INFECTION AND IMMUNITY, Jan. 1971, p. 59-65

Vol. 3, No. 1
Printed in U.S.A.

Effect of Ca^{2+} on the Synthesis of Deoxyribonucleic Acid in Virulent and Avirulent *Yersinia*[1]

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Received for publication 13 August 1970

Virulent and potentially virulent cells of *Yersinia* (*Pasteurella*) *pestis* produce virulence or V and W antigens (VW^+) but remain static at 37 C during aeration in enriched Ca^{2+}-deficient media containing 0.02 M Mg^{2+}. 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^{2+}. Furthermore, rates of protein and ribonucleic acid synthesis were essentially identical in dividing and static VW^+ cells and in mutant VW^- organisms. However, the rate of deoxyribonucleic acid (DNA) synthesis in static cells was about 10% of that observed in dividing organisms. Accordingly, bacteriostasis of VW^+ cells in the simulated intracellular environment is evidently caused by reactions directly associated with cessation of DNA synthesis rather than by alterations in the regulatory capacity of the cytoplasmic membrane.

The dependence of the chlamydiae, rickettsiae, and plasmodia upon living cells of their hosts and the highly restricted ability to parasitize such cells are presumably closely related phenomena (16); elucidation of one of these mechanisms should facilitate gaining an understanding of the other. To minimize difficulties associated with the preparation of obligate intracellular bacterial or protozoan parasites, which are easily damaged during the process of purification (16, 17, 23), we have examined the metabolism of facultative intracellular parasites of the genus *Yersinia* (*Pasteurella*). These organisms exhibit distinct physiological responses in media which simulate mammalian plasma or intracellular fluid.

Cells of wild-type *Y. pestis* and *Y. pseudotuberculosis* produce the virulence or V and W antigens (VW^+) described by Burrows and Bacon (5), but fail to divide when aerated at 37 C in enriched Ca^{2+}-deficient medium containing 0.02 M Mg^{2+} (8, 14); these concentrations of Ca^{2+} and Mg^{2+} duplicate the values reported for mammalian intracellular fluid (12). In contrast, the addition of physiological levels of Ca^{2+} (0.0025 M) to such a medium promotes cell division and inhibits the production of V and W antigens (3, 8). Mutants which fail to produce detectable amounts of these antigens (VW^-) arise at a rate of 10^{-4}, do not exhibit a nutritional requirement for Ca^{2+} at 37 C, and are avirulent (9).

Brubaker (2) suggested that stasis with expression of the virulence antigens, and division with apparent repression of these antigens, reflected metabolic patterns which are essential for intra- and extracellular growth, respectively. The purpose of this study was to define the physiological block in static VW^+ cells which occurs upon cultivation in a simulated artificial intracellular environment. Identification of the nature of this block, which appears to be at the level of deoxyribonucleic acid (DNA) replication, should permit a greater understanding of virulence in *Yersinia* and may be of interest to those concerned with the metabolism of the obligate intracellular parasites.

MATERIALS AND METHODS

Bacterial cultures. Unless stated otherwise, VW^+ and VW^- cells of *Y. pestis* strain EV76 were used in this study. VW^+ organisms of this strain are potentially virulent owing to loss of ability to form pigmented colonies on hemin-agar (11); this mutation is independent of that resulting in expression of the VW^- phenotype (4). VW^+ and VW^- cells of *Y. pseudotuberculosis* strain PBl were also employed in certain experiments. VW^- isolates of both species were selected on the magnesium oxalate (MGOX) agar of Higuchi and Smith (9). Stock cultures were preserved in buffered glycerol at −20 C as described previously (2).

Cultivation. After incubation for 2 days at 26 C on slopes of Blood Agar Base (BAB; purchased from

[1] This paper is based on portions of a dissertation submitted by G. C. H. Yang to the Department of Microbiology and Public Health of Michigan State University, in partial fulfillment of the requirements for the Ph.D. degree.

