TEMPERATURE - DEPENDENT REGULATION BY Ca^{2+} OF MACROMOLECULAR SYNTHESIS IN YERSINIA PESTIS

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ABSTRACT

TEMPERATURE-DEPENDENT REGULATION BY Ca^{2+} OF MACROMOLECULAR SYNTHESIS IN *YERSINIA PESTIS*

By

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Growth of wild-type cells of *Yersinia pestis* is known to be dependent at 37 C, but not 26 C, upon the presence of physiological concentrations of Ca^{2+}. During logarthmic growth at 26 C without Ca^{2+} or at 37 C with Ca^{2+} (permissive conditions), and 37 C without Ca^{2+} (restrictive condition), the increase in bacterial mass paralleled that of total deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein. This increase in mass was also proportional to the rates of DNA, RNA, and protein synthesis under permissive conditions. At the restrictive condition, however, the rate of DNA synthesis rapidly decreased whereas the rates of RNA and protein synthesis essentially remained constant. A net loss of radioactivity occurred in cells cultivated under permissive conditions but not in those at the restrictive condition following a label-chase with uracil-5-^{3}H and excess unlabeled uracil; static cells (restrictive condition)

maintained a constant level of radioactivity. These observations indicate degradation of RNA during logarithmic growth and either rapid turnover or conservation of RNA during bacteriostasis. The demonstration of an unstable RNA fraction in growing cells (26 C) but not in static cells (37 C) following rifampin treatment implicates the synthesis of stable mRNA during bacteriostasis. Upon shift from restrictive to permissive conditions, further increase in mass occurred which corresponded to an immediate increase in rates of protein, but not RNA synthesis. Following return to 26 C, DNA synthesis was initiated after mass had doubled but synthesis of new DNA was not detected at 37 C, following addition of Ca^{2+}, even though mass almost tripled.

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By

Roselyn Valjean Little

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DEDICATION

To my parents who have given me understanding, encouragement, and support in all endeavors, present and past.

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GENERAL INTRODUCTION

Wild type cells of Yersinia pestis provide a simple and unique system for the study of cell division because of their response to changes in temperature when cultivated in Ca^{++}-deficient media containing 0.02 M Mg^{++}. There is no Mg^{++}- dependent requirement for Ca^{++} when yersiniae are cultivated at 26 C. At 37 C, however, these cells become static unless physiological levels of Ca^{++} (0.0025 M) are present (5,7,32,42). Virulence or V and W antigens are expressed by wild-type (VWA^{+}) cells only under conditions favorable for bacteriostasis (7,32,48), and since these conditions simulate mammalian intracellular fluid this response may be an adaptive mechanism necessary for the survival of these organisms within the intracellular environment of the host (7,32,42-43). Sr^{+++} and Zn^{++} (0.0025 M) can substitute for Ca^{++} in relieving bacteriostasis at 37 C (32) but the role of these divalent cations in cell division has not been defined.

The response of Yersinia pestis to a change in the temperature of its environment mimics that of a DNA-A or C type temperature sensitive mutant of Escherichia coli; the latter are blocked in the initiation of DNA synthesis and cell division at the restrictive condition.

Bacteriostasis in yersiniae, in contrast to that in temperature sensitive bacteria, does not result in the formation of filaments but the static cells instead attain a size about twice that of dividing cells (28). A significant difference does, however, exist between yersiniae and temperature-sensitive bacteria, and should be kept in mind in making analogies between the two. The conditional state of yersiniae does not result from an alteration at the genetic level which is only expressed at the restrictive temperature as is observed in a temperature sensitive mutant. The conditional state of yersiniae appears to be related to changes in genetic expression that are influenced by the environment of these cells since the temperature sensitivity of this organism is only expressed during cultivation at the restrictive temperature in the presence of high concentrations of Mg^{++} (0.02 M) and the absence of physiological levels of Ca^{++} (0.0025 M). Accordingly, changes in genetic expression in yersiniae which are effected by environmental conditions can be studied by comparing the molecular mechanisms that are operative in dividing and static yersiniae; this is the basis for the experimental research in this dissertation.

LITERATURE SURVEY

The Yersinia pestis Model

Only a summary of the properties of the Yersinia pestis system will be given; for a more detailed discussion of this system see the review by R. R. Brubaker (7).

Taxonomy

Yersinia pestis, the causative agent of bubonic plague, was formerly classified as Pasteurella pestis. Following the recommendation by the Xth International Congress of Microbiology (1970), reclassification as Yersinia pestis placed the organism in a genus named for its discoverer, Yersin (7,39,71). The genus Yersinia contains Y. pestis, Y. pseudotuberculosis, and Y. enterocolitica (7). Yersiniae show a taxonomic relationship with enteric bacteria based on common antigens (7,49), common sensitivity to phages (7,26,50,70,76), similarities detected by Addisonian analysis (7,72), acceptance by yersiniae of F-lac$^+$ (7,55), and RTF (7,25,40) from Escherichia coli, sensitivity of certain strains of E. coli to pesticin (a bacteriocin produced by Y. pestis) (4,7,70), DNA homology (7,66), and G-C content (7,49,54).

Physiology

Yersinia pestis will grow within a temperature range of -2 C to 45 C, and will tolerate extremes of pH between 5.0 and 9.6 (7,73-74). For the cultivation of yersiniae *in vitro* the optimum temperature is 28 C, and the optimum pH is between 7.2 and 7.6 (7,73-74). The organism is less fastidious in its nutritional requirements at 26 C than at 37 C. At 26 C there is a requirement for L-methionine and L-phenylalanine; L-isoleucine, L-valine, and glycine will enhance growth (7,11,20,33). At 37 C yersiniae are extremely sensitive to high concentrations of free amino acids (6-7), and require biotin, pantothenate, thiamin, and glutamic acid for growth (7).

The fermentation of glucose in *Y. pestis* takes place via the Embden-Meyerhof pathway and the fermentation products are lactate, ethanol, acetate, and formate (7,21,69). Acetate is oxidized to CO_2 by the tricarboxylic acid cycle in aerated cultures (7,68). The hexose-monophosphate pathway is not operative in this organism because of the absence of glucose-6-phosphate dehydrogenase (1,7,19,57-58), and pentose is probably synthesized by the rearrangement of 3 C and 6 C fragments by transketolase and transaldolase (7). An adaptive Entner-Doudoroff pathway, and remaining enzymes of the hexose-monophosphate pathway metabolize gluconate (7,57).

Virulence Determinants

The five established determinants of virulence in Y. pestis involved in the process of infection are V and W antigens, F1 antigen, pesticin I, pigmentation, and the de novo synthesis of purines (7). The expression of these virulence determinants in vitro depends upon either the temperature of cultivation or the presence or absence of divalent cations.

Virulence or V and W antigens are a protein and lipoprotein (7,48) respectively that are always produced together by wild type strains (VWA^+) (7,9,12); avirulent mutants (VWA^-) do not produce either antigen (7,9,12). Virulent organisms (VWA^+) show a Mg^{++} dependent requirement for Ca^{++} at 37 C for growth whereas avirulent mutants (VWA^-) do not (7); Ca^{++} dependence is, therefore, implicated in pathogenicity. V and W antigens are only expressed in vitro under conditions favorable for bacteriostasis (7,32, 48).

Fraction 1 antigen (F1) is a protein antigen associated with the envelope produced by wild type (FRA^+) strains (7,23). Organisms that are FRA^- lack the ability to produce fraction 1 antigen, and are of reduced virulence in guinea pigs but not mice (7,10). The optimum temperature for expression of F1 antigen is 37 C; at 26 C small amounts of the antigen are present in a bound state (7,23).

Pesticin, a bacteriocin, is produced by wild type (PST^+) cells of Y. pestis simultaneously with fibrinolysin and coagulase (7). Loss of one of these determinants results in the concomitant loss of the other two, and cells lacking these three determinants are of reduced virulence in mice (7). Coagulase and fibrinolysin are associated with invasive properties of the organism, and whether the role of pesticin itself is associated with virulence is not certain (7). Pesticin inhibits the growth of certain strains of Y. pseudotuberculosis, E. coli, Y. enterocolitica, and PST^- strains of Y. pestis (4,7,70). The optimum temperature for production of pesticin I is 26 C (7).

Wild type cells of Y. pestis that are PGM^+ can absorb exogenous hemin and basic aromatic dyes, and grow as pigmented colonies on solid media (7). Strains of Y. pestis that are PGM^- do not absorb pigments, grow as non-pigmented colonies, and are avirulent (7). Optimum conditions for the expression of pigmentation are a temperature of 26 C and a pH of 8.0 (7).

Wild type yersiniae are able to carry out the de novo synthesis of purines and are virulent; purine auxotrophs are of reduced virulence in mice, and the degree of reduction in virulence depends on the location of the metabolic block (7). A block prior to the formation of IMP causes only a slight reduction in virulence in mice ($LD_{50} \sim 10^2$ cells) whereas a block between IMP and GMP results in

an LD_{50} of > 10^8 cells (7). The avirulence of purine auxotrophs is thought to be due to the inability to obtain purines from the host (7).

Bacteriostasis

Bacteriostasis in Yersinia pestis occurs during cultivation at 37 C in Ca^{++} deficient media containing 0.02 M Mg^{++} but the regulatory mechanisms responsible for stasis are not known. Observations by Yang and Brubaker (7,80) show that static cells remain viable, and there is no difference in the membrane permeability of static and dividing cells based on permeation of L-isoleucine, oxygen uptake, or release of preloaded ^{32}P. Based on total synthesis of macromolecules by the incorporation of labeled isotope, protein and RNA synthesis are essentially identical in both static and dividing cells but DNA synthesis stops in static cells about 4 hours after the removal of Ca^{++} (7,80). This residual DNA synthesis in static cells is sufficient to complete the current round of chromosome replication since the DNA content of static cells is greater than that of dividing cells (7,80), and static cells returned to 26 C in the presence of mitomycin C were able to perform one division (80). In the absence of mitomycin C, static cells returned to 26 C underwent at least two synchronous divisions (80).

Control of Macromolecular Synthesis

An understanding of the regulation of macromolecular synthesis within the cell is critical for a definitive solution to the enigma of cell division because a cell divides subsequent to completion of a round of replication (14,30) or attainment of a critical ratio of cell mass to DNA (15). The regulatory mechanisms that govern the synthesis of macromolecules within the cell are not entirely understood but the flow of genetic information is known to proceed in a unidirectional manner. The genetic information encoded in DNA molecules is transcribed by a DNA-dependent RNA polymerase into 3 classes of RNA: messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). Following transcription, mRNA is translated into protein on polyribosomal structures by interaction with charged tRNA molecules.

Kinetics of Macromolecular Synthesis

Maaløe and Kjeldgaard (52) stated as a general principle of regulation that "the overall production of DNA, RNA, and protein is regulated by mechanisms that control the frequencies with which the synthesis of individual nucleotide and amino acid chains are initiated." This principle has been substantiated not only by the classic "shift experiments" performed by Kjeldgaard, Maaløe, and

Schaechter (37) but also by Yoshikawa et al. (82), Helmstetter and Cooper (29), Bremer and Yuan (2), Manor et al. (53), and Winslow and Lazzarini (78).

Shift-Up Experiments.--In shift-up experiments (37, 52) bacteria in balanced growth were transferred from a simple defined medium to an enriched medium resulting in an increase in growth rate. The physiological changes accompanying a shift-up were analyzed.

The main feature of the shift-up is the dissociation of RNA synthesis from other cellular events. There is an immediate increase in the rate of RNA synthesis while other activities initially continue at the pre-shift rate. Mass synthesis increases rapidly with a lag of about 5 minutes before the post-shift rate in the enriched medium is attained. DNA synthesis continues at the pre-shift rate for 20 minutes before the definitive rate in the enriched medium is reached. The average number of nuclei per cell remains constant at about 1.5 for 35 minutes and increases to 3.0 by 50-55 minutes after the shift. Cell division is maintained at the pre-shift rate for about 70 minutes before an increased rate is observed. This dissociation pattern is interpreted by Maaløe and Kjeldgaard (37,52) to implicate the operation of separate control mechanisms for macromolecular synthesis.

