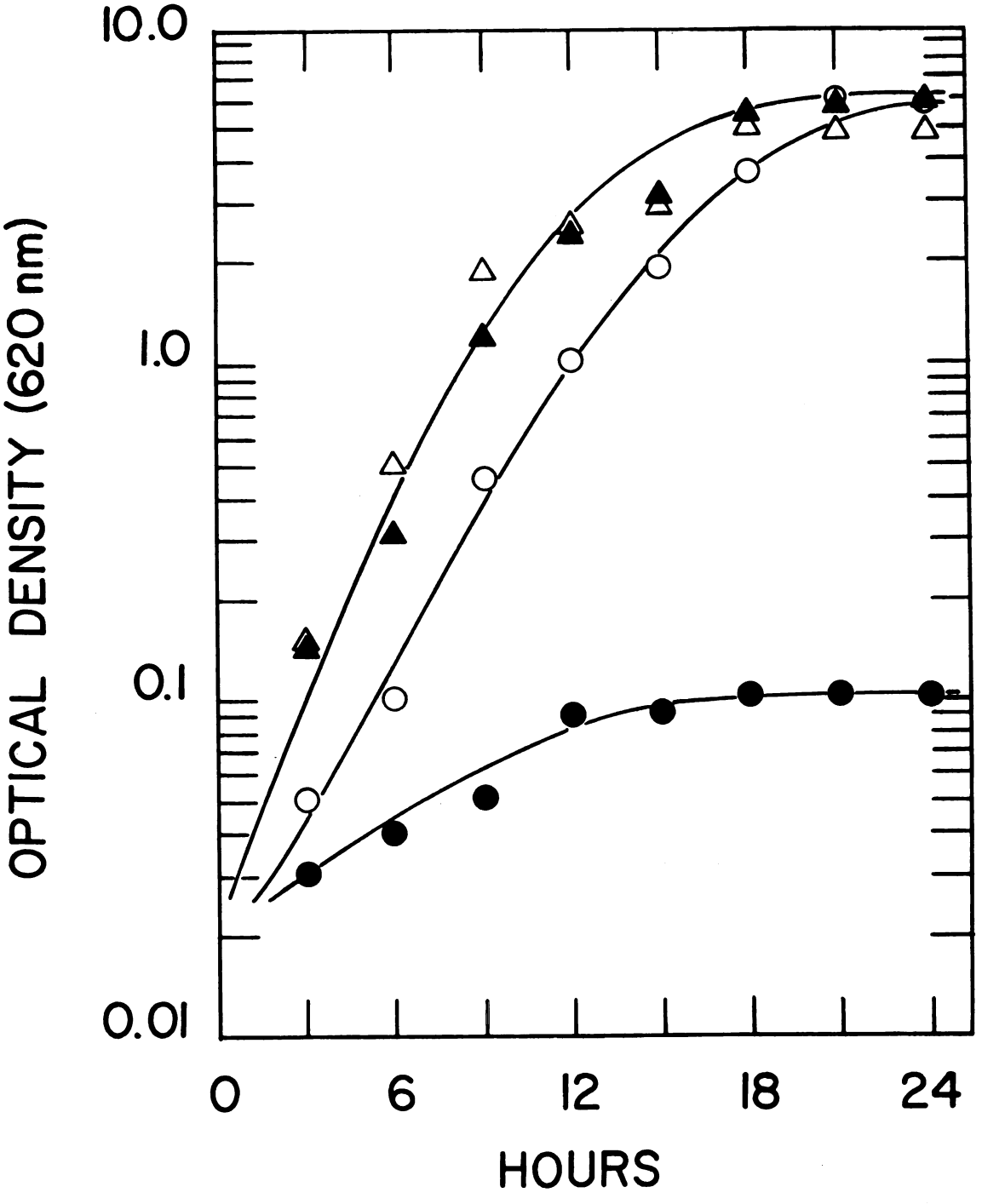
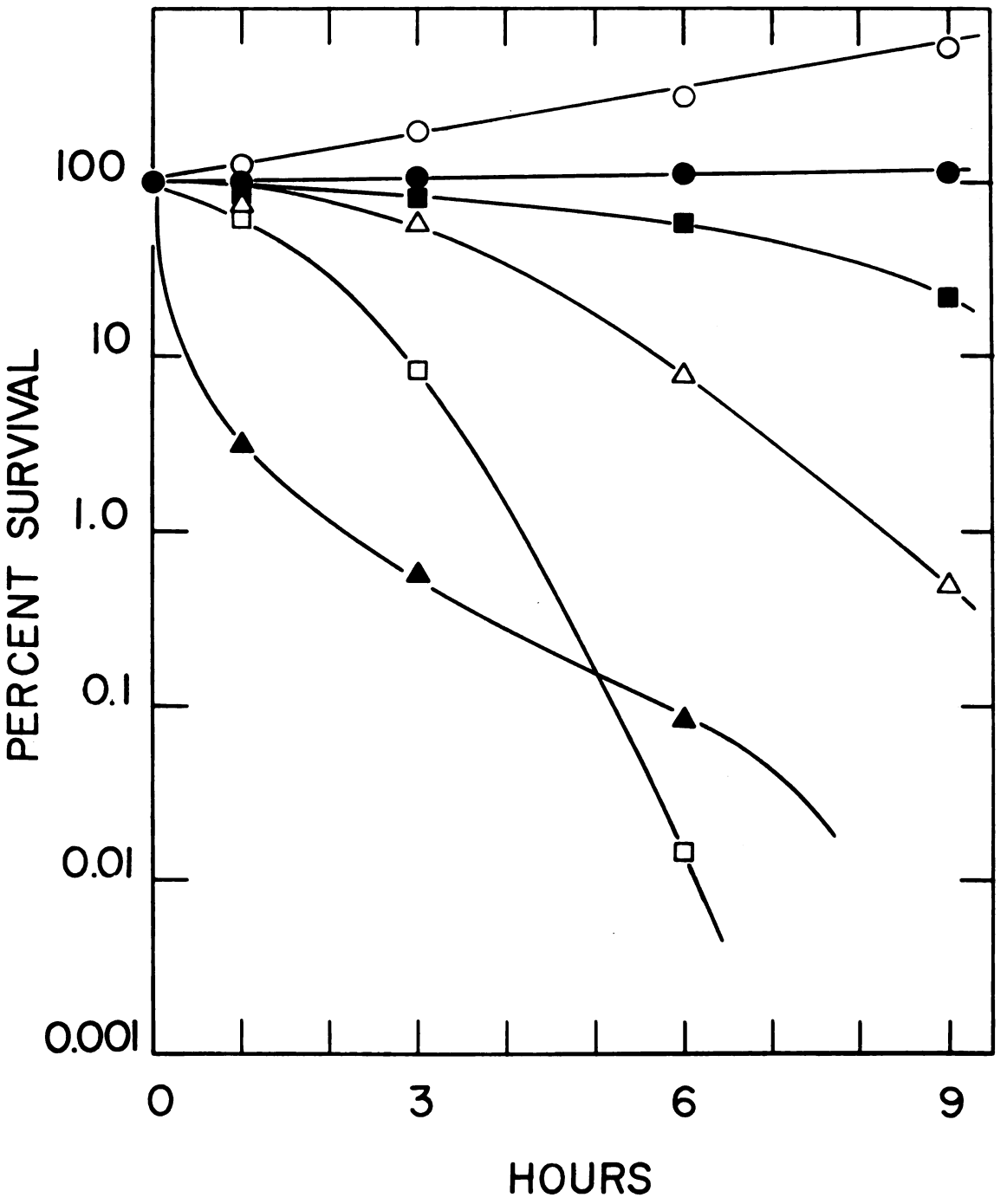

10.0
1.0
0.1
0.01
OPTICAL DENSITY (620 nm)
0
6
12
18
24
HOURS

Fig. 3. Effect of antibiotics on the viability of growing and static VW^+ cells of Y. pestis strain EV76; (●) static cells without addition, (○) growing cells without addition, (■) static cells plus 250 units of penicillin per ml of medium, (□) growing cells plus 250 units of penicillin per ml of medium, (▲) static cells plus 25 units of streptomycin per ml of medium, and (△) growing cells plus 25 units of streptomycin per ml of medium.


100
10
1.0
0.1
0.01
0.001
PERCENT SURVIVAL
0
3
6
9
HOURS

Limited attempts to obtain selective killing of dividing or static VW^+ organisms by the use of other antibiotics with known modes of action were only partially successful. For example, attempts to inhibit synthesis of DNA with mitomycin C and nalidixid acid failed to kill the cells of Y. pestis in the synthetic medium at concentrations of 10 and 100 ug per ml respectively (Table 2). However, static VW^+ cells were extremely sensitive to as little as 25 units of streptomycin per ml (Fig. 3).

Comparisons of Regulatory Capacity of Cytoplasmic Membrane

The previous reports of Brubaker (8) and Surgalla, Andrew and Cavanaugh (110) suggested a distinction in membrane function of VW^+ dividing and static cells. This speculation was tested by the following experiments.