BBL), cells were removed in 0.033 M potassium phosphate buffer, *p*H 7.0 (phosphate buffer), centrifuged at 27,000 × *g* for 10 min, washed with phosphate buffer, and inoculated at a concentration of 3×10^8 to 5×10^8 per ml into 500-ml Erlenmeyer flasks containing 50 ml of a modification of the chemically defined medium of Higuchi, Kupferberg, and Smith (8). The composition of this medium was altered from that originally described by removal of all of the D-amino acids except D-alanine (synthetic medium); 0.02 M Mg^{2+} and either 0.0025 M or no Ca^{2+} were included. After aeration for 12 hr at 37 C on a model BB wrist action shaker (Burrell Corp., Pittsburgh, Pa.), either radioisotopes were added to the cultures or, in other experiments, the cells were collected by centrifugation, washed in phosphate buffer, and used for comparative studies of bacteriostasis. Appropriate steps, including routine plating on BAB and MGOX agar, were taken to assure that cultures were not contaminated or overgrown with VW^- cells. Rates of growth were expressed as the product of the optical density at 620 nm and the dilution factor or as counts of viable cells. The latter were obtained by spreading samples, appropriately diluted in phosphate buffer, on plates of BAB; the plates were incubated for 2 days at 26 C.

Regulatory capacity of the cytoplasmic membrane. In studies of amino acid uptake, washed cells which had been incubated with or without Ca^{2+} for 12 hr at 37 C were aerated in phosphate buffer containing 0.025 M potassium gluconate and 10 μM ^{14}C-isoleucine (2 μCi per μmole). Samples of 1 ml were withdrawn at brief intervals and immediately passed through 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.); the residue was washed twice with 3 ml of prewarmed phosphate buffer. After the filter had been dried at 80 C for at least 6 hr, radioactivity was measured in a model Mark I liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.); counting vials contained 10 or 15 ml of a mixture of 0.4% 2,5-diphenyloxazole and 0.005% 1,4-di[2-(5-phenyloxazolyl)]-benzene in toluene.

The ability of dividing and static cells to retain internal ^{32}P was determined by cultivating the organisms for 12 hr at 26 C in synthetic medium containing $KH^{32}PO_4^-$ (0.04 μCi per μmole). After this process of preloading, the organisms were washed three times in nonradioactive phosphate buffer containing 0.07 M NaCl, to remove exchangeable isotope, and were then inoculated into synthetic medium in the presence or absence of Ca^{2+}. Samples were then removed at intervals of 90 min and passed through 0.45-μm membrane filters; after the filters had dried, radioactivity in the filtrate was estimated on planchets with a model 447 gas-flow counter (Nuclear-Chicago Corp.).

Oxygen uptake was determined in the presence of 0.1 M potassium gluconate, which was present as a component of synthetic medium, by standard manometric methods (22). Prior to introduction of substrate, the cells were washed for 30 min in 0.05 M potassium phosphate (*p*H 7.0) to reduce endogenous metabolism.

Synthesis of macromolecules. The synthesis of protein was estimated by measuring the incorporation of ^{14}C-L-isoleucine into trichloroacetic acid-insoluble precipitates. Samples of 1 ml were removed at intervals of 10 min from cultures in synthetic medium containing a reduced concentration (0.15 mM) of radioactive isoleucine (1.3 μCi per μmole), mixed with 1 ml of cold 10% trichloroacetic acid, incubated for 30 min at 5 C, and then passed through 0.45-μm membrane filters. After an additional wash with 5 ml of 5% trichloroacetic acid, the membranes were mounted and dried, and radioactivity was determined in the gas-flow counter.

A similar procedure was used to estimate the synthesis of ribonucleic acid (RNA). In this case, 0.05 mM ^{14}C-uracil (1.0 μCi per μmole) was added to the cultures in the presence of cytosine (20 μg per ml) to minimize the incorporation of radioactivity into DNA (13). Samples were collected, and radioactivity was determined by procedures used for the estimation of protein synthesis.