RNA synthesis following a shift-up is unique because not only is the increase in rate immediate but also because the initial rate in broth exceeds the definitive rate. This observation was made using chemical assays and further study of this phenomenon was made following the kinetics of RNA synthesis by the incorporation of ^{32}P or ^{14}C-uracil. The results of these experiments showed that the rapid increase in RNA synthesis is maintained for 25-30 minutes before the definitive rate characteristic for broth is reached. Slow growing cells were concluded to have a latent capacity for RNA production because of the immediate increase in the rate of RNA synthesis following a shift-up (52), and the large and instantaneous rate of RNA synthesis in the presence of high concentrations of chloramphenicol (24,44). Consideration of the components necessary for RNA synthesis led to the hypothesis by Maaløe and Kjeldgaard (52) that the overall rate of RNA synthesis is governed by the frequency of initiation of new chains. The frequency with which new chains are initiated was postulated to be controlled at the DNA level by a general, nonspecific repression mechanism. That is, RNA synthesis depends on the availability of "receptive" DNA sites or derepressed operons.

The kinetics of protein synthesis during the period following a shift-up was followed by the incorporation

of labeled amino-acids and the results indicated a lag of 20 minutes before an increase in the post-shift rate is attained. The correlation between rRNA and growth rate (38, 52), the number of ribosomes and growth rate (18), and the number of ribosomes and protein synthesis (77) were the bases for the conclusion by Maaløe and Kjeldgaard that the post-shift increase in the rate of protein synthesis was due to an increased concentration of ribosomes in the enriched medium.

The autoradiography of DNA by Cairns (13), the semiconservative nature of DNA replication demonstrated by Meselson and Stahl (56), genetic evidence provided by Yoshikawa and Sueoka (81) resulting from transormation of DNA markers from Bacillus subtilis, Maaløe and Kjeldgaard's autoradiograms of fast and slow growing cells (52), and the multifork replication pattern observed in rapidly growing cells by Oishi et al. (61) and Pritchard and Lark (65) formed the bases for interpretations by Maaløe and Kjeldgaard (52) which are given in the following statements. (i) "Replication is normally initiated at a specific site and proceeds continuously through the entire length of the genome; at a given temperature, this unit process takes approximately the same time whatever the growth rate." (ii) "During slow growth, DNA synthesis is discontinuous, and a full round of replication takes less than one division time." (iii) " At the growth rate

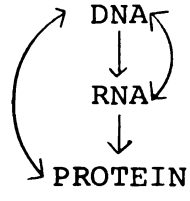
characteristic of glucose-minimal medium, synthesis is almost continuous, and one round of replication can just be completed during the division cycle." (iv) "At higher growth rates, synthesis is also continuous, but less than one round of replication is accomplished between successive divisions; the necessary doubling of the total DNA content of the cell is achieved by having more than one growing point per nucleus."

Shift-Down Experiments.--A shift-down experiment (37,52) is the correlative of a shift-up experiment, and involves the transfer of bacteria from an enriched medium to a simple defined medium accompanied by a decrease in growth rate.

The effects of a shift-down on DNA synthesis and cell division are similar to that observed following a shift-up in that the pre-shift rates are maintained for some time before the post-shift rates characteristic of the new medium are attained. RNA and protein synthesis, however, stop immediately following a shift-down. The immediate effects of protein and RNA synthesis are followed after about 60 minutes by a gradual increase in rates that leads to balanced growth at reduced rates that are characteristic of the new medium. The immediate cessation of protein and RNA synthesis after a shift-down were suggested by Maaløe and Kjeldgaard to simulate the direct and indirect effects respectively of amino acid starvation (52).

Interdependence of DNA, RNA, and Protein

There is evidence for an interrelationship between the synthesis of DNA and that of protein and RNA. The overall cycle of macromolecular synthesis, therefore, appears to be regulated by mechanisms that are interdependent. Stated simply: DNA is needed as a template for the synthesis of RNA (mRNA, rRNA, and tRNA); RNA serves as a template (mRNA), workbench (rRNA), and amino acid carrier (tRNA) for the synthesis of protein; protein and RNA are required for the synthesis of DNA.



The role of DNA in RNA synthesis (35,75), and RNA is protein synthesis (3,27,34,59-60,63) is well documented, and will not be discussed in this review. The involvement of protein and RNA synthesis in the synthesis of DNA is of current interest and will be considered.

The requirement for protein synthesis in the initiation of DNA replication was first suggested by Maaløe and Hanawalt (51). Experimental verification of a requirement for protein synthesis to initiate replication,

but not to maintain it, was provided by Lark, Repko, and Hoffman (45). This evidence lends support to the postulation by Jacob, Brenner, and Cuzin (36) of a hypothetical protein initiator coded for by a specific region of the DNA template in their "replicon" model. This positive control of the initiation of replication is also embodied in the models of Helmstetter et al. (31) and Donachie and Masters (16). A negative control of replication is set forth in the model by Pritchard et al. (64) in which a repressor of initiation is synthesized and diluted below a critical concentration during growth allowing initiation to take place.

The demonstration of a period before initiation in which protein synthesis is not needed in E. coli 15 T^- by Lark and Renger (46) indicates the involvement of some other event in initiation. The possible involvement of transcription in the initiation of DNA synthesis was first suggested by Dove et al. (17) based on observations of the replication of phage λ. Subsequently, Brutlag, Schekman, and Kornberg (8) demonstrated the necessity for transcription in the conversion of M13 single-stranded DNA to the double-stranded replicative form and suggested the possible role of RNA as a primer for DNA synthesis. Stable replication in the absence of protein synthesis observed by Rosenberg et al. (67) and by Kogoma and Lark (41) requires

transcription for its establishment but not for its maintenance because it can continue in the presence of rifampicin or streptolydigin. This observation favors a model of replication in which RNA is needed as a stable product rather than as a primer which would need to be synthesized before each round of replication (62). Lark (47) has demonstrated a requirement for RNA synthesis in E. coli 15 T^- even after the requirement for protein synthesis has been satisfied. Initiation of replication was sensitive to the rifampicin or streptolydigin mediated inhibition of RNA synthesis at a time when it was insensitive to high concentrations of chloramphenicol. The requirement for RNA synthesis was not, therefore, an indirect consequence of blocking the synthesis of protein(s) needed for initiation. Based on this evidence, Lark has proposed a model for replication whereby an RNA core with assembled proteins constitute an initiation complex for DNA synthesis. Worcel and Burgi (79) have proposed a model for the folded chromosome of E. coli based on the sedimentation properties of DNA following intercalation with ethidium bromide or treatment with DNase. This model depicts the chromosome as a looped structure with a core consisting of an RNA species which stabilizes the chromosome through DNA-RNA interactions. According to this model, before each initiation event 2 RNA cores would need to be synthesized, around which newly replicated DNA strands could fold.

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ARTICLE

TEMPERATURE-DEPENDENT REGULATION BY Ca^{2+} OF MACROMOLECULAR SYNTHESIS IN *YERSINIA PESTIS*

By

R. V. Little and R. R. Brubaker

(Manuscript to be published)

ABSTRACT

Growth of wild-type cells of Yersinia pestis is known to be dependent at 37 C, but not 26 C, upon the presence of physiological concentrations of Ca^{2+}. During logarithmic growth at 26 C without Ca^{2+} or at 37 C with Ca^{2+} (permissive conditions), and 37 C without Ca^{2+} (restrictive condition), the increase in bacterial mass paralleled that of total deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein. This increase in mass was also proportional to the rates of DNA, RNA, and protein synthesis under permissive conditions. At the restrictive condition, however, the rate of DNA synthesis rapidly decreased whereas the rates of RNA and protein synthesis essentially remained constant. A net loss of radioactivity occurred in cells cultivated under permissive conditions but not in those at the restrictive condition following a label-chase with uracil-5-^{3}H and excess unlabeled uracil; static cells (restrictive condition) maintained a constant level of radioactivity. These observations indicate degradation of RNA during logarithmic growth and either rapid turnover or conservation of RNA during bacteriostasis. The demonstration of an unstable RNA fraction in growing cells (26 C) but not in static cells

(37 C) following rifampin treatment implicates the synthesis of stable mRNA during bacteriostasis. Upon shift from restrictive to permissive conditions, further increase in mass occurred which corresponded to an immediate increase in rates of protein, but not RNA synthesis. Following return to 26 C, DNA synthesis was initiated after mass had doubled but synthesis of new DNA was not detected at 37 C, following addition of Ca^{2+}, even though mass almost tripled.

TEMPERATURE-DEPENDENT REGULATION BY Ca^{2+} OF MACROMOLECULAR SYNTHESIS IN *YERSINIA PESTIS*

Wild-type cells of *Yersinia pestis* (a facultative intracellular parasite) fail to grow *in vitro* at temperatures characteristic of the mammalian hosts unless Ca^{2+} is present. Mg^{2+} potentiates the nutritional requirement for Ca^{2+} which is not observed during cultivation at room temperature. The concentrations of Ca^{2+} and Mg^{2+} that promote growth or bacteriostasis *in vitro* are identical to those reported to exist within mammalian extracellular (2.5 mM Ca^{2+}; 1.2 mM Mg^{2+}) and intracellular (no Ca^{2+}; 20 mM Mg^{2+}) fluids, respectively (13). Although this relationship may be coincidental, the response of yersiniae to Ca^{2+} is unique in nature and presumably reflects an adaptation to these distinct *in vivo* environments (4).

Ca^{2+} can serve as a cofactor for certain membrane-associated enzymes (8) and exoenzymes (17) and can also exist as a structural component of the gram negative cell surface (6,7,19). Previous attempts to equate bacteriostasis with loss of these functions were not successful. Ca^{2+}-starved yersiniae were about twice the mass of normal organisms but were otherwise similar in morphology; both

types of cells exhibited comparable rates of oxidation, transport, and retention of endogenous ^{32}P (4). These findings suggested that the cell membrane was not grossly altered during bacteriostasis. In this case, catabolic pathways and possibly anabolic reactions might also remain functional. In order to test this possibility, a comparative study of macromolecular synthesis was initiated. Preliminary experiments, as expected, failed to disclose the occurrence of significant deoxyribonucleic acid (DNA) synthesis during bacteriostasis. However, the apparent rates of ribonucleic acid (RNA) and protein synthesis per static organism closely approximated those detected per normal cell (20).

The purpose of this report is to define the kinetics of DNA, RNA and protein synthesis in static and normal yersiniae and to relate these findings to the accumulation of mass.

MATERIALS AND METHODS

Bacteria

The live vaccine strain EV76 of Y. pestis was used throughout this investigation. Cells of this isolate exhibit the nutritional requirement for Ca^{2+} typical of wild-type organisms but are avirulent due to the mutational loss of a determinant said to regulate iron metabolism in vivo. No genetic or phenotypic relationships exist between this property and the expression of Ca^{2+}-dependence (4).

Cultivation

Modified Higuchi medium (20) was used in all experiments. The composition of this medium, which was also used as a diluent, is shown in Table 1.

The organisms were incubated for 36 to 48 hr at 26 C on slopes of blood agar base (Baltimore Biological Labs, Baltimore, Md.), removed in diluent, centrifuged at 27,000 x g for 10 min at 5 C, resuspended, and added to 100 ml of medium per 1 liter flask. Inoculation was performed to yield an optical density of about 0.08 at 620 nm which corresponded to 7.5×10^7 viable cells per ml.