Uptake of Amino Acid

^{14}C-L-isoleucine was used for the measurement of amino acid uptake since this amino acid apparently is not catalizable by Yersinia cells (9). Essentially no difference in ability to accumulate ^{14}C-isoleucine was observed between static and dividing organisms (Fig. 4).

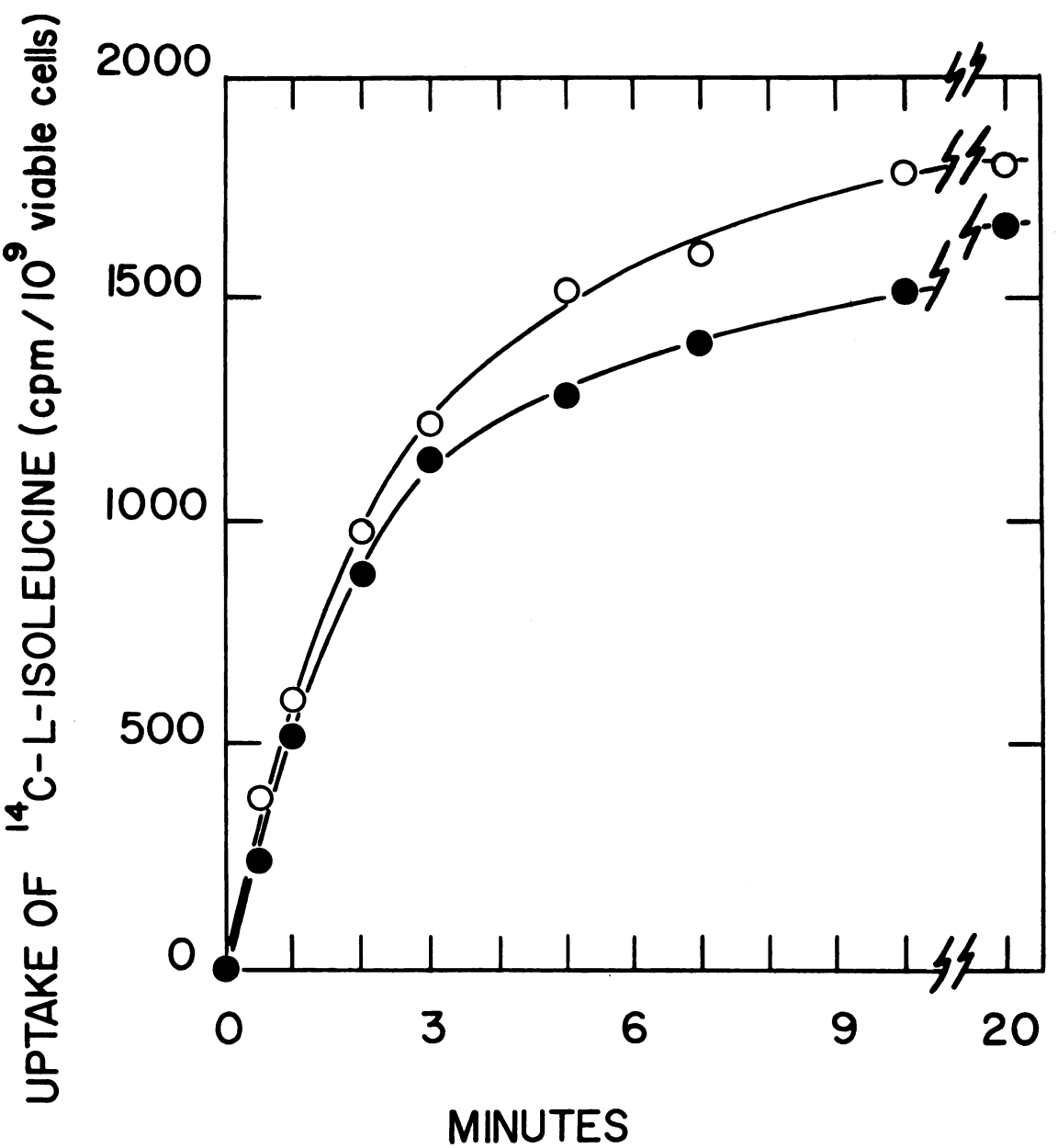
Uptake of Oxygen

Since gluconate is readily metabolized by Y. pestis (see LITERATURE REVIEW), this substance was used as substrate for the measurement of the rates of oxygen uptake.

TABLE 2.--Effect of inhibitors of DNA synthesis on the growth of VW^+ Yersinia pestis.

Added Antibiotic	Added 0.0025M Ca^{++}	Viable cells per ml x 10^8				
		0 hr	4 hr	8 hr	12 hr	16 hr
None	0	2.1	1.8	1.6	1.8	2.2
None	+	2.6	2.1	7.8	14.0	30.0
Nalidixic acid	0	2.4	2.2	1.4	.87	.15
Nalidixic acid	+	3.0	4.6	6.2	10.00	18.00
Mitomycin C	0	1.8	1.6	1.2	.87	.42
Mitomycin C	+	2.0	2.4	4.6	8.40	18.00

Fig. 4. Incorporation of ^{14}C-L-isoleucine into the internal pool of growing (○) and static (●) VW^{+} cells of Y. pestis strain EV76 suspended in neutral potassium phosphate buffer plus gluconate ions.


UPTAKE OF ^{14}C-L-ISOLEUCINE (cpm / 10^9 viable cells)
2000
1500
1000
500
0
0
3
6
9
20
MINUTES

On the basis of dried weight, the rate of oxygen uptake determined for VW^+ growing cells was slightly greater than that obtained for VW^+ static cells (Fig. 5). This difference did not appear to be significant because the size of the static cell is larger than the dividing cell (16). The difference would therefore be reduced if the rate of oxygen uptake was computed on a per cell basis.

Direct Measurement of Membrane Leakage

No differences were observed between the rates of release of non-exchangeable ^{32}P by static and dividing VW^+ cells (Fig. 6). In separate experiments it was found that both static and dividing cells released 260 nm absorbing substance at equal rates (data not shown).

These observations suggested that the cytoplasmic membranes of static organisms retained considerable regulatory capacity. Furthermore, it appeared that a considerable amount of macromolecular synthesis occurred in static cells as judged by the acute sensitivity to streptomycin and release of macromolecules at a rate equivalent to that of dividing organisms. Accordingly, a study of macromolecular synthesis was initiated.

Comparison of Rates of Macromolecular Synthesis

Essentially no difference in rates of RNA synthesis by static and dividing cells was observed (Fig. 7B). Protein was synthesized at slightly reduced but not significantly

Fig. 5. The consumption of oxygen expressed in terms of microliters per mg of dry weight, by washed static and growing cells of Y. pestis strain EV76; (●) static cells plus gluconate, (▲) static cells less gluconate, (O) growing cells plus gluconate, and (△) growing cells less gluconate.