Owing to an inability of yersiniae to accumulate thymine or thymidine, the synthesis of DNA was determined with ^{14}C-uracil by a procedure employed by Roodyn and Mandel (19). Samples of 1 ml were removed from cultures containing 0.05 mM radioactive uracil (1.0 μCi per μmole), added to 0.1 ml of 5.5 N NaOH, and incubated at 37 C for 18 hr. The preparations then received 0.1 ml of 6 N HCl and 1.2 ml of cold 10% trichloroacetic acid containing nonradioactive uracil (50 mg per ml). After incubation at 5 C for at least 30 min, the samples were filtered and radioactivity was determined as described previously. RNA, but not DNA, is hydrolyzed by this procedure (20).

Reagents and radioisotopes. All amino acids and vitamins were products of Calbiochem (Los Angeles, Calif.), and inorganic compounds of highest available purity were obtained from J. T. Baker Chemical Co. (Phillipsburg, N.J.). Radioisotopes were purchased from New England Nuclear Corp. (Boston, Mass.).

RESULTS

A lag period of about 4 hr occurred before growth of VW^+ cells of *Y. pestis* commenced in cultures containing Ca^{2+} (Fig. 1). After further incubation for 6 hr, such organisms had entered the logarithmic growth phase, whereas stasis was complete by this time in parallel cultures lacking added Ca^{2+}. Accordingly, cells used in subsequent experiments were preincubated for 12 hr to permit the expression of maximal phenotypic differences related to growth and stasis.

As noted by Higuchi, Kupferberg, and Smith (8), the synthetic medium was toxic to cells of *Y. pestis* inoculated at concentrations of less than 10^8 cells per ml. This effect was not observed in the case of *Y. pseudotuberculosis*, which could initiate growth without lag from inocula of 10^7 VW^- cells per ml. Unlike VW^+ cells of *Y. pestis*, those of *Y. pseudotuberculosis* were able to undergo about three divisions in Ca^{2+}-deficient medium before the onset of stasis (Fig. 2); ability of VW^+ organisms of the latter species to multiply in the

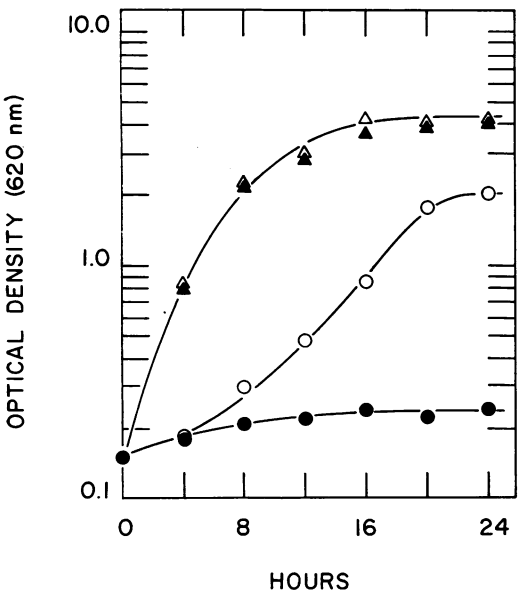

FIG. 1. *Growth of Y. pestis strain EV76 in the modified synthetic medium of Higuchi, Kupferberg, and Smith (8) after inoculation at a level of* 3×10^8 *cells per ml. (●) VW+ cells without added* Ca^{2+}, (○) *VW+ cells plus 0.0025 M* Ca^{2+}, (▲) *VW− cells without added* Ca^{2+}, *and (△) VW− cells plus 0.0025 M* Ca^{2+}.