Flasks were aerated in a model G76 gyrotatory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 26 C (a permissive condition) for 3 to 5 hr until the optical

density approximated 0.1. By this time the cells had generally commenced logarithmic growth and the cultures were either retained at 26 C, shifted to another shaker set at 37 C following addition of 2.5 mM $CaCl_2$ (another permissive condition), or shifted to 37 C without addition of Ca^{2+} (restrictive condition). In some experiments, cultures were maintained under the restrictive condition just described for 10 hr and then shifted to one or the other permissive condition.

Routine plating was performed on blood agar base and oxalate agar (12) in order to monitor the incidence of Ca^{2+}-independent mutants in the cultures. In no case did the latter contribute significantly to the total bacterial population.

Net Synthesis of Macromolecules

Samples of 2.0 ml were periodically removed from cultures and immediately frozen in an alcohol-dry ice bath. Prior to analysis, the samples were thawed and then precipitated by addition of an equal volume of chilled 10% trichloroacetic acid. After storage for 30 min in the cold, the precipitates were collected by centrifugation, washed with 5% trichloroacetic acid, and then resuspended in 0.5 ml of 10% perchloric acid. Nucleic acids were quantitatively extracted (9) by treatment at 70 C for 10 min followed by overnight incubation at room temperature. After centrifugation, the

supernatant fluids were removed and the precipitates were again extracted by the same procedure. Both supernatant fractions were pooled and assayed for DNA and RNA by the diphenylamine method of Giles and Myers (10) and the orcinol procedure of Dische (5), respectively. Precipitates remaining after the second perchloric acid extraction were dissolved by boiling in 1.0 ml of 1 N NaOH for 10 min; protein in these samples was determined by the method of Lowry et al. (14). Standards were calf thymus DNA, yeast RNA, and Bovine serum albumin obtained from the Sigma Chemical Co. (St. Louis, Mo.).

Kinetics of Macromolecular Synthesis

The uptake of radioactive thymine or thymidine by yersiniae was not inducible thus carrier-free isotopes were used to determine the kinetics of DNA synthesis. In preliminary experiments, a 3 min lag was observed and then linear incorporation into DNA was directly proportional to cell mass over the range encountered during growth under permissive conditions. The incorporation of thymine was about 4 times that of thymidine thus the former was used in pulse-labeling experiments; incorporation of thymine was not significantly influenced by the temperature of incubation.

The kinetics of DNA synthesis were determined by adding 1.0 ml of culture to tubes (20 x 150 mm) containing 0.1 ml of an aqueous solution of carrier-free thymine-methyl-^{3}H

(10 μ Ci). The tubes were aerated for 7 min in a water bath set at the same temperature as the parent culture. Incorporation of isotope was stopped by addition of 2 ml of cold 5% trichloroacetic acid and the samples were stored for 1 hr in an ice bath. Following centrifugation, the supernatant fluids were carefully decanted and the precipitates were resuspended in 0.2 ml of 5.5 N NaOH. The tubes then received 3 ml of cold 5% trichloroacetic acid and, after 30 min in an ice bath, the precipitates were collected by filtration through membrane filters (Arthur H. Thomas, Co., Philadelphia, Pa.). After washing with an additional 20 ml of 5% trichloroacetic acid, the membranes were dried and prepared for counting. All solutions of trichloroacetic acid contained 0.004% nonradioactive thymine.

Variations in growth were sometimes noted when exogenous uracil or L-histidine was added to the medium. In order to avoid these variables, carrier-free uracil-5-^{3}H (10 μ Ci per ml) and uniformly labeled ^{14}C-L-histidine (0.1 μ Ci per ml) were used to determine the kinetics of RNA and protein synthesis, respectively. Histidine was chosen as a precursor of protein because this compound was the only noncatabolizable amino acid not already present at high concentration in the medium. Under the conditions used in pulse-labeling experiments, the incorporation of radioactive uracil and histidine into trichloroacetic acid-insoluble material was linear and proportional to mass over the range encountered during

growth at permissive conditions. However, the incorporation of uracil during cultivation at 37 C was significantly greater than that observed at 26 C. RNA synthesis per unit of cell mass at 37 C was therefore greater than that determined at 26 C. In contrast, the incorporation of histidine per unit of cell mass was slightly greater at 26 C than at 37 C.

The procedure used for determining the kinetics of RNA and protein synthesis was similar to that described for DNA except that pulses were of 5 and 6 min duration, respectively, and, following storage of trichloroacetic acid-precipitated material for 1 hr, the precipitates were directly collected on membrane filters.

After drying, the membranes were placed in vials which received 10 ml of toluene base containing 0.4% 2,5-diphenyl-oxazole (PPO) and 0.005% 1,4-di-2-(5-phenyloxazolyl)-benzene (dimethyl POPOP). Radioactivity was determined in a Packard Tricarb scintillation counter. Radioisotopes were purchased from New England Nuclear (Boston, Mass.).

Degradation of RNA

Logarithmically growing cells (26 C) were radio-actively labeled in modified Higuchi medium containing uracil-5-^{3}H (0.1 μ Ci per ml) and unlabeled uracil (1 μg per ml) for 12 hr. Following this labeling period, excess cold uracil (5 μg per ml) was added and the label was chased for 4 hr. The cells were collected by centrifugation at

27,000 x g for 20 min at 5 C, washed once in 0.033 M potassium phosphate (pH 7.0), and resuspended in modified Higuchi medium containing unlabeled uracil (1 µg per ml) to yield an optical density of 0.1. Resuspended cultures were aerated under the restrictive and permissive conditions. Samples (1.0 ml) were removed from cultures at hourly intervals for 11 hr and treated in the same manner described for the kinetics of RNA synthesis.

Rifampin Treatment

Growing (26 C without Ca^{2+}) and static (37 C without Ca^{2+}) yersiniae were treated with sodium phosphate-ethylene dinitrilotetraacetic acid (0.1 M phosphate, 10^{-3} M EDTA, pH 6.8) as described by Bremer et al.(3) to facilitate the uptake of rifampin (18). The EDTA treatment was terminated by dilution with 26 C or 37 C medium lacking Ca^{2+} (1 vol). At 4 min subsequent to the termination of EDTA treatment 1.0 ml aliquots of the treated cells were added to tubes containing 0.1 ml of uracil-2-C^{14} (10 µ Ci) and 0.1 ml of rifampin (100 µg). After various periods of incubation (26 C or 37 C with aeration) with uracil-2-C^{14} and rifampin, 0.5 ml samples were removed and processed to obtain the alkaline hydrolysate of the acid precipitate following the procedure of Bremer et al. (3); this procedure completely excludes radioactive DNA. The alkaline hydrolysate (0.5 ml) containing the radioactive ribonucleotides was counted in 5 ml of Bray's

liquid scintillation fluid (2). Rifampin was purchased from Calbiochem (San Diego, Calif.).

Mass

Cell mass was determined as a direct function of optical density at 620 nm in 1 ml cuvettes with a model 2000 spectrophotometer (Gilford Instrument Labs, Oberlin, Ohio). Uninoculated medium was used as a blank.

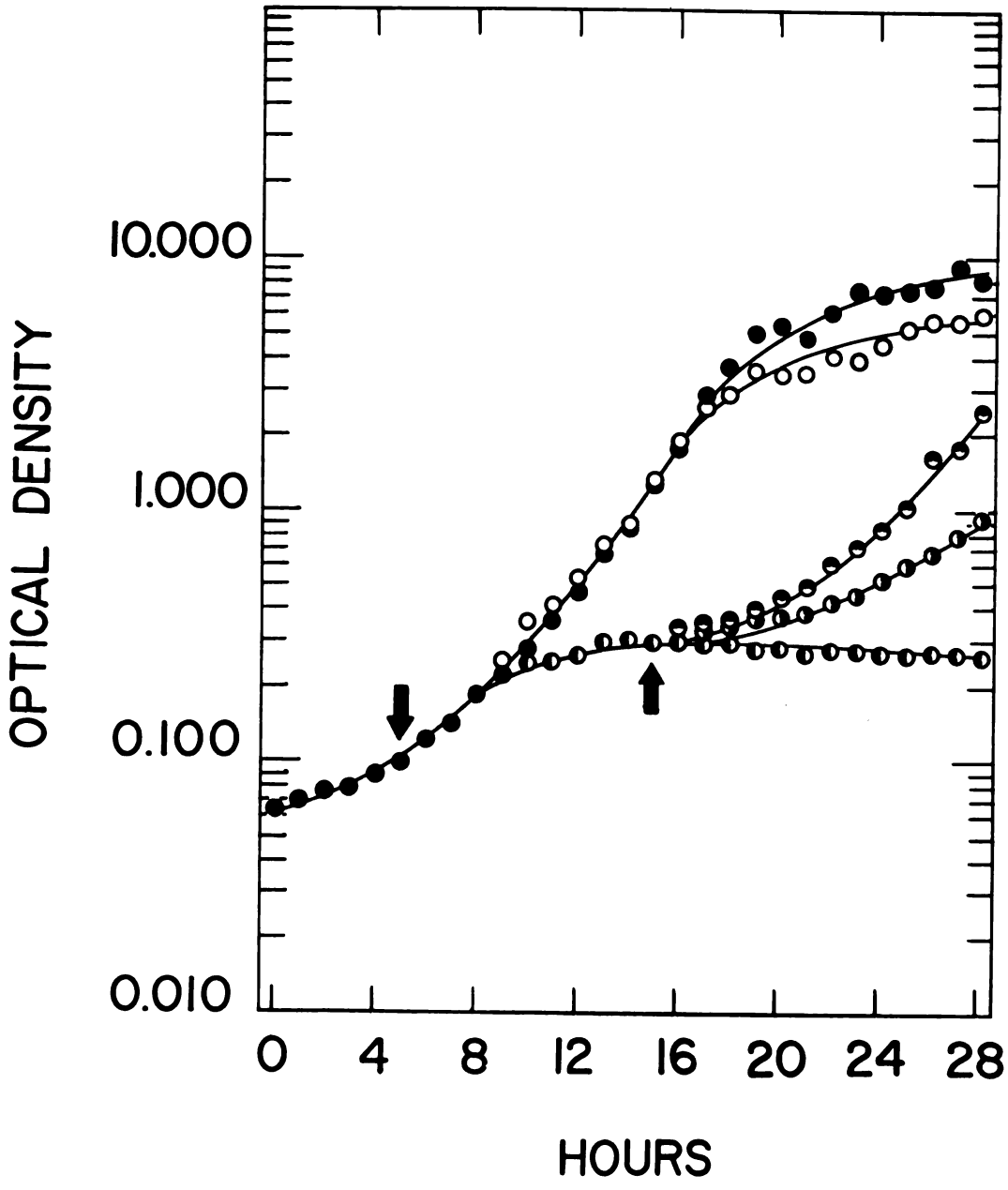
RESULTS

Mass

The increase in cell mass during cultivation at 26 C and at 37 C in the presence and absence of Ca^{2+} is shown in Figure 1. After a lag, the rates of increase were similar under the 2 sets of permissive conditions but the logarithmic growth phase was extended for about 2 hr during incubation at 26 C. After the first shift to restrictive conditions, mass doubled and then decreased slightly towards the end of the determination. A significant lag again occurred after the second shift to permissive conditions; this phase was most prominent in the culture which received Ca^{2+} at 37 C.

This determination of mass defines the experimental system used in subsequent investigations of macromolecular synthesis. The responses shown in Figure 1 were generally reproducible although some variation was noted upon initial shift to 37 C in the presence of Ca^{2+} and in the duration of the lag period following recovery from bacteriostasis at 37 C following addition of Ca^{2+}. In order to facilitate direct comparisons, mass is shown in subsequent illustrations depicting total synthesis and rates of synthesis of macromolecules.

Figure 1.--Effect of Ca^{2+} and temperature on the growth of Y. pestis strain EV76. A series of cultures were inoculated and aerated at 26 C; one culture remained at this temperature throughout the experiment (●). At the point shown by the first arrow, one culture received 2.5 mM $CaCl_2$ and was shifted to 37 C (○), and 3 cultures were shifted to 37 C without addition of Ca^{2+} (◐). One of the latter was maintained under this condition and, at the point shown by the second arrow, the second was shifted to 26 C without addition of Ca^{2+} (◓) while the third culture was retained at 37 C after receiving 2.5 mM Ca^{2+} (◑).