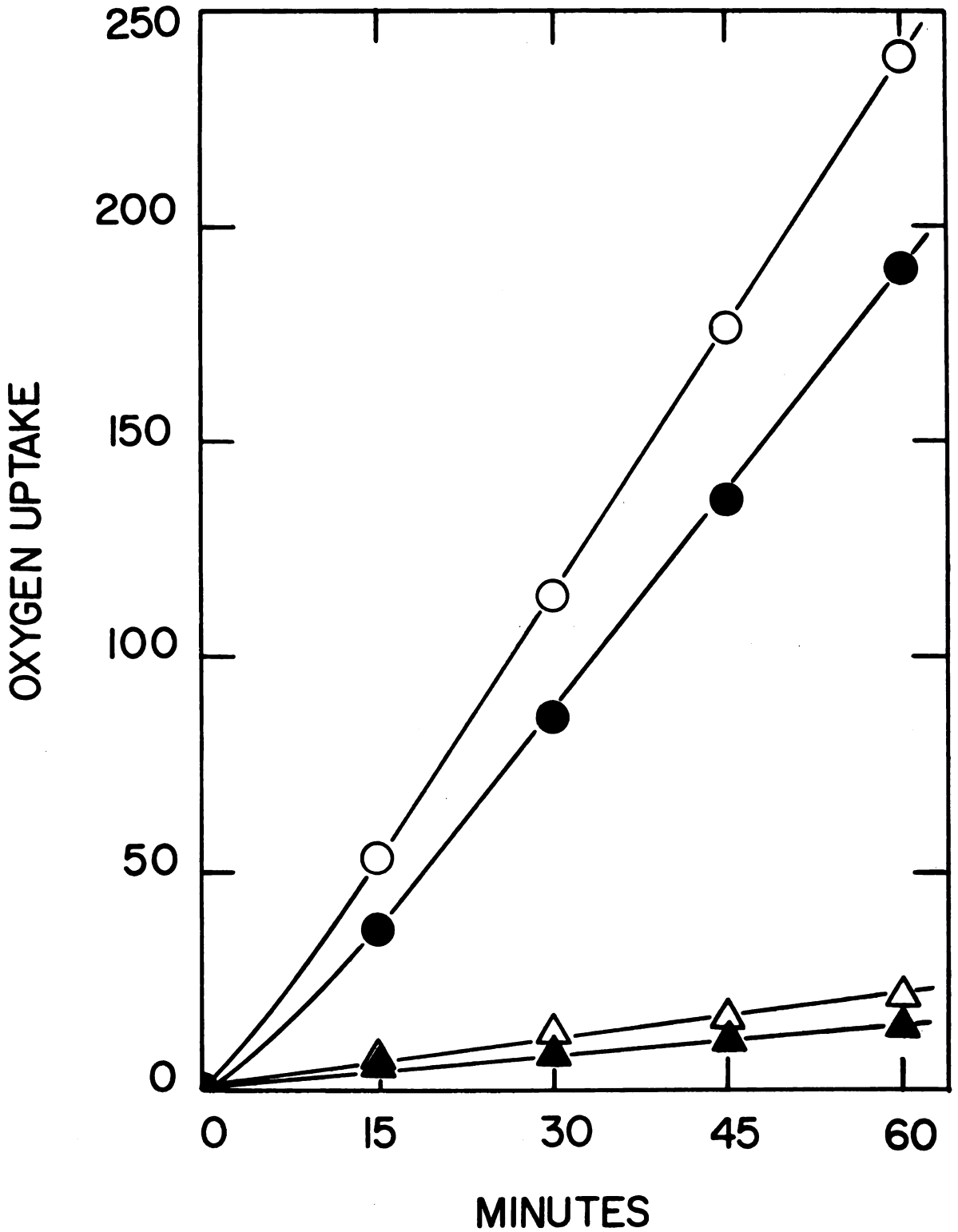
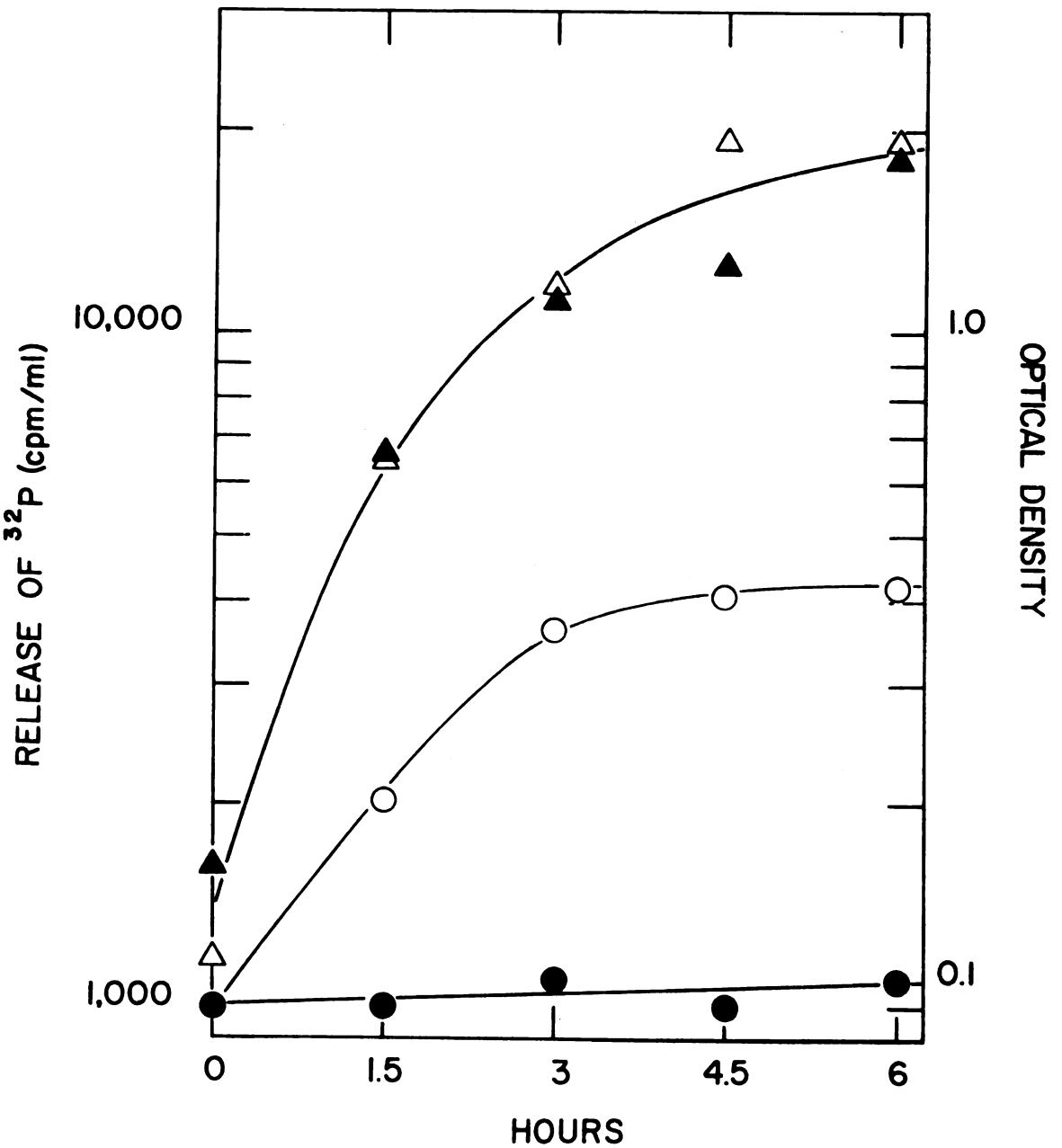

250
200
150
100
50
0
OXYGEN UPTAKE
0
15
30
45
60
MINUTES

Fig. 6. Release of ^{32}P from VW^+ cells of Y. pestis strain EV76 preloaded with $KH^{32}PO_4^-$ at 26 C containing 180,000 cpm per 10^8 organisms; (●) optical density of static cells, (○) optical density of growing cells, (▲) radioactivity released from static cells, and (△) radioactivity released from growing cells.


RELEASE OF ^{32}P (cpm/ml)
10,000
1,000
OPTICAL DENSITY
1.0
0.1
0
1.5
3
4.5
6
HOURS

different rates in static cells than in dividing cells (Fig. 7A). However, the rate of DNA synthesis in static cells was markedly reduced as compared to that in the dividing organisms (Fig. 7C). Comparative rates of macromolecular synthesis, corrected from the data illustrated in Figure 7, are shown in Table 3. To obtain an explanation to account for this selective inhibition of DNA synthesis, the following series of experiments was conducted.

Comparison of DNA Polymerase Activity

DNA polymerase, which is presumably necessary for chromosome replication, was examined to see if a defect in this enzyme activity was the major cause of inhibition of DNA synthesis in static organisms. The results of Figure 8 indicated that both VW^+ and VW^- cells possessed the same levels of DNA polymerase, whether or not Ca^{++} was present in the growth medium. The validity of the assay method is demonstrated in Table 4. In the absence of enzyme of dGTP, very low amounts of radioactivity were incorporated into the trichloroacetic acid precipitable fraction. About an equal amount of radioactivity was incorporated into the complete system and that lacking "activated" primer DNA. This finding appeared to indicate that the crude cell-free extracts of VW^+ static and growing cells already contained degraded primer DNA and that additional "activated" DNA was not required for DNA polymerization.

Fig. 7. Rates of synthesis of macromolecules by static and growing cells of Y. pestis strain EV76; (●) VW^+ cells without added Ca^{++}, (○) VW^+ cells plus 0.0025 M Ca^{++}, (▲) VW^- cells without added Ca^{++}, and (△) VW^- cells plus 0.0025 M Ca^{++}. A - incorporation of ^{14}C-L-isoleucine into protein; B - incorporation of ^{14}C-uracil into ribonucleic acid; and C - conversion of ^{14}C-uracil into thymidine triphosphate and subsequent incorporation into deoxyribonucleic acid.