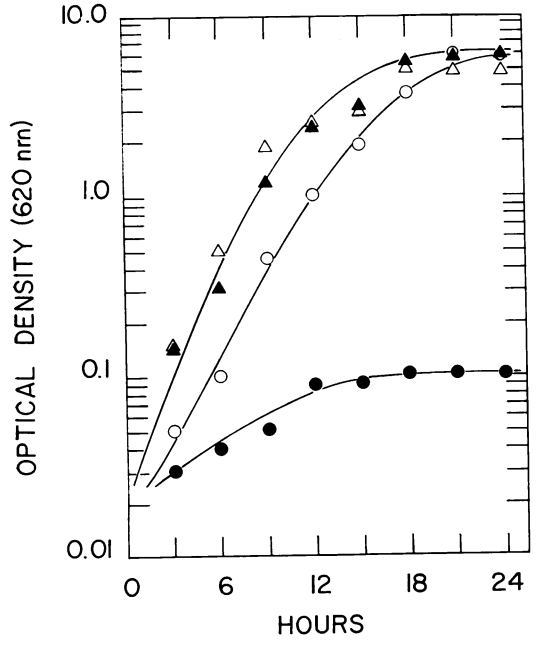

FIG. 2. *Repetition of the experiment of Fig. 1 with Y. pseudotuberculosis strain PB1. The inocula were* 2×10^7 *cell per ml.*

absence of Ca^{2+} was more pronounced in cultures receiving higher inocula (*not illustrated*). In addition, the lag period exhibited by VW^+ cells of *Y. pseudotuberculosis* cultivated with Ca^{2+} was not as pronounced as that shown in Fig. 1 for *Y. pestis*.

Penicillin was introduced into cultures 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 Fig. 3, static VW^+ cells were not significantly influenced by as much as 250 units of penicillin per ml of medium; organisms growing in the presence of Ca^{2+} and penicillin were rapidly killed. This finding demonstrates that there was no significant turnover within the population of static VW^+ cells.

Limited attempts to obtain selective killing of dividing or static VW^+ organisms by use of other antibiotics with known modes of action were only partially successful. For example, inhibitors of DNA synthesis such as mitomycin C or nalidixic acid failed to kill cells of *Y. pestis* in synthetic medium at standard concentrations. How-

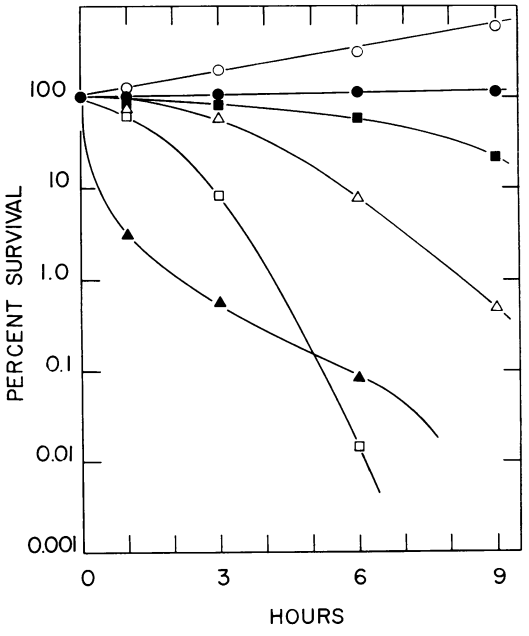

FIG. 3. *Effect of antibiotics on the viability of growing and static VW+ cells of Y. pestis strain EV76. (●) Static cells without addition, (○) growing cells without addition, (■) static cells plus 250 units of penicillin per ml of medium, (□) growing cells plus 250 units of penicillin per ml of medium,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

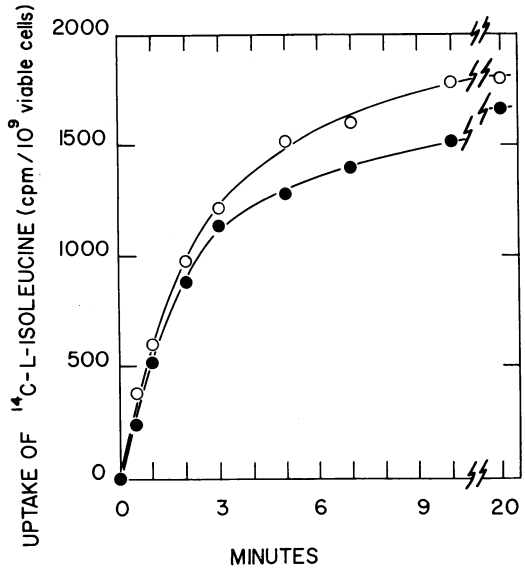

FIG. 4. *Incorporation of ^{14}C-L-isoleucine into the internal pool of growing (○) and static (●) VW$^+$ cells of Y. pestis strain EV76 suspended in neutral potassium phosphate buffer plus gluconate ions.*