OPTICAL DENSITY
10.000
1.000
0.100
0.010
0
4
8
12
16
20
24
28
HOURS

Total Synthesis of Macromolecules

Increases in net DNA, RNA and protein paralleled that of mass during growth under permissive conditions (Figure 2). Similar results were obtained during cultivation under restrictive conditions indicating that bacteriostasis was not a function of gross change of macromolecular composition.

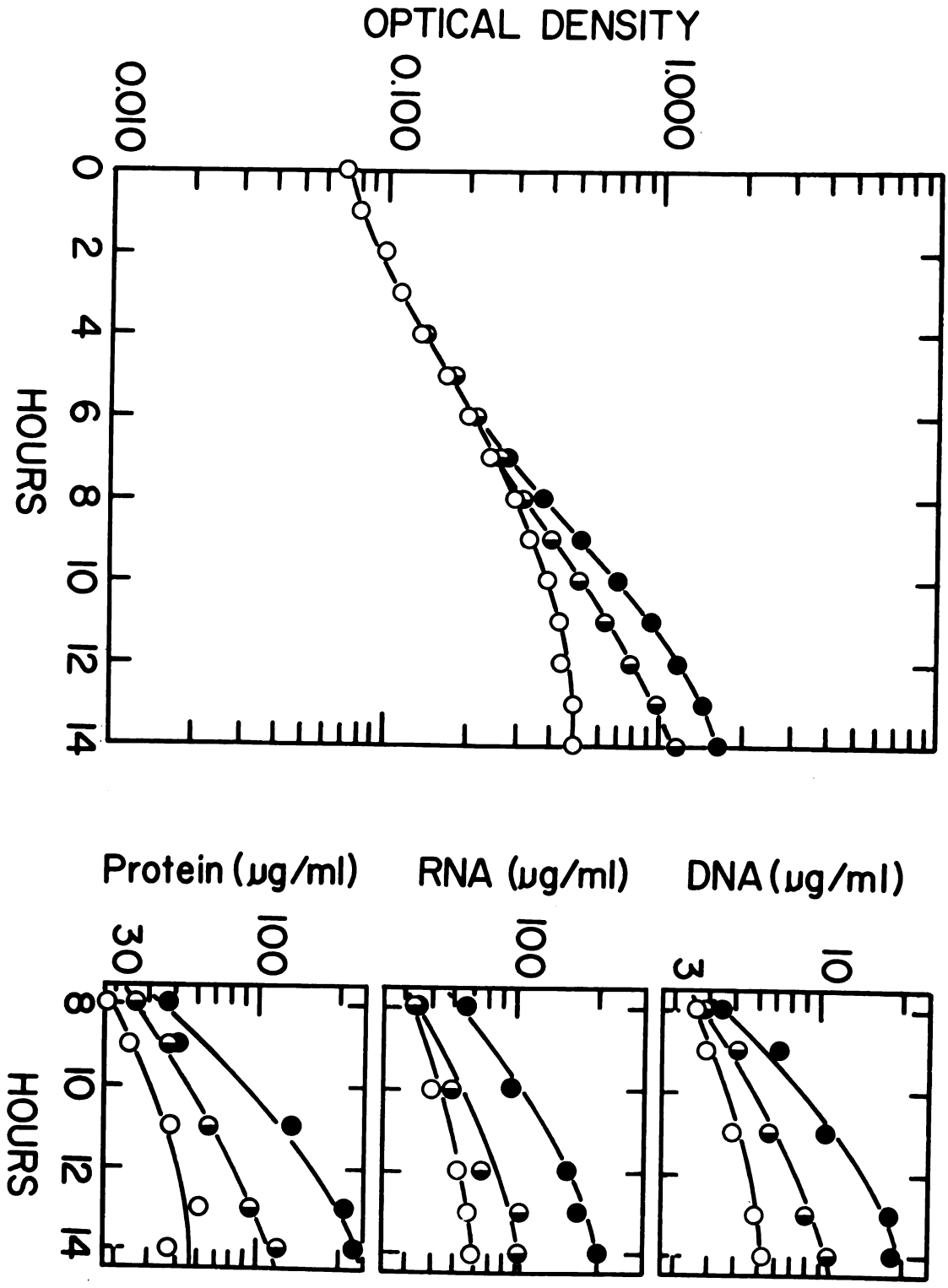
Kinetics of Macromolecular Synthesis During the Onset of Stasis

In control cultures incubated under permissive conditions, DNA synthesis closely paralleled mass until the cells approached the stationary phase whereupon a decrease became evident (Figure 3A, 3C). DNA synthesis under restrictive conditions also initially followed mass and then fell rapidly following the onset of bacteriostasis (Figure 3B).

Similarly, the ratio of RNA synthesis to mass remained nearly constant during cultivation under permissive conditions (Figure 4A, 4C). However, unlike the results obtained with DNA, RNA synthesis under restrictive conditions was maintained relative to mass following the onset of bacteriostasis (Figure 4B).

Constant ratios of mass to protein synthesis were also observed during growth under permissive conditions (Figure 5A, 5C). In contrast, protein synthesis under restrictive conditions fell to a reduced but constant level relative to mass as the organisms became static (Figure 5B).

Figure 2.--Total DNA, RNA and protein, as determined by chemical assay, in cultures of *Y. pestis* strain EV76 cultivated at 26 C without Ca^{2+} (●), at 37 C without Ca^{2+} (○), and at 37 C with 2.5 mM Ca^{2+} (◑). All cultures were incubated for 4 hr at 26 C without Ca^{2+} before shift to the indicated conditions.


OPTICAL DENSITY
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HOURS
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2
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6
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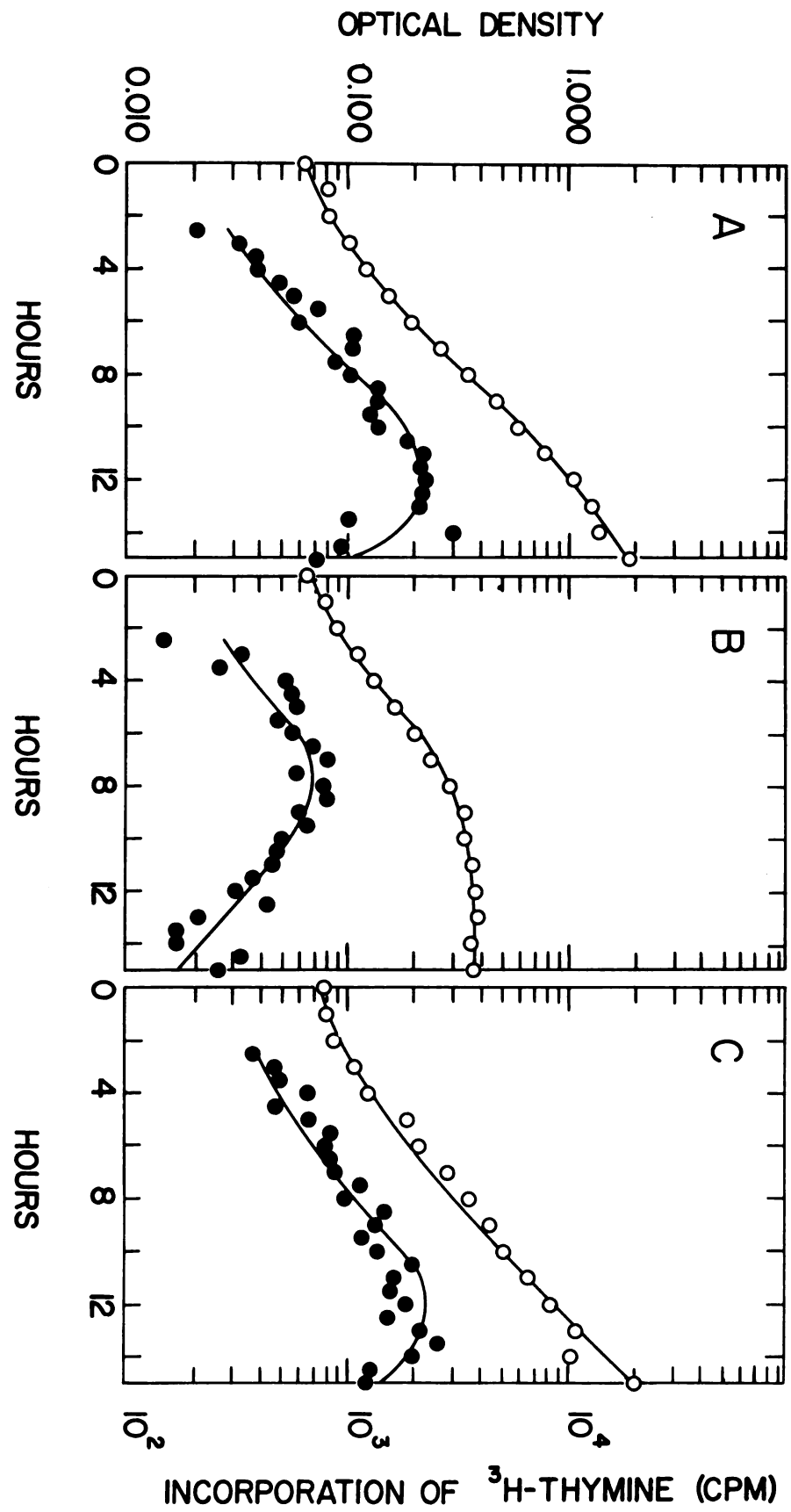
10
12
14
Protein (µg/ml)
RNA (µg/ml)
DNA (µg/ml)
30
100
100
3
10
HOURS
8
10
12
14