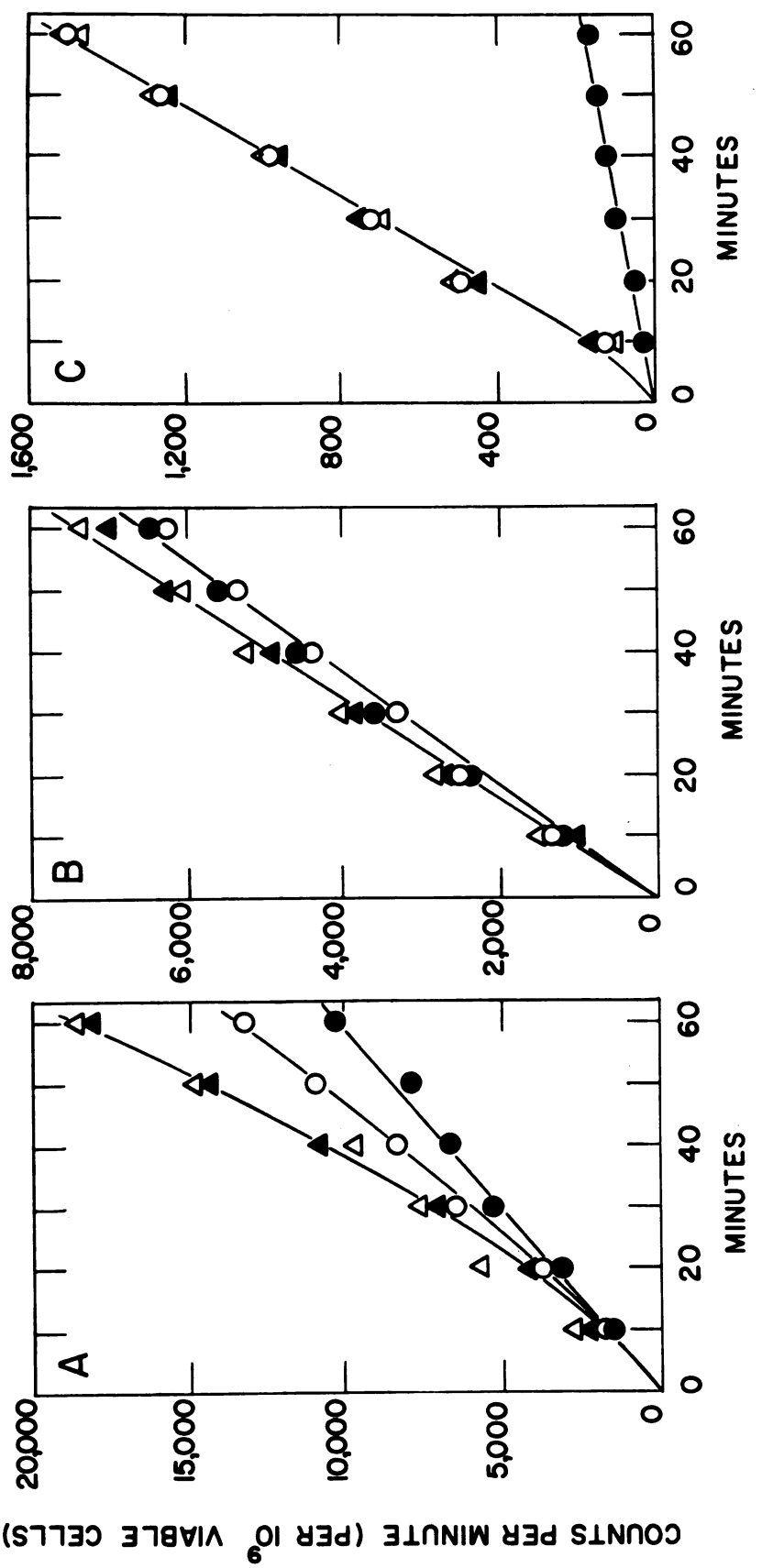

COUNTS PER MINUTE (PER 10^9 VIABLE CELLS)
A
B
C
MINUTES
MINUTES
MINUTES
0
5,000
10,000
15,000
20,000
0
2,000
4,000
6,000
8,000
0
400
800
1,200
1,600
0
20
40
60

TABLE 3.--Comparison of rates of macromolecular synthesis in 10^9 VW^+ and VW^- *Y. pestis* cultivated in the presence (0.0025 M) and absence of Ca^{++}.

Macromolecule[a]	Genotype							
	VW^-				VW^+			
	Plus 0.0025 M Ca^{++}		No added Ca^{++}		Plus 0.0025 M Ca^{++}		No added Ca^{++}	
	nmoles per hr[b]	%	nmoles per hr	%	nmoles per hr	%	nmoles per hr	%
Protein	14.5	100	14.5	100	12.3	85	10.7	74
RNA	8.7	100	8.7	100	7.8	90	7.8	90
DNA	2.3	100	2.3	100	2.3	100	0.2	10

[a]Represented by L-isoleucine, uracil, and thymine incorporated into protein, RNA, and DNA, respectively.

[b]Values taken as 100 per cent.

Fig. 8. DNA polymerase activities of growing and dividing cells of Y. pestis strain EV76; (●) VW^+ cells without added Ca^{++}, (○) VW^+ cells plus 0.0025 M Ca^{++}, (▲) VW^- cells without added Ca^{++}, and (△) VW^- cells plus 0.0025 M Ca^{++}.

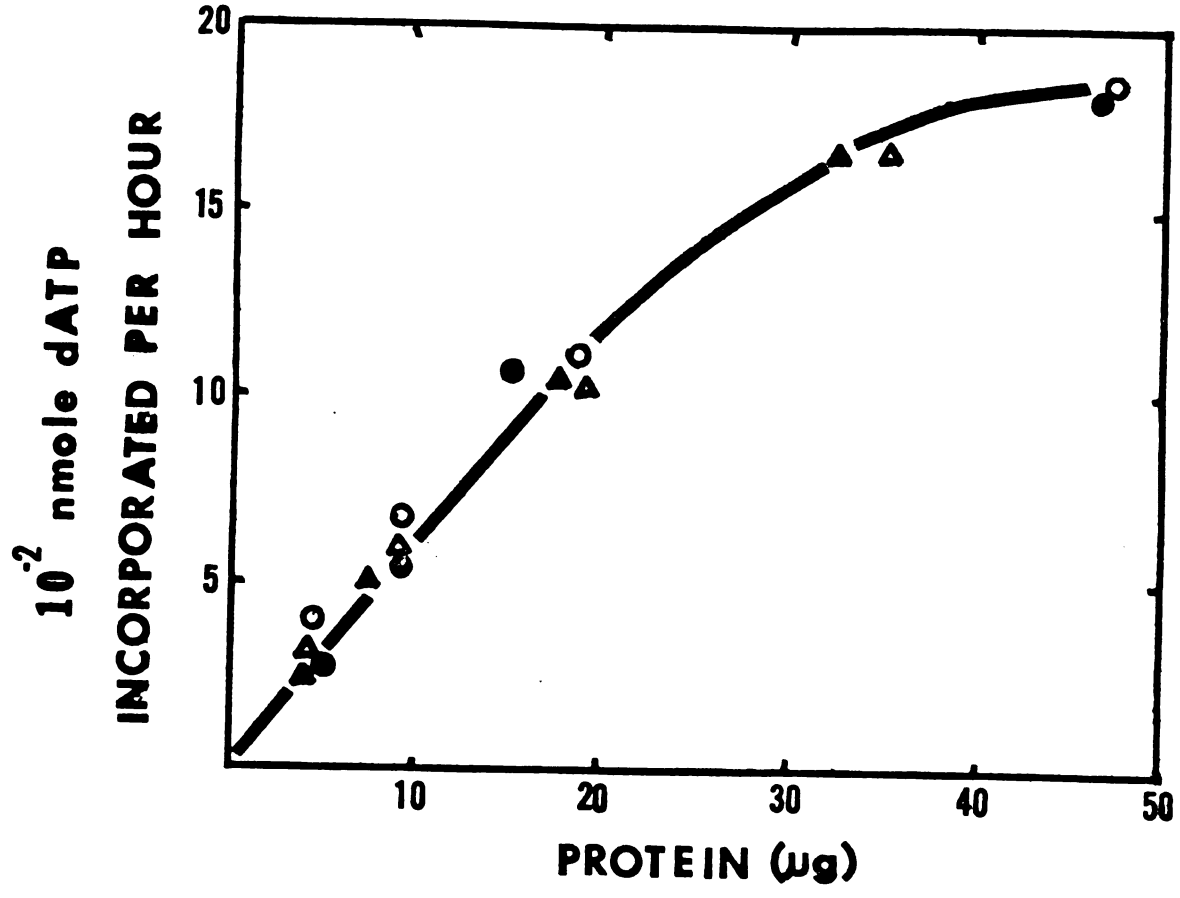

20
15
10
5
10
20
30
40
50
10^{-2} nmole dATP
INCORPORATED PER HOUR
PROTEIN (µg)

TABLE 4. Control experiment of DNA polymerase assay.

Components in the assay system	^{14}C-dATP incorporated[a]	
	VW^+ + Ca	VW^+ - Ca
Complete system	2389	2295
Less enzyme	303	313
Less dTTP	334	379
Less "Activated DNA" (calf thymus)	2116	2064