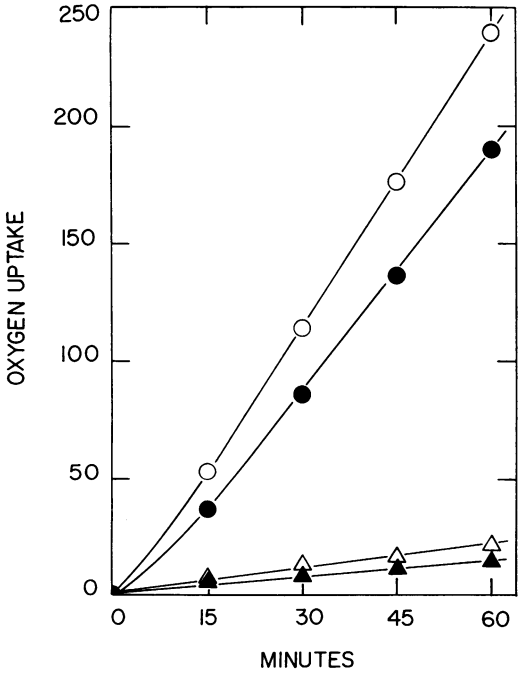

FIG. 5. *Consumption of oxygen, expressed in terms of microliters per milligram of dry weight, by washed static and growing cells of Y. pestis strain EV76. (●) Static cells plus gluconate, (▲) static cells less gluconate, (○) growing cells plus gluconate, and (△) growing cells less gluconate.*

ever, static VW$^+$ organisms were extremely sensitive to as little as 25 units of streptomycin per ml of medium (Fig. 3).

Essentially no differences in ability to accumulate ^{14}C-L-isoleucine were observed between static and dividing organisms (Fig. 4). The rate of oxygen uptake determined for dividing VW$^+$ cells was slightly greater than that obtained for static organisms (Fig. 5). This difference did not appear to be significant and may reflect damage caused by aeration in buffer prior to addition of substrate. Similarly, no distinctions were noted between the rates of release of nonexchangeable ^{32}P by static and dividing VW$^+$ cells (Fig. 6). These findings suggested that the cytoplasmic membranes of static VW$^+$ organisms retained considerable regulatory capacity. Furthermore, it appeared that a considerable amount of macromolecular synthesis occurred in static cells as judged by acute sensitivity to streptomycin and release of ^{32}P at a rate equivalent to that of dividing organisms. Accordingly, a study of macromolecular synthesis was initiated.

Essentially no difference in rates of RNA synthesis by static and dividing organisms was observed (Fig. 7B). Protein was produced at a slightly reduced rate in static cells, but the rate was not significantly different from that in dividing cells (Fig. 7A). However, the rate of DNA syn-

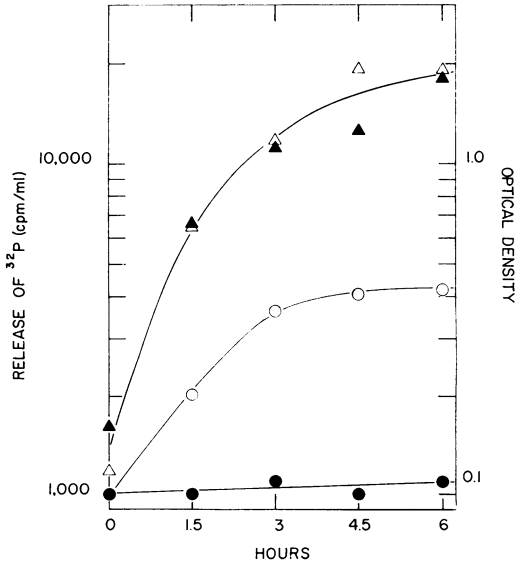

FIG. 6. *Release of ^{32}P from VW$^+$ cells of Y. pestis strain EV76 preloaded with KH^{32}PO$_4^-$ at 26 C containing 180,000 counts per min per 10^8 organisms. (●) Optical density of static cells, (○) optical density of growing cells, (▲) radioactivity released from static cells, and (△) radioactivity released from growing cells.*