Figure 3.--Kinetics of DNA synthesis, determined by pulse-labeling with ^{3}H-thymine, in cells of *Y. pestis* strain EV76 cultivated at 26 C without Ca^{2+} (A), at 37 C without Ca^{2+} (B), and at 37 C with 2.5 mM Ca^{2+} (C). All cultures were incubated for 3 hr at 26 C without Ca^{2+} before shift to the indicated conditions: (○), optical density; (●), radioactivity.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HOURS
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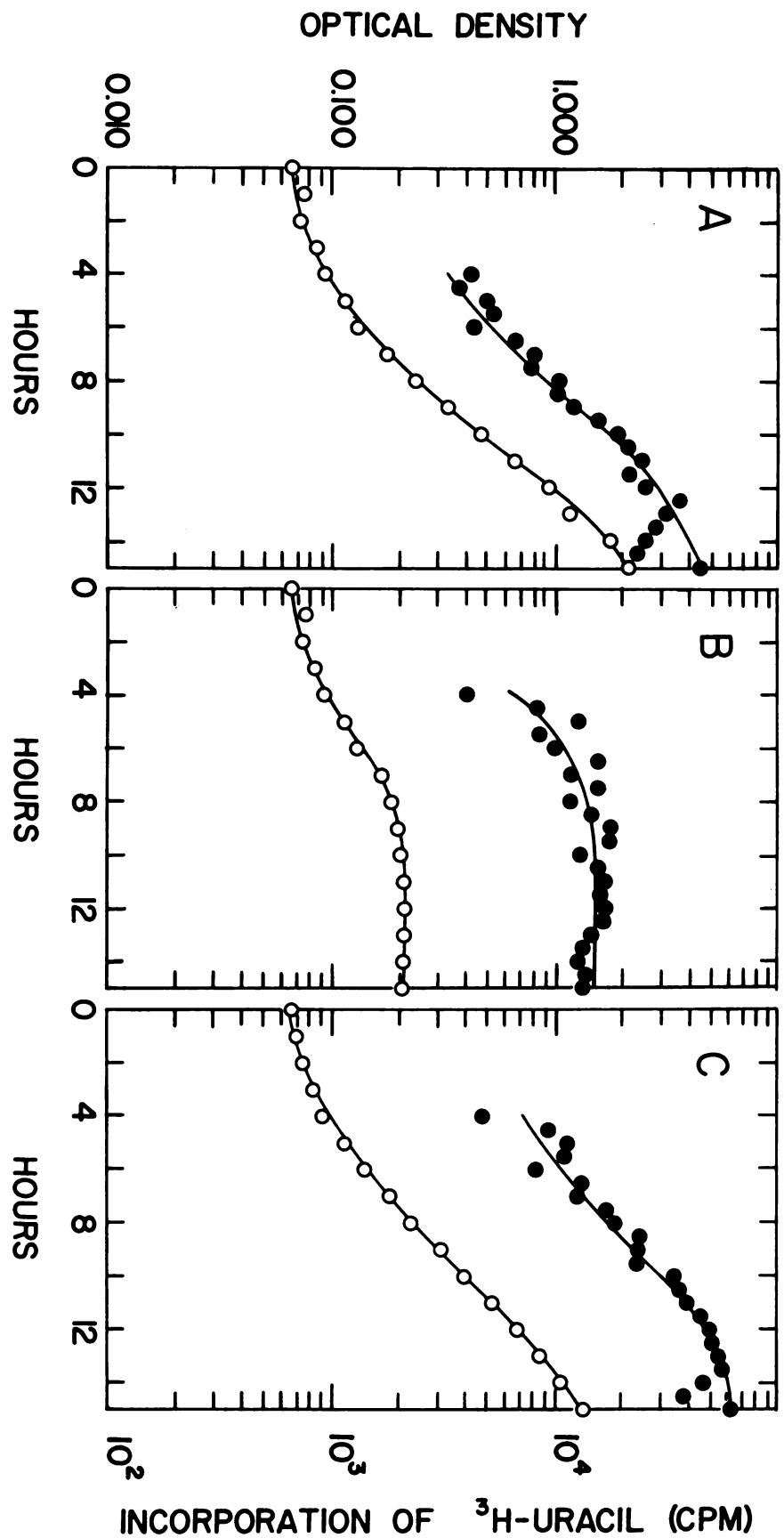
8
12
10^2
10^3
10^4
INCORPORATION OF ^{3}H-THYMINE (CPM)

Figure 4.--Kinetics of RNA synthesis, determined by pulse-labeling with ^{3}H-uravil, in cells of Y. pestis strain EV76 cultivated at 26 C without Ca^{2+} (A), at 37 C without Ca^{2+} (B), and at 37 C with 2.5 mM Ca^{2+} (C). All cultures were incubated for 4 hr at 26 C without Ca^{2+} before shift to the indicated conditions: (○), optical density; (●), radioactivity.


OPTICAL DENSITY
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HOURS
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10^2
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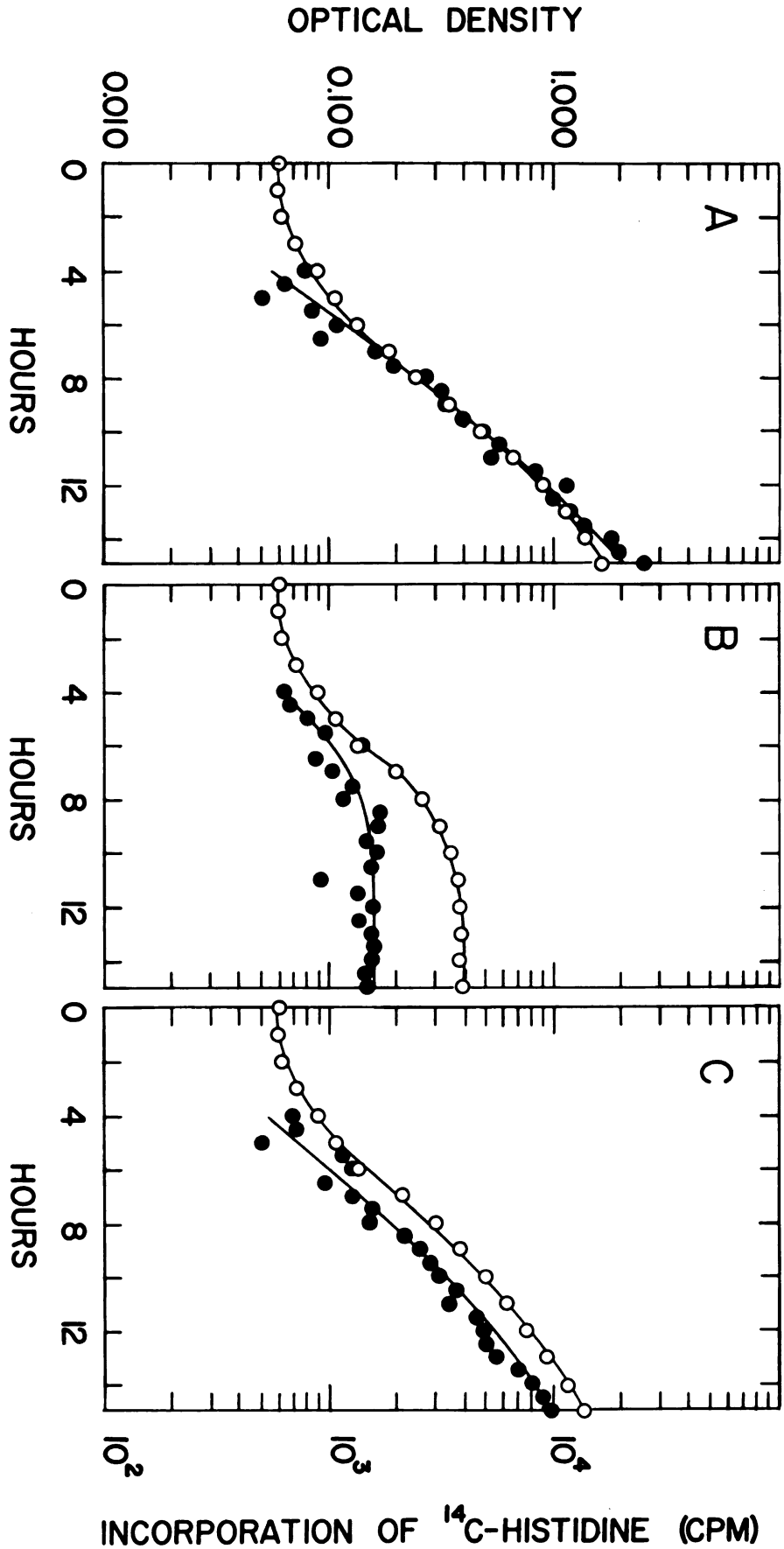
10^4
INCORPORATION OF ^{3}H-URACIL (CPM)

Figure 5.--Kinetics of protein synthesis, determined by pulse-labeling with ^{14}C-histidine, in cells of Y. pestis strain EV76 cultivated at 26 C without Ca^{2+} (A), at 37 C without Ca^{2+} (B), and at 37 C with 2.5 mM Ca^{2+} (C). All cultures were incubated for 5 hr at 26 C without Ca^{2+} before shift to the indicated conditions: (○), opitcal density; (●), radio-activity.


OPTICAL DENSITY
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10^4
INCORPORATION OF ^{14}C-HISTIDINE (CPM)

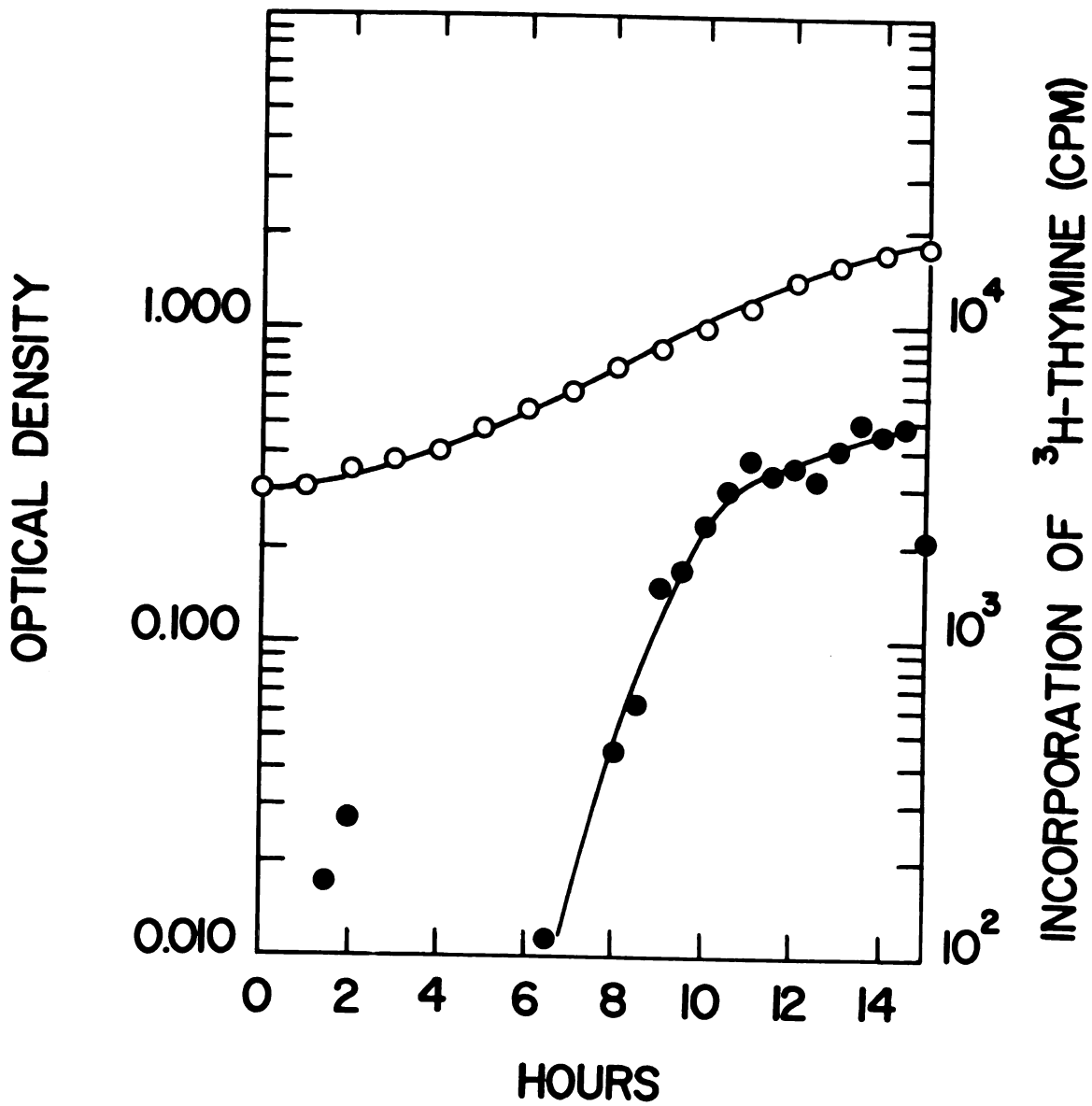
Kinetics of Macromolecular Synthesis Upon Recovery from Stasis

In the following experiments, sets of 3 parallel cultures were incubated for 10 hr under restrictive conditions; a culture was then shifted to each of the permissive conditions and the remainder was maintained as a control at 37 C without added Ca^{2+}. Increase in mass and macromolecular synthesis were followed as the cells recovered from bacteriostasis.

Significant synthesis of DNA was delayed for about 8 hr following return of static organisms to 26 C (Figure 6). At this time, mass has approximately doubled and a rapid synthesis of DNA was maintained until a second doubling occurred. Thereafter, synthesis paralleled the increase of mass. New DNA was not produced in the control culture retained under restrictive conditions. Similarly, synthesis of DNA was not initiated in cells maintained at 37 C after addition of Ca^{2+} even though mass had almost tripled at an optical density of 0.8 when the experiment was terminated (not illustrated).