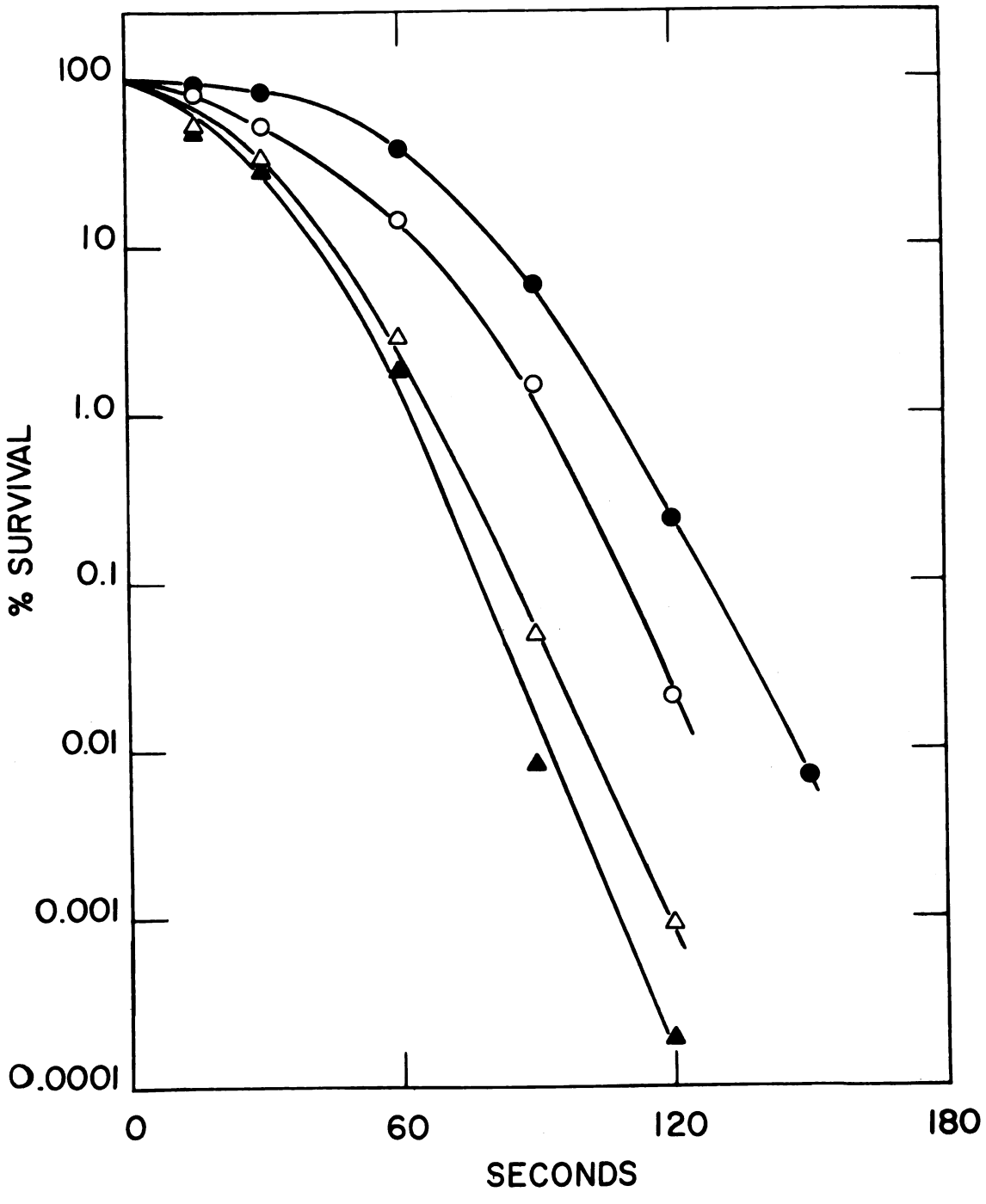
[a]Activity expressed as cpm per 10 ug of protein 30^{-1} min. (4×10^5 cpm equivalent to 1 nmole of d-ATP incorporated).

Sensitivity to Ultraviolet Light

As an indirect approach to determining the number of nuclei per cell, the UV sensitivity of the static and dividing cells were compared. As expected, the results showed that static cells are more resistant to UV radiation than dividing cells (Fig. 9). The VW^+ dividing cells, cultivated with Ca^{++}, were more resistant to UV radiation than were VW^- cells, but were less resistant than were VW^+ static cells.

Since the preceeding experiment had demonstrated that both static and dividing cells contained equal levels of DNA polymerase (Fig. 8), the distinct responses to UV radiation appeared to reflect a quantitative gradient of nuclear substance in the static and dividing cells.

Fig. 9. Sensitivity to ultraviolet light of growing and static cells of Y. pestis strain EV76; (●) VW^+ cells grown without Ca^{++}, (○) VW^+ cells grown with 0.0025 M Ca^{++}, (▲) VW^- cells grown without Ca^{++}, and (△) VW^- cells grown with 0.0025 M Ca^{++}.


100
10
1.0
0.1
0.01
0.001
0.0001
% SURVIVAL
0
60
120
180
SECONDS

Accordingly, the DNA concentration of these cells were compared by chemical determination.

Quantitative DNA Determination

The results of diphenylamine assays revealed that static cells contained more than twice the amount of DNA than that present in dividing cells (Table 5). This result was consistent with microscopic observation that static cells contained more nuclear staining bodies than did dividing cells (P. J. Hall, personal communication). The fact that dividing VW^+ cells did not contain more nuclei than did VW^- dividing cells, but were more resistant to UV radiation than the latter, may be related to the reports of Surgalla (109) and Brubaker (8) that production of V and W antigens may be regulated by episomal determinants which can often promote resistance to UV (31, 59, 103).

The foregoing experiments demonstrated that after 12 hr incubation at 37 C, the static cells were not synthesizing DNA, but had already accumulated twice the amount of DNA as had the dividing cells. In order to determine the cause of of the termination of DNA synthesis, the rates of DNA synthesis prior to the 12 hr incubation period were examined.

Mode of Inhibition of DNA Replication

A culture of VW^+_1 organisms which had been incubated at 26 C for 12 hr in synthetic medium was redistributed into

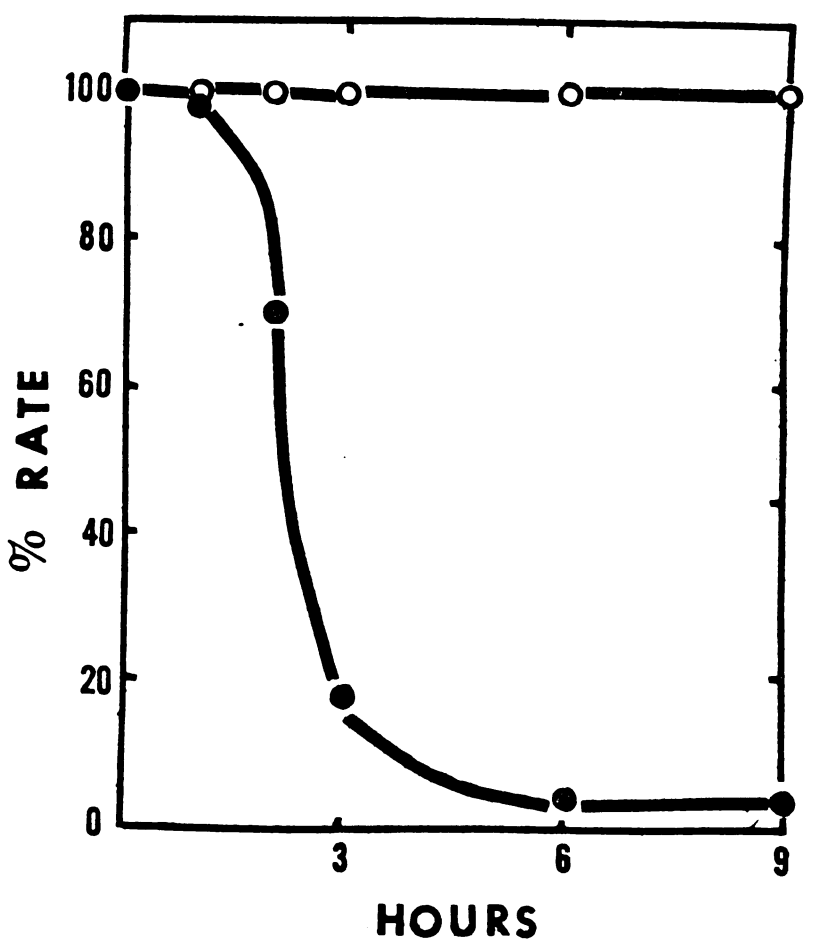
TABLE 5.--DNA content of Y. pestis cells cultivated in the synthetic medium for 12 hr.*

Cell type	Added Ca^{++} (0.0025 M)	ug of DNA per 10^9 cells
VW^+	0	42.32
VW^+	+	18.89
VW^-	0	18.52
VW^-	+	17.92

*Determined by diphenylamine assay.

several flasks. In order to minimize the chance of contamination during a long period of incubation the kinetics of DNA synthesis was determined from these flasks at intervals during incubation at 37 C. The absolute rates of synthesis in VW^+ dividing cells were similar in all of these experiments. The per cent rate of DNA synthesis was calculated by taking the value of dividing cells at each interval as 100%. The results, as illustrated in Figure 10, indicated that both growing and static cells synthesized DNA at almost equal rates during the first hr of incubation. Subsequently, the rate of synthesis in static cells became progressively reduced. Termination of synthesis in static cells occurred after about 4 hr of incubation; a period which was incidentally equivalent to one generation time of VW^+ cells growing in the presence of Ca^{++} (Fig. 1).

Fig. 10. Per cent rate of DNA synthesis in static (●) and growing (○) cells of VW^{+} Y. pestis strain EV76 cultivated at 37 C.


100
80
60
40
20
0
% RATE
3
6
9
HOURS

Effect of Ca^{++} on the Growth of Static Cells

When VW^{+} cells were plated on MGOX agar, no colonies were observed after 40 hr of incubation at 37 C. However, colonies appeared when the plates were further incubated at 26 C for 2 days (Brubaker, personal communication). Although Ca^{++} was found to promote the growth of VW^{+} cells at 37 C (53) it was not certain that this cation could initiate division in cells which had remained static at 37 C for long periods of time.