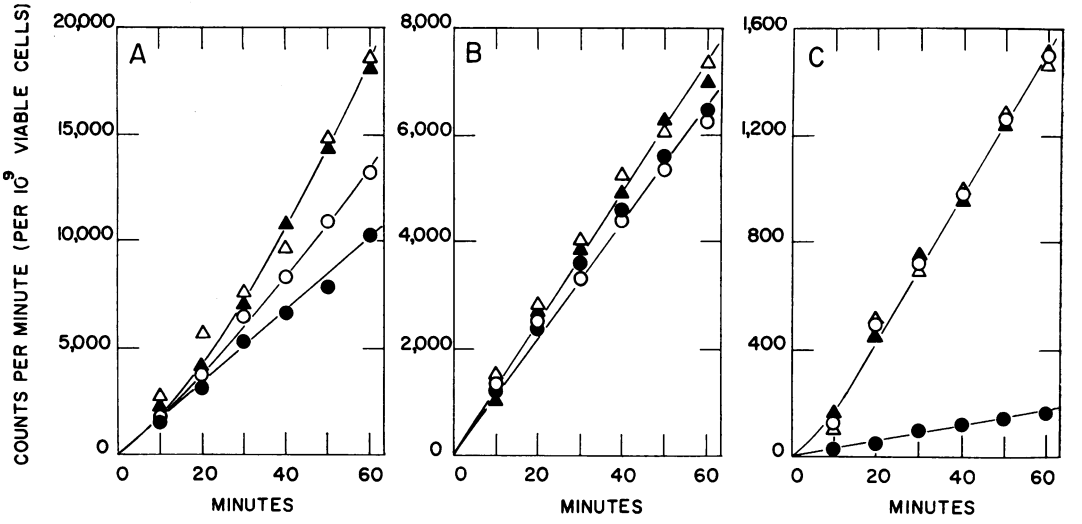

FIG. 7. *Rates of synthesis of macromolecules by static and growing cells of Y. pestis strain EV76. (●) VW^+ cells without added Ca^{2+}, (●) VW^+ cells plus 0.0025 M Ca^{2+}, (▲) VW^- cells without added Ca^{2+}, and (△) VW^- cells plus 0.0025 M Ca^{2+}. (A) Incorporation of ^{14}C-L-isoleucine into 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.*

TABLE 1. *Comparison of rates of macromolecular synthesis in 10^9 VW^+ and VW^- Y. pestis cells cultivated in the presence (0.0025 M) and absence of Ca^{2+}*

Macromolecule[a]	Genotype							
	VW⁻				VW⁺			
	Plus 0.0025 M Ca^{2+}		No added Ca^{2+}		Plus 0.0025 M Ca^{2+}		No added Ca^{2+}	
	Amt (nmoles/hr)[b]	Per cent	Amt (nmoles/hr)	Per cent	Amt (nmoles/hr)	Per cent	Amt (nmoles/hr)	Per cent
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%.

thesis in static cells was markedly reduced in comparison with that in dividing organisms (Fig. 7C). Comparative rates of macromolecular synthesis, corrected from the data illustrated in Fig. 7, are shown in Table 1.