Upon return to 26 C, the ratio of RNA synthesis to mass appeared to decrease initially (Figure 7A); this change was caused in part by the effect of temperature on uracil-transport noted previously. The decreased ratio observed following addition of Ca^{2+} to cells maintained at 37 C (Figure 7C) cannot be explained on this basis. A similar initial decrease in synthetic rate was also determined in control cells retained under restrictive conditions (Figure 7B).

Figure 6.--Reinitiation of DNA synthesis, determined by pulse-labeling with ^{3}H-thymine, in cells of *Y. pestis* strain EV76 upon shift from 37 C to 26 C in Ca^{2+}-deficient medium. The organisms had previously been maintained at 37 C for 12 hr: (○), optical density; (●), radioactivity.


OPTICAL DENSITY
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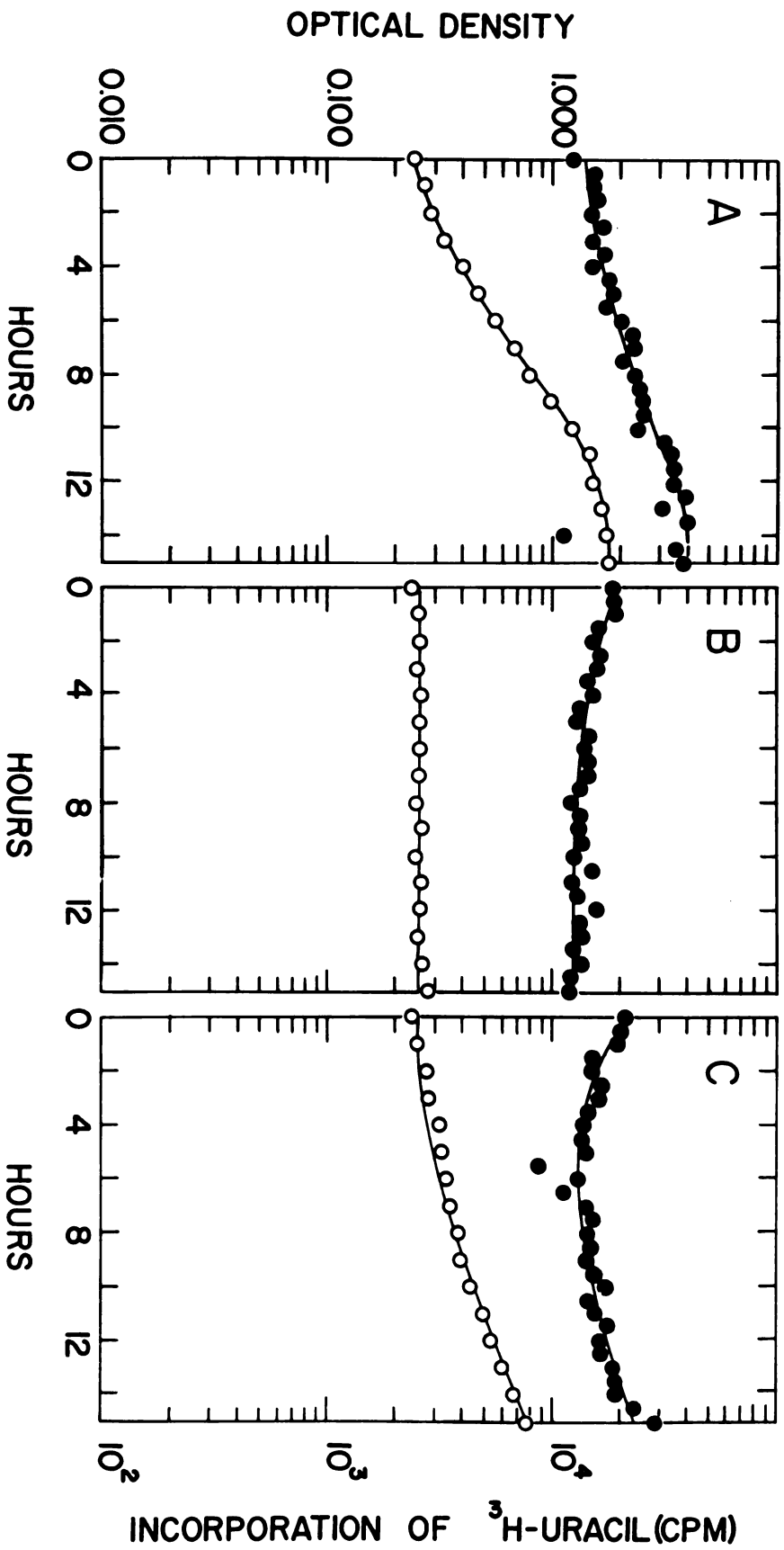
HOURS
10^4
10^3
10^2
INCORPORATION OF ^{3}H-THYMINE (CPM)

Figure 7.--Kinetics of RNA synthesis, determined by pulse-labeling with ^{3}H-uracil, in cells of *Y. pestis* strain EV76 cultivated at 26 C without Ca^{2+} (A), at 37 C without Ca^{2+} (B), and at 37 C with 2.5 mM Ca^{2+} (C). All cultures were incubated for 10 hr at 37 C without Ca^{2+} before shift to the indicated conditions: (○), optical density; (●), radioactivity.


OPTICAL DENSITY
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INCORPORATION OF ^{3}H-URACIL(CPM)

In contrast, the ratio of protein synthesis to mass increased following return to permissive conditions. This increase was more apparent following return to 26 C (Figure 8A) than after addition of Ca^{2+} to cells maintained at 37 C (Figure 8C). In both cases, however, the ratios eventually approached those observed during initial growth under permissive conditions (Figure 5A, 5C). A further decrease in protein synthesis relative to mass was observed in control cultures maintained under restrictive conditions (Figure 8B).

The results that have been given for the kinetics of macromolecular synthesis by pulse-labeling of DNA, RNA, and protein correspond to those obtained by replotting these data to give the rates of macromolecular synthesis during the onset of stasis (Figure 9) and upon recovery from stasis (Figure 10).

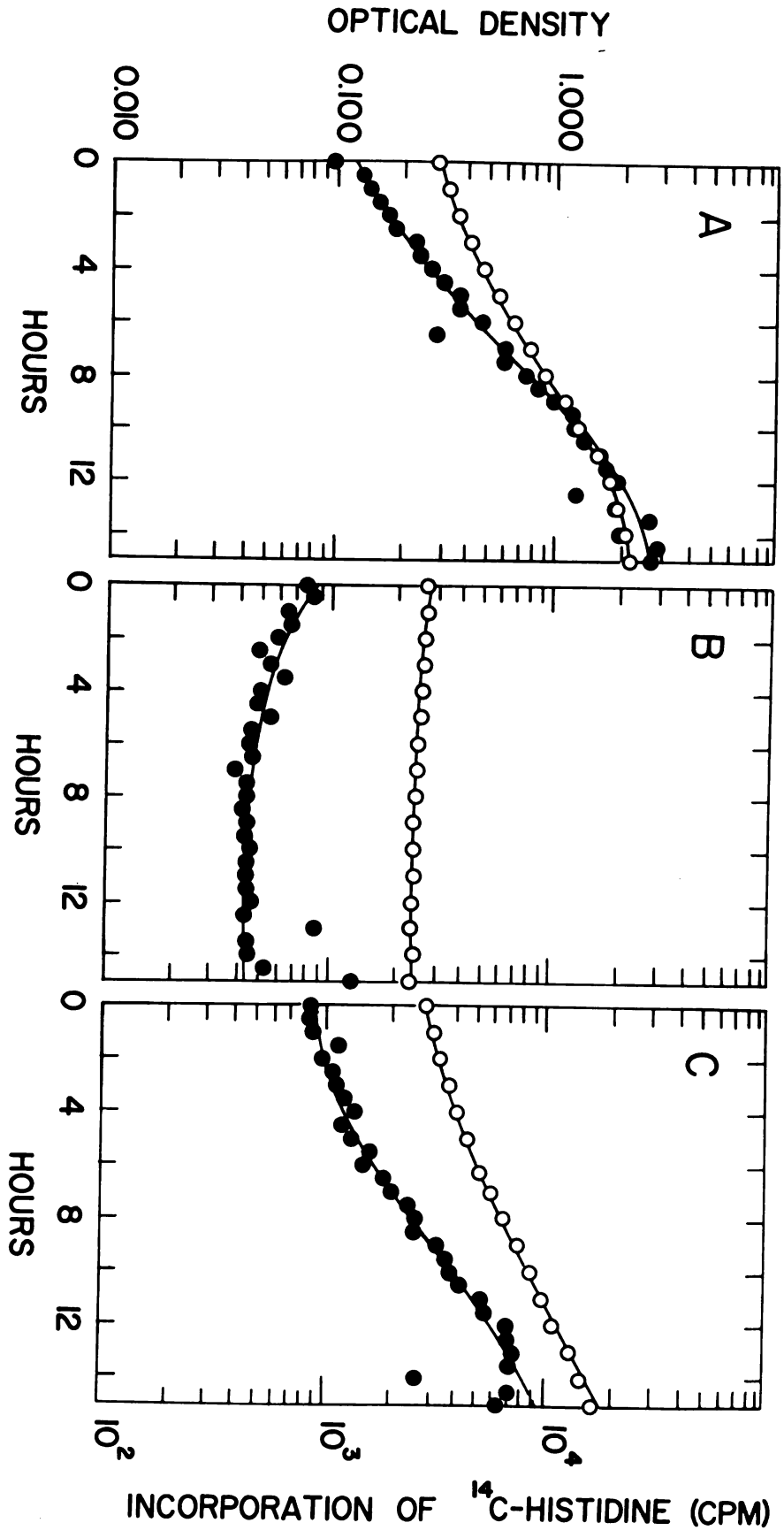
Degradation of RNA

Following a 12 hr labeling period of RNA with uracil-5-^{3}H and a 4 hr chase with excess unlabeled uracil, loss of radioactivity was observed only under permissive conditions and not at the restrictive condition upon resuspension in unlabeled modified Higuchi medium (Figure 11).

Rifampin Treatment

Treatment of growing cells (26 C) with rifampin resulted in an immediate burst of RNA synthesis which reached a maximum at 30 sec, and subsequently declined to a constant

Figure 8.--Kinetics of protein synthesis, determined by pulse-labeling with ^{14}C-histidine, in cells of Y. pestis strain EV76 cultivated at 26 C without Ca^{2+} (A), at 37 C without Ca^{2+} (B), and at 37 C with 2.5 mM Ca^{2+} (C). All cultures were incubated for 10 hr at 37 C without Ca^{2+} before shift to the indicated conditions: (○), optical density; (●), radioactivity.


OPTICAL DENSITY
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C
HOURS
0
4
8
12
10^2
10^3
10^4
INCORPORATION OF ^{14}C-HISTIDINE (CPM)

Figure 9.--Rates of macromolecular synthesis during the onset of stasis in cells of Y. pestis strain EV76 cultivated at 26 C without Ca^{2+} (1), at 37 C without Ca^{2+} (2), and at 37 C with 2.5 mM Ca^{2+} (3). The data shown in Fig. 3-5 was redrawn to illustrate the rates of DNA synthesis (A), RNA synthesis (B), and protein synthesis (C).