Further study showed that when VW^{+} cells were cultivated in the Ca^{++}-deficient medium and aerated at 37 C for 2 to 6 hr, the addition of Ca^{++} initiated growth after a significant lag period (Fig. 11). The length of this period was in proportion to the time of Ca^{++}-starvation and the total amount of growth of the starved cells was considerably decreased. Growth could not be initiated by supplementation of Ca^{++} if the cells were starved for Ca^{++} for periods longer than 8 hr (Fig. 12). However, decreasing the temperature of incubation to 26 C in the absence of Ca^{++} permitted growth to occur following a short lag period (Fig. 11F, 12).

Thermal Induction of Growth Inhibitor in VW^{+} Cells

Witkin (120) has proposed a model for filament formation in Escherichia coli B in which UV action on the chromosome was assumed to promote formation of a division inhibitor. By analogy, attempts were made to demonstrate

Fig. 11. Effect of Ca^{++} on the growth of VW^{+} cells of Y. pestis strain EV76 at 37 C. Cells were preincubated in synthetic medium lacking added Ca^{++} for 12 hr at 26 C and then incubation was continued at 37 C with addition of Ca^{++} (0.0025 M) at 0 hr (A), 2 hr (B), 4 hr (C), 6 hr (D), or was not added (E); growth following reduction of temperature to 26 C after 6 hr is shown in F.

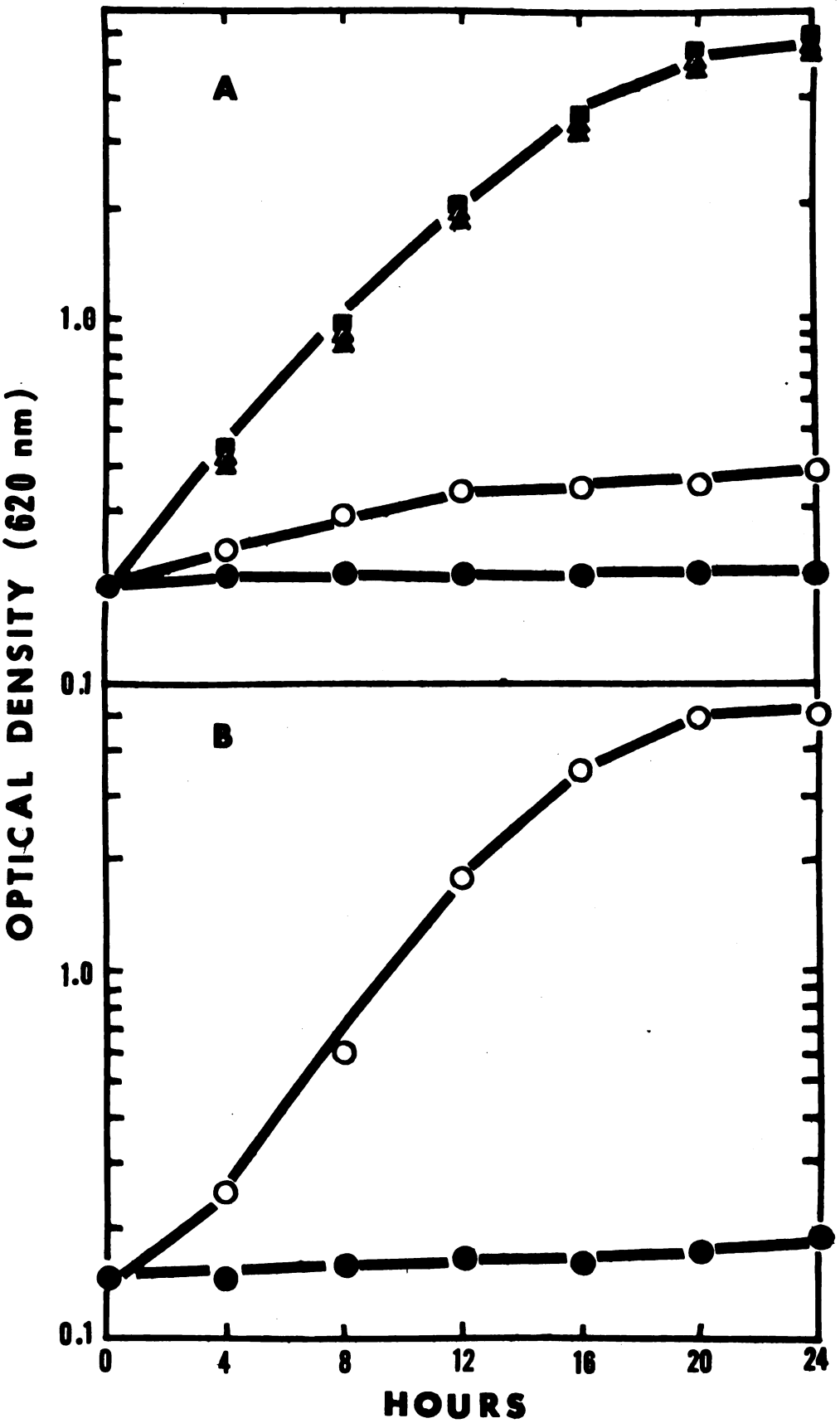
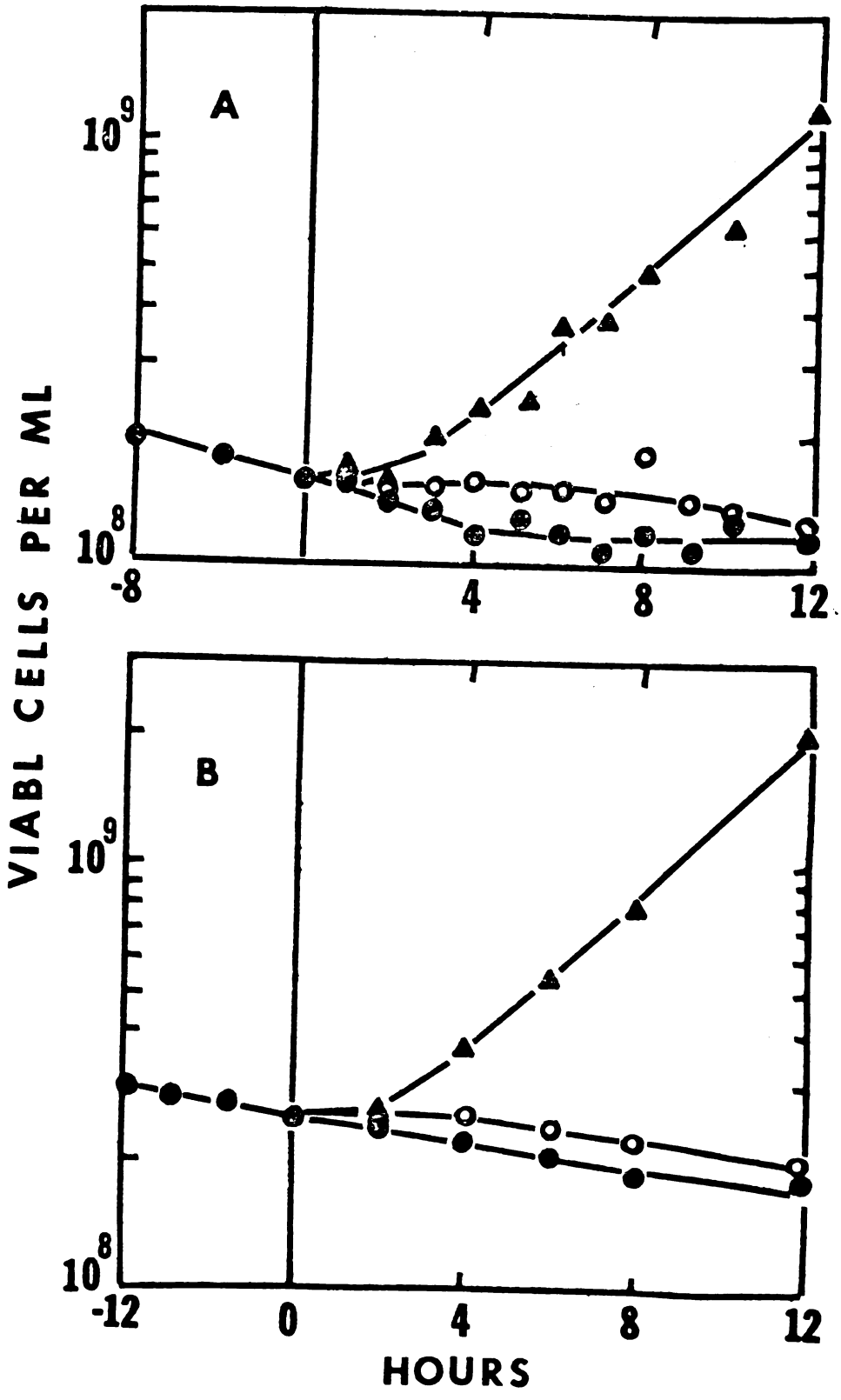

A
B
1.0
0.1
1.0
0.1
OPTICAL DENSITY (620 nm)
0
4
8
12
16
20
24
HOURS

Fig. 12. This figure represents the repetition of the experiment of Figure 11 except that VW^{+} cells were starved for Ca^{++} for 8 hr (A) or 12 hr (B) at 37 C; (●) continuous incubation at 37 C without further alteration, (○) addition of Ca^{++} (0.0025 M), and (▲) reduction of temperature to 26 C.