DISCUSSION

Initially virulent cultures of yersiniae often become avirulent after aeration at 37 C because of the emergence of VW^- cells (6–8, 18). This shift in population can be prevented by cultivation at high *p*H (18) or by addition of compounds such as $NaHCO_3$, pyrimidines, 2,4-dinitrophenol, potassium iodide, salicylate ions, and biliverdin, which appear primarily to retard the growth of VW^- organisms (1, 6, 21). The shift to avirulence may also be prevented by addition of Ca^{2+}, Sr^{2+}, Zn^{2+}, KSCN, potassium oleate, and sodium deoxycholate, which generally act by selectively stimulating the growth of VW^+ cells (8, 21). As noted by Surgalla, Andrews, and Cavanaugh (21), the majority of these substances can directly interact with typical cell membranes and surfaces. Furthermore, Brubaker (2) showed that a high concentration of Na^+ and a low ratio of ionic strength to osmolarity were toxic to VW^+ organisms during initial cultivation at 37 C. This set of conditions, which may have been responsible

for the lag periods shown in Fig. 1 and 2, was assumed to cause 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, we initiated with some optimism a study designed to detect functional defects in the cytoplasmic membranes of static yersiniae.

If the amount of radioactivity released by dividing VW^+ cells, after preloading with $KH^{32}PO_4^-$, had been less than that released by static cells, a loss of regulatory capacity in the cytoplasmic membranes of the latter would have been suspected. The reverse would have indicated that the static cells were metabolically inert or dormant. However, as shown in Fig. 6, the rates of release from both types of organisms were identical, suggesting that significant turnover of organic phosphates occurred in the static cells and that these compounds were retained by a functional cell membrane.

In a more direct test of membrane function, isoleucine was concentrated by static cells at a rate commensurate with that of growing organisms. The finding that one amino acid can be accumulated by static organisms does not, of course, prove that remaining amino acids or other substances were similarly transported. However, the observation that both static and dividing cells could synthesize protein at comparable rates suggests that stasis is not caused by a general or specific block in permeation of either amino acids or potential sources of energy. Similarly, the fact that static and dividing organisms consumed about the same amounts of oxygen does not prove that uptake of this gas was directly coupled with generation of adenosine triphosphate via the cytochrome system of the cell membrane. Nevertheless, the discovery that the rates of the energy-dependent synthesis of RNA and protein in dividing and static cells were similar indicates that stasis is not caused by an inability to form high-energy compounds.

The major difference noted between dividing and static organisms was that the latter failed to synthesize significant amounts of DNA. This finding in itself is not surprising; it is inconceivable that such cells can complete an unlimited number of rounds of replication without releasing extracellular DNA. Of significance was the finding that the rates of RNA and protein synthesis were not similarly reduced, as would occur if stasis was a consequence of, or analogous to, a shift-down to a minimal medium or to one lacking some critical nutrient (15). Continued RNA and protein synthesis presumably accounts for the previous observation that static organisms are somewhat larger than dividing cells (3). It will be of interest to determine the number of nuclei per static cell; if this number is greatly in excess of that in dividing cells, it is conceivable that termination of DNA synthesis is caused by an abnormal value of the ratio of DNA to mass (15). In this case, the primary cause of stasis would be a block in the process of cell division per se rather than in inhibition at the level of DNA replication. Temperature-sensitive mutants of this phenotype have been isolated from *Escherichia coli* (10). Perhaps an understanding of why static organisms are especially sensitive to streptomycin, which is generally known to inhibit the function of ribosomes, will facilitate definition of the metabolic block which prevents cell division.

In any event, the results reported in this communication indicate that the cell membranes of VW^+ organisms possess considerable regulatory capacity during cultivation in the simulated intracellular environment. The possibility remains, of course, that newly replicated DNA may fail to attach to the cell membrane or that the cell membrane may be unable to serve as a template for formation of new membrane subunits. This aspect of the division process, as well as DNA replication immediately after removal of Ca^{2+}, will be discussed in a subsequent communication.

The findings reported here may relate to the similar situation which 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 (16). However, these limitations do not always exist, as illustrated by the rickettsiae which, when carefully purified, can generate endogenous high-energy phosphate and possess typical cytoplasmic membranes (17). The phenomenon of stasis in these organisms may thus be analogous to that in *Yersinia*.

ACKNOWLEDGMENTS

This investigation was supported by the Michigan Agricultural Experiment Station (article no. 5156) and by Public Health Service grant AI 08468-02 from the National Institute of Allergy and Infectious Diseases.

The technical assistance of Prudence J. Hall is gratefully acknowledged.

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