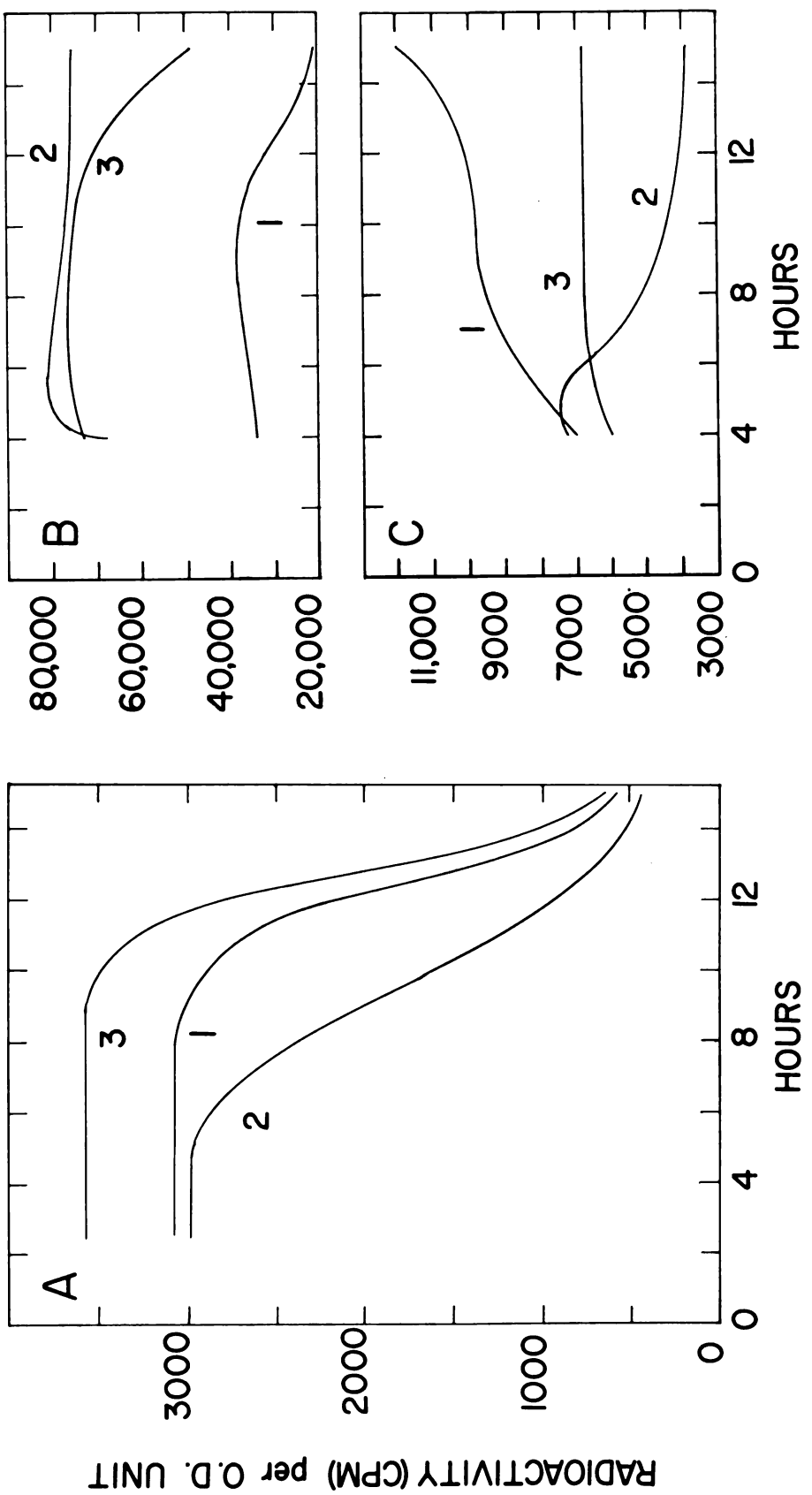

A
B
C
1
2
3
HOURS
0
4
8
12
3000
2000
1000
80,000
60,000
40,000
20,000
11,000
9000
7000
5000
RADIOACTIVITY (CPM) per O.D. UNIT

Figure 10.--Rates of macromolecular synthesis upon recovery from stasis in cells of Y. pestis strain EV76 cultivated at 26 C without Ca^{2+} (1), at 37 C without Ca^{2+} (2), and at 37 C with 2.5 mM Ca^{2+} (3). The data shown in Figures 6-8 was redrawn to illustrate the rates of DNA synthesis (A), RNA synthesis (B), and protein synthesis (C).

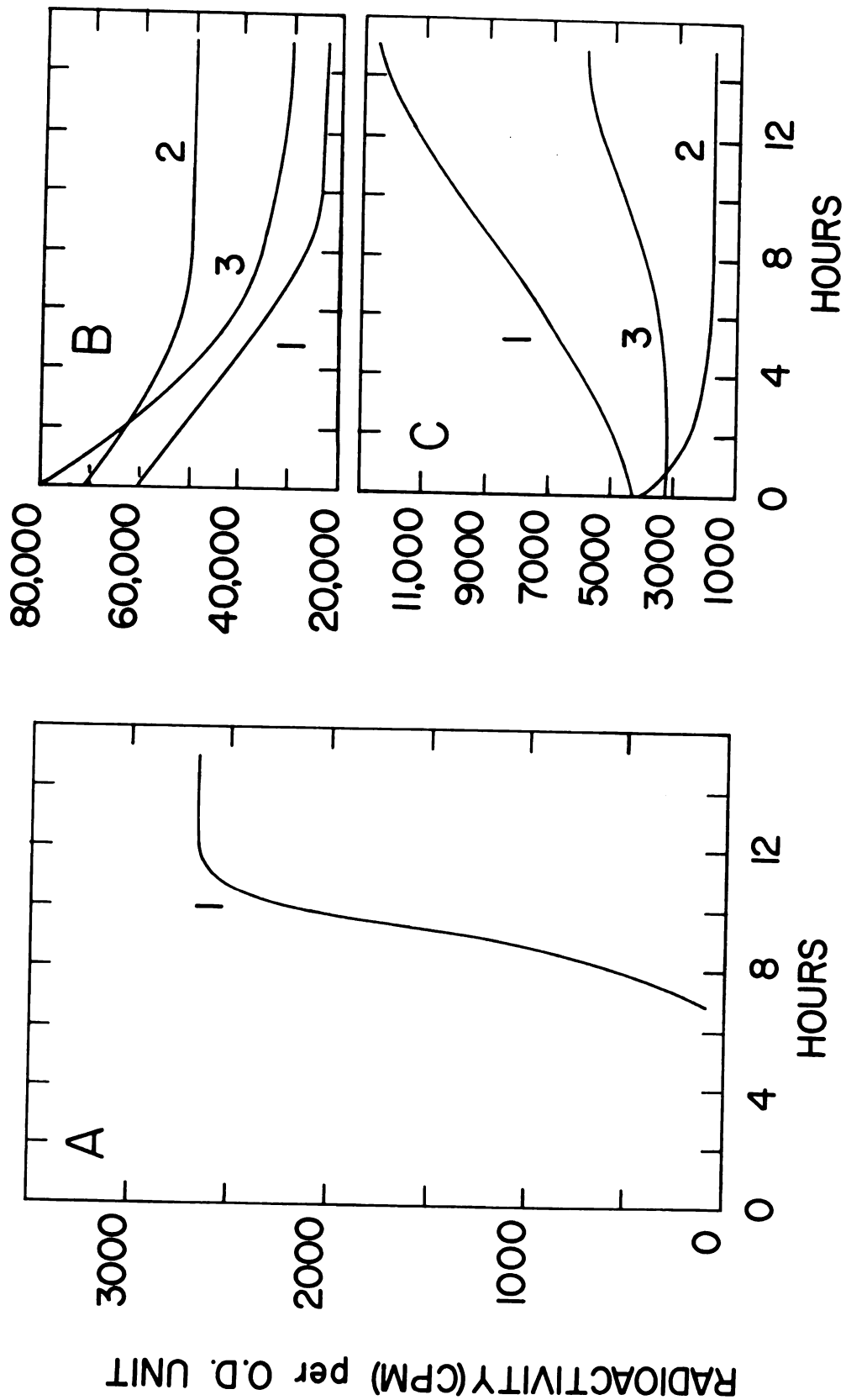
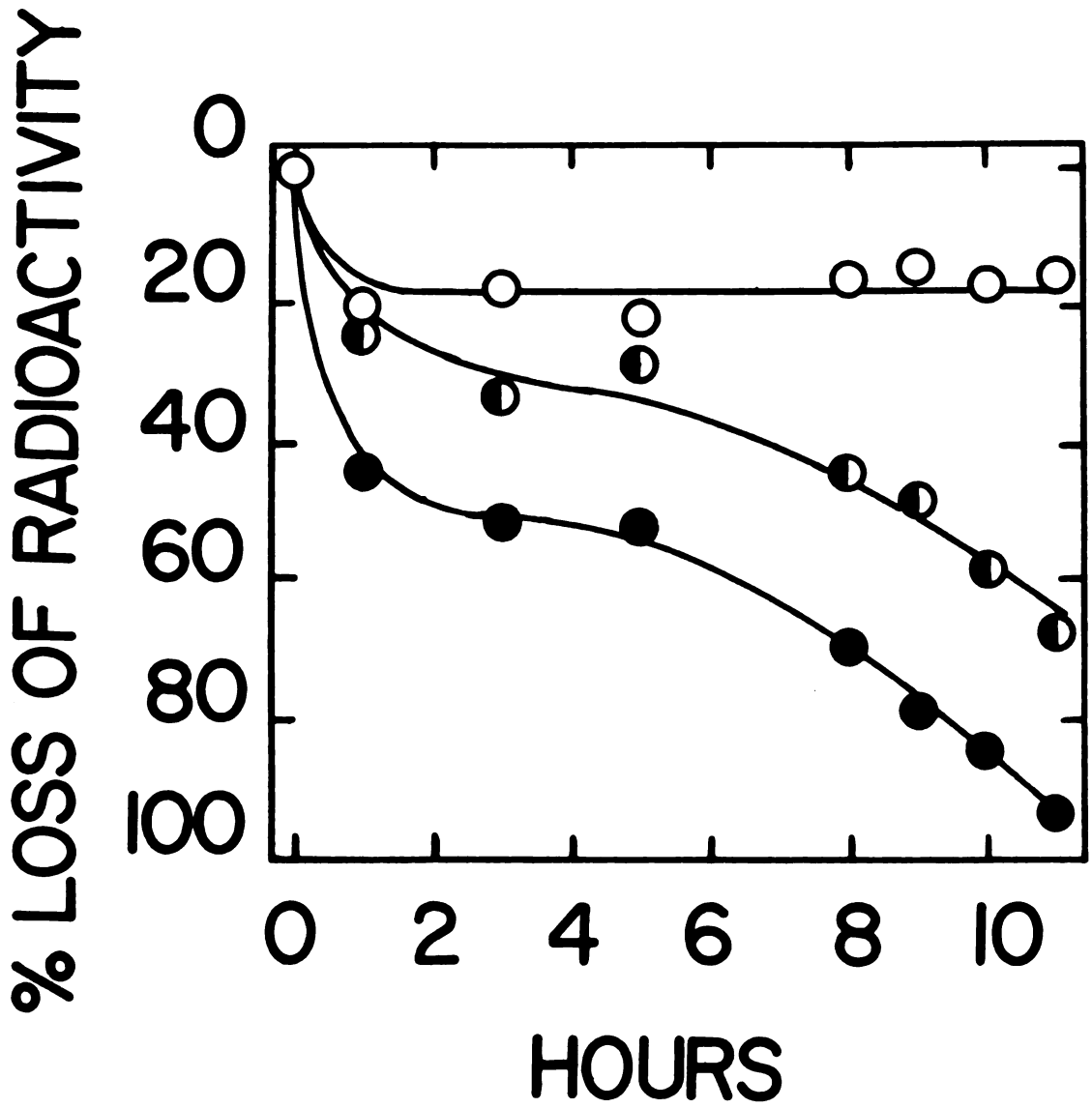
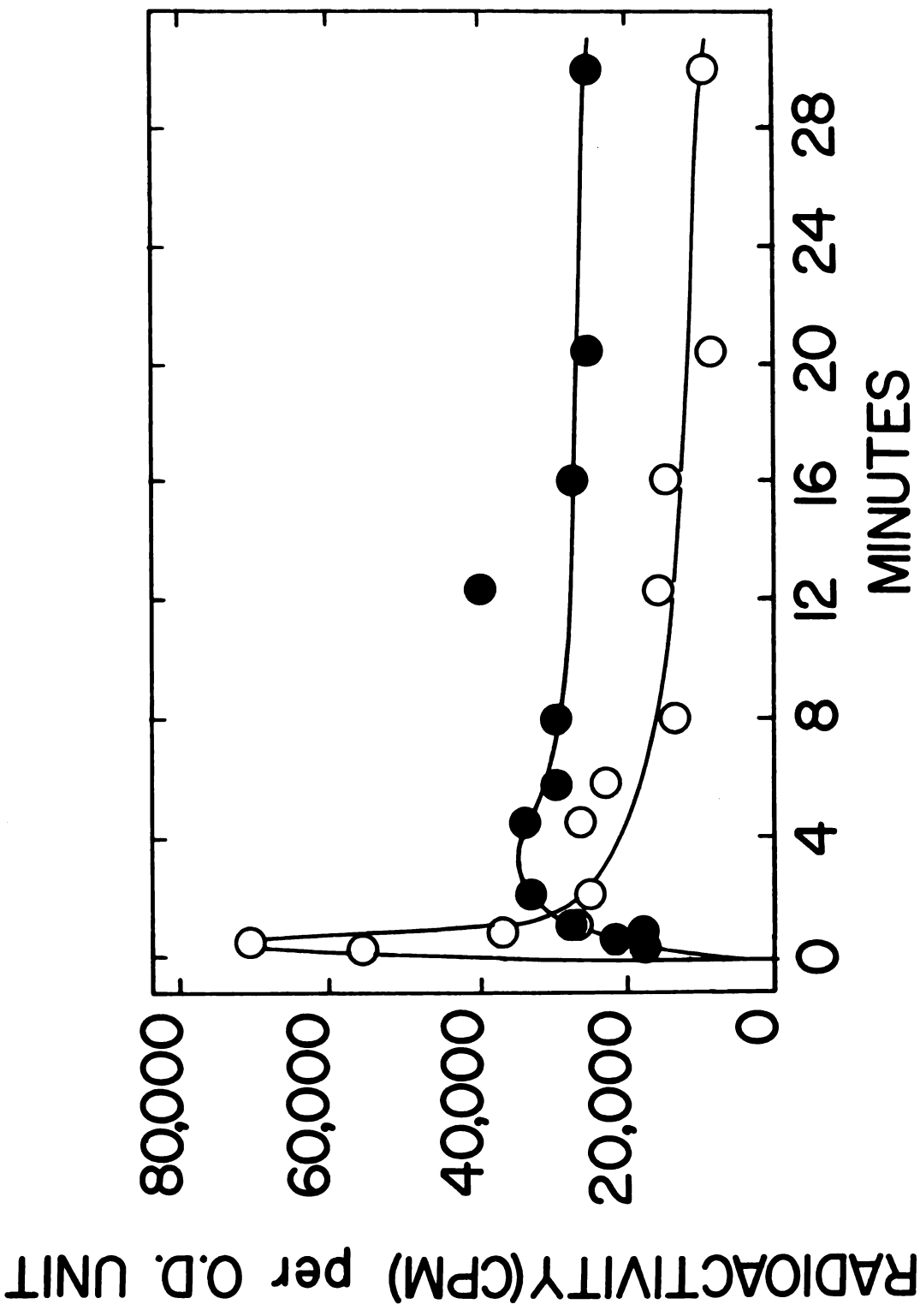