A
B
10^9
10^8
VIABL CELLS PER ML
-8
4
8
12
-12
0
HOURS

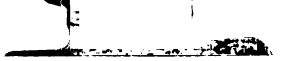
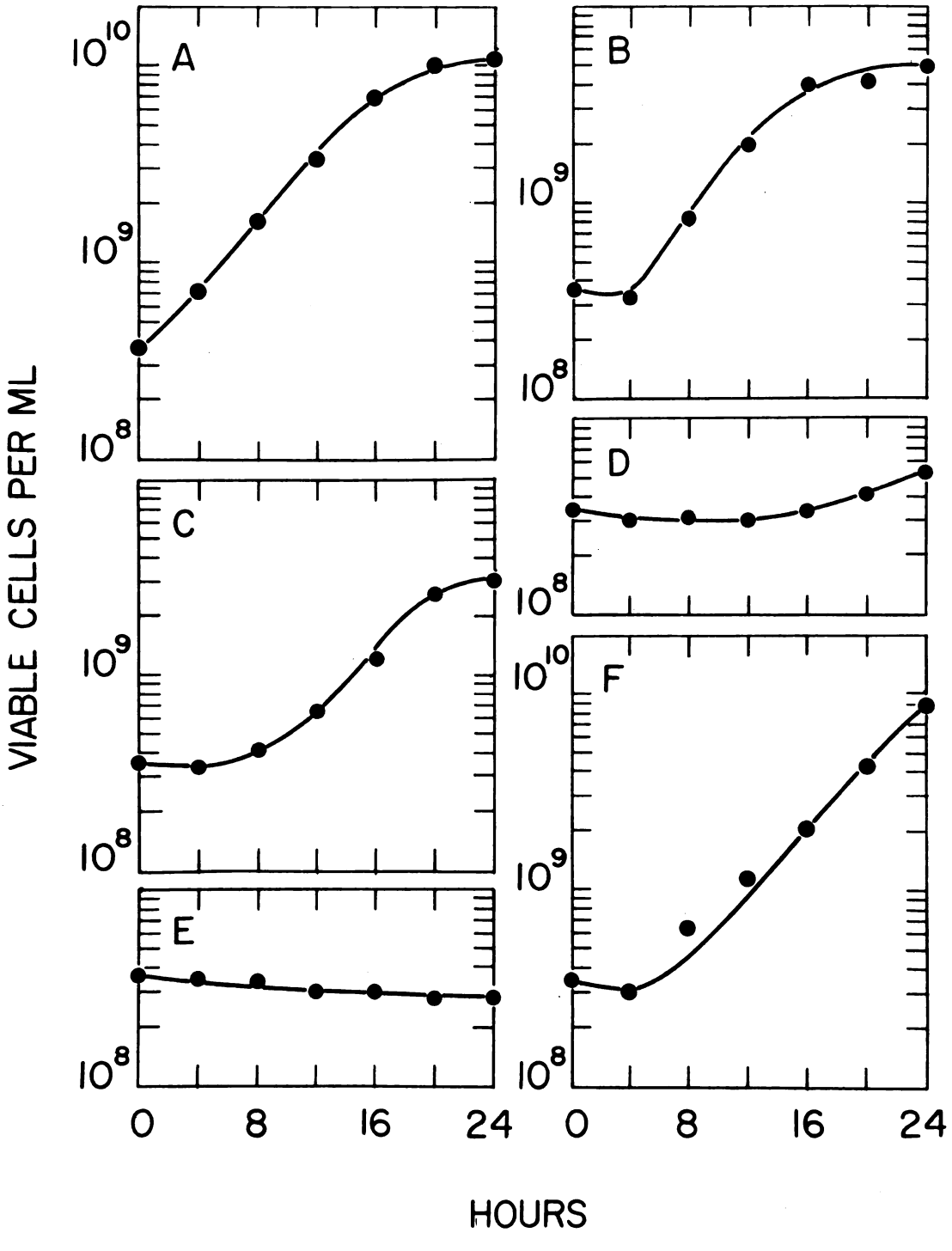


Fig. 13. Growth of VW^+ cells of Y. pestis strain EV76 following preincubation at 26 C for 12 hr and treatment in A for 30 sec at 58 C (●), 55 C (■), 53 C (△), and not treated (▲); the cells were then incubated at 26 C except for one culture (○) which was incubated at 37 C. In B the cells were preincubated at 37 C and were retained at 37 C (●) or were incubated at 26 C (○).


A
B
C
D
E
F
10^{10}
10^{9}
10^{8}
VIABLE CELLS PER ML
0
8
16
24
HOURS

the possible existence of an analogous growth inhibitor in VW^+ cells which could be induced by heat. The VW^+ cells were grown at 26 C for 12 hr in synthetic medium without Ca^{++} and were then heated for 30 sec at various temperatures; incubation was subsequently continued at 26 C. The results of this determination, shown in Figure 13, indicated that the brief treatment with heat did not result in inhibition of growth at 26 C. Incidentally, organisms apparently were quite sensitive to heat since treatment at 58 C for 30 sec was completely bactericidal.

CHAPTER V

DISCUSSION

Compounds which can interact with cytoplasmic membranes are known to influence selectively the rates of growth of both VW^+ and VW^- organisms (see LITERATURE REVIEW). Furthermore, Brubaker (8) reported that a high concentration of Na^+ and a low ratio of ionic strength to osmolarity were toxic to VW^+ organisms. This set of conditions, which may have been responsible for the lag period shown in Figure 1, was assumed to promote a disruption of normal membrane function. In view of these findings, as well as the reports noted previously concerning the loss of metabolites from obligate intracellular parasites (see INTRODUCTION), a study designed to detect the possible functional defects in the cytoplasmic membranes of static Y. pestis cells was initiated.

If the amount of 260 nm absorbing material or preloaded ^{32}P released by dividing VW^+ cells had been less than that released by static cells, a loss of regulatory capacity of the cytoplasmic membranes of the latter would have been suspected. The reverse case would have suggested that the static cells were metabolically inactive, at least with regard to macromolecular synthesis. However, as shown in Figure 6, the rates of release of ^{32}P by both types of

cells were identical suggesting that significant turnover of organic phosphate occurred in the static and dividing cells and that these compounds were retained by a functional cell membrane.

In another direct test of membrane function, isoleucine was accumulated by static cells at a rate commensurate with that of growing organisms (Fig. 4). The finding that one amino acid can be accumulated by static organisms does not, of course, prove that the remaining required amino acids or other substances were similarly transported. However, the observation that both static and dividing cells could synthesize protein at comparable rates (Table 3) indicated that stasis is not caused by decreased rates of uptake of amino acids. The rates of the energy-dependent synthesis of RNA and protein in dividing and static cells were similar (Table 3) indicating that stasis is not caused by a limitation in energy supply. The similar rates of 0_2 consumption (Fig. 5) are consistent with this conclusion.

The major difference noted between the dividing and static cells was that the latter failed to synthesize significant amounts of DNA (Table 3). This finding in itself would not be surprising if the rates of RNA and protein synthesis were similarly reduced as occurs when stasis is a consequence of or analogous to a shift-down to a minimal medium or to one lacking some critical nutrient (82). Continued RNA and protein synthesis presumably accounts for

the previous observation that static organisms are larger than dividing cells (16).

Figure 8 shows that inhibition of DNA synthesis in static cells is not due to a lack of the polymerizing enzyme. The possibility that inhibition of DNA synthesis was due to deficiency in the formation of deoxynucleoside triphosphates (dNTP) was not tested. However, thermo-inhibition of DNA synthesis in certain E. coli mutants was not due to this reason (37).

Selective inhibition of DNA synthesis has also been characterized in several thermosensitive mutants of E. coli and B. subtilis (37, 44, 57). At high temperatures, these mutants stop DNA synthesis either immediately or progressively but continue to synthesize RNA and protein. As a result, the cells become elongated and fail to divide. These mutants contain normal levels of DNA polymerase, synthesize the four dNTP (37), and repair UV damage normally (26). Due to their inability to complete a round of DNA replication, the viability of these mutants declines rapidly.

Despite the similarities shared by these mutants and the static cells of Y. pestis, the fundamental differences between the two are that the latter appears to have completed a round of DNA replication before the sensitivity of DNA synthesis to high temperature becomes apparent (Table 5),and also the cells remain viable even in the absence of DNA replication (Fig. 11E). It is conceivable,

therefore, that the cause of stasis is a block in the process of septation with concomitant termination of DNA synthesis. However, thermosensitive mutants defective in septum formation usually continue to replicate DNA for several generations and form long multi-nuclear filaments (57). Therefore, by analogy, it seems unlikely that the termination of DNA synthesis in static cells is due to the inhibition of septation alone. Rather it appears as if there is another block at the reinitiation of DNA replication.

Initiation of a new round of replication appears to be associated with four factors: 1) the availability of an initiator complex which includes the initiator pro-replicator and replicator described by Lark (72); 2) with exception of fast growing organisms, the completion of an old round of replication; 3) nick formation (creation of 3' hydroxyl ends of DNA by endonuclease action) and the initial breakage of one chromosome strand so as to permit strand separation and initiation of replication (68, 72); 4) and the crucial value of cellular mass/DNA ratio (29, 82).

The finding that static cells have completed a round of replication obviously eliminates the second factor as being associated with inhibition of replication. Nick forming ability by static cells was not tested directly. However, the finding that cell-free extract of static cells

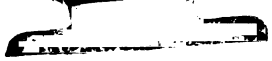
already contained primer DNA (Table 4) indicates that endonuclease activity was present within static cells. Due to lack of information concerning the normal values of cellular mass and DNA in the growing cells, it is difficult to evaluate whether an abnormality of the mass/DNA ratio could lead to inhibition of reinitiation of replication. However, one might expect to observe reinitiation of DNA synthesis after termination of replication because static cells apparently synthesized RNA and protein (mass) at normal rates (Table 3). The discovery that static cells did not reinitiate DNA synthesis after 4 hr incubation (Fig. 10), suggests that the fourth possibility is not the major cause of replication inhibition. This leaves an alteration of the first factor as being the most likely explanation for stasis.

As reported by Hirota, Ryter and Jacob (57), Helmstetter (48), and Smith and Pardee (105), the cell membrane-associated protein required for cellular division and the initiator complex of cell replication are both heat labile. Therefore, raising the temperature from 26 to 37 C could possibly result in the inactivation of both systems. Similarly, the same phenotypic lesion may be responsible for the inhibition of septation and initiation of replication since it is still unclear whether the replicator and the site of septum formation are the same cell membrane components (98). Inouye and Guthrie (60) reported that a membrane-associated

protein of E. coli apparently coordinated the cell division and replication through the membrane. Furthermore, it has been shown in gram-positive organisms that mesosomes serve as the sites of chromosome attachment and septum formation (98, 99). These findings may suggest that the same membrane component is responsible for initiation of replication and septation. On the other hand, Smith and Pardee (105) demonstrated that heat labile proteins required for cell division and DNA replication were different since heat inactivation did not inhibit these two processes to the same degree.

The foregoing discussion leads to the prediction that cell division would be observed if static cells were cultivated under unrestricted conditions (such as addition of Ca^{++} or temperature shift back to 26 C) prior to the synthesis of new DNA. The best method to demonstrate this would, of course, be to show that one cell division could occur when the synthesis of DNA was blocked by thymine starvation or antibiotics such as mitomycin C or nalidixic acid. However, thymine auxotrophs which retained all other genetic characters of the VW^{+} cell could not be obtained and attempts to use nalidixic acid and mitomycin C were not successful (Table 2).

An alternative explanation exists. Although attempts to induce a thermo-dependent inhibitor of growth were not successful (Fig. 13), the possibility that such an inhibitor was produced after a longer period of incubation at 37 C was



not excluded. The existence of a growth inhibitor may account for the observation that the period of delay between addition of Ca^{++} to static cells and commencement of division increases in proportion to the length of the period of Ca^{++} starvation (Figs. 11 and 12). When the concentration of this hypothetical inhibitor exceeds the threshold level, Ca^{++} might no longer be able to initiate growth. In this case, it is necessary to assume that the phenotypic expression of this inhibitor is temperature dependence and that its production is inhibited by Ca^{++}, since growth was observed if Ca^{++} was included in the initial culture medium or if the temperature was reduced to 26 C. Assuming that the presumptive inhibitor is freely permeable, it seems likely that spent culture medium of static organisms would also inhibit the growth of VW^{-} cells at 37 C. This phenomenon may have been observed in the experiments reported by Brubaker and Surgalla (15) where static VW^{+} cells inhibited the growth of streptomycin-resistant VW^{-} organisms in the presence of antibiotic.

At present it is difficult to define with certainty the role of Ca^{++} in the process of cell division. Grula and Grula (45) reported that Ca^{++} and Mn^{++} could overcome the inhibitory effect of cycloserine. Burgoyne, Wagar and Atkinson (18) found that Ca^{++} was required for the action of endonuclease in rat liver nuclei. As discussed previously, the priming of DNA synthesis in static cells seems

to be normal, thus the possibility that Ca^{++} is required for endonuclease activity seems unlikely. Considering that this cation is mostly known for its function in the stabilization of protein structure (46, 115) and that the effect of Ca^{++} in Yersinia cells is replaceable by Zn^{++} and Sr^{++} (53), it seems possible that these divalent cations are required to stabilize the subunits of heat sensitive initiators of cell division and DNA replication (57); they could also be required for the binding of membrane-associated replicator and septum forming site(s). These possibilities cannot be distinguished on the basis of current knowledge.

It is known that V and W antigens are readily secreted by static cells cultivated in the Ca^{++}-deficient medium (16), and that addition of Ca^{++} results in suppression of the release of V and W antigens (14) with concomitant growth. These findings, as well as the fact that free W antigen presumably originates from the cell wall or membrane (8) suggests that these antigens are membrane constituents which are essential for cell replication and division. In this case, Ca^{++} would be required to maintain the structural integrity of the cell membrane at high temperature.

If these considerations are valid, a qualitative or quantitative difference between static and dividing cells would be detectable by using the double-labeling technique of Inouye and Guthrie (60) or by means of fractionation of

membrane proteins. Moreover, monospecific antibody against V or W antigen, should, in the presence of membrane components of VW^- cells, fix complement providing that these proteins are not inhibitory to complement activity. Further investigation of this kind should clarify the roles of V and W antigens.

Ogg et al. (94) reported that spent culture filtrates of VW^- mutants promoted the growth of VW^+ organisms. Similarly, Delaporte (27) found that UV irradiated populations of filamentous E. coli, which evidently lack the ability to form septum (114), could be induced to divide in the presence of diffusable substances from normal growing cells. Subsequent study by Adler et al. (1) indicated that these division-promoting substances consisted of a heat stable and a heat labile cellular constituent. To some extent, these findings support the notion that static cells lack functional sites of DNA replication or cell division.

Assuming that this concept is correct, a very obvious question one may ask is, then, why do VW^+ cells undergo bacteriostasis when introduced into the simulated mammalian intracellular environment? This question is difficult to answer at present, but, as stated by Janssen and Surgalla (66), only VW^+ cells seem to be capable of multiplying within fixed macrophages. It therefore seems likely that the ability of these cells to release V and W antigens and to undergo bacteriostasis is essential to intracellular growth.

The findings reported in this dissertation may relate to the similar situation that occurs in obligate intracellular parasites where, by definition, the expression of stasis *in vitro* is constitutive. The failure of some of these organisms to grow *in vitro* is undoubtedly caused by an inability to generate endogenous high energy compounds or to maintain physiological concentration gradients (91). However, these limitations do not always exist as illustrated by rickettsial parasites (see INTRODUCTION). The phenomenon of stasis in these organisms may thus be analogous to that in *Yersinia*.

CHAPTER VI

SUMMARY

1. Potentially virulent VW^+ cells of Y. pestis EV76 cultivated in simulated mammalian intracellular conditions were static but possessed functional cytoplasmic membranes as judged by concentration of ^{14}C-isoleucine, release of ^{32}P, and consumption of oxygen at rates comparable to those of dividing cells cultivated with Ca^{++}.

2. The rates of protein and RNA synthesis were essentially identical in dividing and static cells. However, the synthesis of DNA ceased in static cells about 4 hr after the removal of Ca^{++}. During this period of time, which corresponded to one generation in the presence of Ca^{++}, the static cells completed one chromosome replication as judged by a two-fold increase in content of DNA and a corresponding degree of resistance to irradiation with UV light.

3. The termination of DNA synthesis in static cells was not due to a deficiency in DNA polymerase, but rather due to an inability to initiate a new round of chromosome replication.

4. Apparently the bacteriostasis of VW^+ cells is caused by thermo-inactivation of membrane-associated proteins

required for the initiation of DNA replication and the formation of septae.

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Appendix A. Modified synthetic medium of Higuchi, Kupferberg and Smith (53).

Constituent	g per L	Constituent	g per L
Amino Acids		Vitamins	
L-Glutamate	12.00	Thiamine HCl	0.001
L-Phenylalanine	0.40	Ca-panthothenate	0.001
L-Methionine	0.24	Biotine	0.0005
L-Valine	0.80	Other Additions	
L-Leucine	0.20	D-xylose*	10.0
L-lysine HCl	0.20	Phenol red	0.01
L-Proline	0.80		
L-Threonine	0.16	Salt Components	Molarity
Glycin	2.00		
DL-Alanine	0.40	K_2HPO_4	0.025
L-Tyrosine	0.20	Citric Acid	0.01
L-Argine HCl	0.20	K-Gluconate	0.01
L-Isoleucine	0.50	NH_4-acetate	0.01
L-Cysteine HCl*	0.175	$MnCl_2$	0.000001
L-Tryptophane*	0.04	$FeCl_2$	0.0001
		$MgCl_2$	0.02

*Separately sterilized. (Vitamins combined as a Single solution).