A
B
C
1
2
3
0
4
8
12
HOURS
3000
2000
1000
0
80,000
60,000
40,000
20,000
11,000
9000
7000
5000
3000
1000
RADIOACTIVITY (CPM) per O.D. UNIT

Figure 11.--Loss of trichloroacetic acid-insoluble radioactivity, following a label-chase with uracil-5-^{3}H and excess unlabeled uracil, in cells of Y. pestis strain EV76 during cultivation in unlabeled modified Higuchi medium at 26 C without Ca^{2+} (●), at 37 C without Ca^{2+} (○), and at 37 C with 2.5 mM Ca^{2+} (◑).


% LOSS OF RADIOACTIVITY
0
20
40
60
80
100
0
2
4
6
8
10
HOURS

level characteristic for stable RNA (Figure 12). Static cells (37 C) showed no decay of an unstable RNA fraction following rifampin treatment and reached a constant plateau characteristic for completion of stable RNA chains (Figure 12).

Figure 12.--Residual RNA synthesis after rifampin treatment, determined by labeling with ^{14}C-uracil, in cells of Y. pestis EV76 cultivated at 26 C without Ca^{2+} (○), and at 37 C without Ca^{2+} (●).


RADIOACTIVITY (CPM) per O.D. UNIT
80,000
60,000
40,000
20,000
0
0
4
8
12
16
20
24
28
MINUTES

DISCUSSION

Although DNA accumulated (Figure 2), rates of syntheses fell during the onset of bacteriostasis (Figure 3B) in a manner similar to that observed during terminal growth under permissive conditions (Figure 3A, 3C). In the latter, this decrease in rate presumably reflects a step-down, caused by exhaustion of nutrients, resulting in a reduction of the number of replication forks per chromosome (15). A similar failure to initiate new rounds of replication could account for the early reduction in rate of DNA synthesis which occurred following shift to restrictive conditions. In this case, the decreased rate would represent the progressive termination of rounds of replication already in progress during the onset of bacteriostasis. Accordingly, the chromosomes would become aligned at the terminus and, following return to permissive conditions, an abrupt reinitiation of synthesis might occur as shown in Figure 6. It seems unlikely that the decreased rate of DNA synthesis represents a progressive inactivation of some component of the replication system per se. A gradual resumption of DNA synthesis upon return to 26 C might be expected in this case. Furthermore, chromosome replication during bacteriostasis would

cease at random points prior to the terminus, a situation which can be lethal in bacteria (15). By appropriate use of existing procedures (1), it should be possible to directly identify the correct alternative.

It is significant that DNA synthesis resumed upon return to 26 C after mass had doubled but, following addition of Ca^{2+} at 37 C, new synthesis was not detected even though mass almost tripled during the course of the experiment. Further attempts will be made to determine if DNA synthesis can be reinitiated at 37 C following Ca^{2+}-starvation. Also, it would be of interest to determine if the increase in mass observed during this period reflects normal cell division with segregation of preformed chromosomes or elongation of the organisms without cell division.

Protein synthesis was significantly reduced during the onset of bacteriostasis relative to its synthesis during logarithmic growth. Following incubation for about 3 hr under restrictive conditions, mass had doubled and continued to increase whereas the rate of protein synthesis fell and then became constant (Figure 5B). Continued synthesis of protein at this rate presumably contributed to a final doubling of mass. Although the latter did not exhibit further increase, the same rate of protein synthesis was maintained for at least 8 hr. The rate of protein synthesis increased immediately following shift of static cells to permissive conditions (Figure 8A, 8C). Nevertheless, considerable time

elapsed before the ratios of synthesis to mass matched those which were observed during the steady state following initiation of growth under permissive conditions (Figure 5A, 5C).

In direct contrast to the results obtained with DNA, bacteriostasis did not significantly affect the rate of RNA synthesis which closely paralleled that of mass during cultivation under both permissive and restrictive conditions (Figure 4). Accordingly, the amount of RNA synthesized per unit of mass is constant regardless of the condition of growth. Under permissive conditions, newly synthesized RNA clearly accumulated as a function of increasing mass. Under the restrictive condition, however, mass failed to increase, thus newly synthesized RNA must either accumulate extracellularly or undergo degradation to a form which is soluble in trichloroacetic acid. Results obtained by chemical assay failed to reveal a significant net increase of RNA during cultivation under restrictive conditions (Figure 2).

In contrast to the degradation of RNA under permissive conditions (indicated by a net loss of trichloroacetic acid insoluble RNA subsequent to a label-chase), there was no net loss of RNA under the restrictive condition. This result, along with those obtained from the chemical assay of RNA and rates of RNA synthesis, indicate the maintenance of a constant level of RNA concomitant with no net synthesis and, therefore, strongly implicate rapid turnover of RNA during

bacteriostasis. The species of RNA which may undergo turnover has not yet been determined nor have the presumptive products of degradation been isolated. If, in fact, a species of RNA does undergo turnover during bacteriostasis there are several possibilities that should be considered. The species of RNA undergoing turnover may be an unstable species, such as mRNA which is selectively synthesized during bacteriostasis. Bacteriostasis, in this instance, would simulate the effects of a shift-down which results in the selective synthesis of mRNA (11). Another possibility is that the regulatory mechanisms that govern bacteriostasis involve the turnover of some stable species of RNA such as rRNA, a new species of stable RNA, or a stable form of mRNA. The inability to demonstrate the existence of an unstable fraction of RNA following rifampin treatment, and the decreased rate of protein synthesis observed in static yersiniae would argue for the synthesis of stable mRNA. Inhibition of translation in E. coli by antibiotics which are believed to immobolize ribosomes has been shown by Pato et al. (16) to protect mRNA from degradation and increase its half-life. The decreased rates of protein synthesis observed in static yersiniae may reflect a partial inhibition of translation which results in the stabilization of mRNA due to the decreased rate of ribosome movement.

A decrease in the rate of RNA synthesis was noted in organisms maintained at 37 C in the presence (Figure 7C) and

absence (Figure 7B) of Ca^{2+} where initial changes in mass were small or nonexistent. Although this decrease in rate was not observed in cells returned to 26 C where mass rapidly accumulated (Figure 7A), the initial relative difference was similar. Upon further incubation at 26 C, the ratio of synthetic rate to mass approximated that previously observed during the steady state at 26 C (Figure 4A). The corresponding ratio (Figure 4C) was not obtained following addition of Ca^{2+} to static cells maintained at 37 C. These findings suggest that prolonged bacteriostasis promotes a sustained inhibition of RNA synthesis which is less readily reversed by addition of Ca^{2+} at 37 C than by reduction of temperature to 26 C.

As shown in this paper, bacteriostasis is associated with an apparent inability to initiate new rounds of chromosome replication, and decreased rates of protein synthesis. The latter may be a function of inhibition at the level of translation resulting in the stabilization of mRNA and accompanied by the eventual turnover of the same or a different species of RNA. The primary event which initiates bacteriostasis remains to be determined. Its identification may contribute towards an understanding of the normal cell cycle and provide an insight into the correlation between Ca^{2+}-dependence and virulence.

TABLE 1.--Composition of Synthetic Medium[a].

Constituent	Concentration	Constituent	Concentration
Amino acids:	g per liter	Vitamins[b]:	mg per liter
L-glutamic acid	12.0	Thiamin	1.0
L-phenylalanine	0.4	Ca-pantothenate	1.0
L-tyrosine	0.2	Biotin	0.5
L-tryptophan[b]	0.02	Salts	mM
L-isoleucine	0.5	K_2HPO_4	25
L-valine	0.8	Citric acid	10
L-leucine	0.2	Na-gluconate	10
DL-alanine	0.4	NH_4-acetate	10
glycine	2.0	$MgSO_4$[c]	20
L-threonine	0.16	$FeSO_4$	0.1
L-proline	0.8	$MnCl_2$	0.01
L-lysine•HCl	0.2	Miscellaneous	g per liter
L-arginine•HCl	0.2	D-xylose[b]	10
L-methionine	0.24	Phenol red	0.01
L-cysteine•HCl[b]	0.175		

[a]Amino acids, phenol red, and salts (except constituents noted) were combined and autoclaved. After cooling, $MgSO_4$ was added and then the medium was neutralized with 5 N NaOH. Remaining components were then added.

[b]Sterilized separately by filtration.

[c]Sterilized separately by autoclaving.

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