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SEQUENTIAL REPLICATION OF THE BACILLUS SUBTILIS CHROMOSOME, III. REGULATION OF INITIATION

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Evidence for the sequential replication of the *Bacillus subtilis* chromosome has been presented for strain W23 in our previous reports. The experiments were based on the comparison of marker frequencies in DNA preparations from the exponential and stationary growth phases, using genetic transformation. If the chromosome replicates in a single sequential order, the frequency of each marker in the exponential phase should be a function of its location on the chromosome. On the other hand, the nonreplicating or completed chromosomes in the stationary growth phase should give uniform frequency of all markers, and DNA from stationary phase cells should provide the standard for measuring marker frequencies in the exponential growth phase by genetic transformation. Previous results[1] indicated that such a polarity does exist in strain W23 of *B. subtilis*. Based on these results, a genetic map was constructed in which the adenine (*ade*) marker is located near the point of origin of chromosome replication, and methionine (*met*) and isoleucine (*ileu*) markers near the terminus. This work also indicated that chromosomes in the stationary phase were in the completed form. The possibility of other models of replication which, although less likely, could account for these results has previously been discussed in detail.[1] All of these conclusions were confirmed more directly by using isotopic transfer experiments.[2] However, the same type of experiments carried out on another strain of *B. subtilis* (W168) revealed an apparent absence of replication polarity of the chromosome.[3] This communication reports experimental results showing that the nonpolarity of strain W168 is only an apparent one. Thus, unlike strain W23, chromosomes of strain W168 in stationary phase cells are not in the completed form but are in various stages of replication.

Therefore, using stationary phase DNA as the standard, the marker frequency analysis of exponential phase DNA failed to reveal the polarity of strain W168. However, when DNA from spores of W168 was used as the standard, a replication polarity became evident, and the replication order of the genetic markers was the same as that of strain W23. The basic difference between the two strains, therefore, seems to lie in the regulation of initiation of chromosome replication and not in the nature of the polarity itself. In connection with the regulation of initiation, we are also reporting evidence for more than one replication point per chromosome (multifork replication) in a rapidly growing cell population.

Materials and Methods.—Two wild-type strains, W23 and W168, were used as the DNA donor strains. Recipient mutants and methods for DNA isolation were the same as described before.[1, 2] Transforming activities were assayed according to a modified method[2] of Anagnostopoulos and Spizizen.[4] The basal medium[4] (medium C) contained 14 gm K_2HPO_4, 6 gm KH_2PO_4, 1 gm Na_3-citrate·$2H_2O$, 2 gm $(NH_4)_2SO_4$, 0.2 gm $MgSO_4 \cdot 7H_2O$, 5 gm glucose in 1000 ml H_2O (pH 7.2). Spores were produced by shaking strain W168 in a spore liquid medium (0.5% Difco potato extract in medium C) for 5 days at 37°C and by plating strain W23 on a spore agar medium (2.5% agar in a spore liquid medium) and incubating the plates at 37°C for 4–5 days. Crude spores were collected by centrifugation and purified by treatment with lysozyme and duponol.[5] Germination of the spores was made in a Penassay medium (1.2% Difco Penassay medium).

Transforming DNA from spores was prepared by grinding purified spores in liquid nitrogen as follows. The spore suspension in H_2O, which had been stored at 4°C, was centrifuged. The pellet of spores was then half dried *in vacuo*. Fourteen mg of the half- dried spores were ground in liquid nitrogen in a prechilled mortar for 5–10 min with the constant addition of liquid nitrogen. The ground spores were suspended in 1.5 ml versene-saline solution (0.1 *M* EDTA and 0.15 *M* NaCl, pH 8.0) with 2.5% duponol for 15 min at 37°C, centrifuged, and the supernatant was blown into 3 ml of 95% ethanol and kept cold for 2 hr. Threads and precipitate were collected by centrifugation and dissolved in 0.5 ml saline citrate solution (0.15 *M* NaCl and 0.015 *M* sodium citrate). $NaClO_4$ was added (final conc. 1 *M*) and shaken with an equal volume of isoamyl alcohol-chloroform (1:24) mixture for 30 min. The aqueous layer was blown into 3 volumes of 95% ethanol (no visible precipitate) and kept cold for 3 hr. After centrifugation at 10,000 rpm for 20 min at 4°C, the invisible precipitate was dissolved in 1 ml saline citrate solution. The optical density at 260 mμ of the solution indicated a nucleic acid concentration of 60 μg/ml. CsCl density gradient centrifugation of the sample in a Spinco model E ultracentrifuge showed a single peak of DNA with a density of 1.703,[6] i.e., identical to the density of *B. subtilis* DNA from vegetative cells. The amount of DNA obtained from 14 mg of spores was estimated to be 10 μg from the ultraviolet absorption picture of banded DNA in CsCl density gradient. Another estimate of the DNA amount, calculated from transforming activity measurements, gave a value of 4 μg assuming that the specific transforming activity is the same as that of DNA prepared from exponential and stationary phase cells. The discrepancy between the two estimates indicated that the specific transforming activity of the spore DNA preparation was lower than that of DNA prepared from exponential and stationary phase cells.

Results.—Polar versus nonpolar replication: Various forms of marker frequency analysis have shown an apparent absence of polarity of the chromosome replication of strain W168 in contrast to strain W23 where polarity is evident.[3] Table 1 gives an example of the evidence for nonpolarity. This apparent nonpolarity of strain W168 has also been shown by isotopic transfer experiments, and two alternative models have been proposed to explain the results.[3] One model states that the control of reinitiation of the W168 chromosome replication is not as rigid as in strain W23; consequently, the replication cycles of chromosomes continue in the stationary phase until the components essential for DNA synthesis become deficient, and thus completed chromosomes are not accumulated. In this model, stationary cells of strain W168, like exponential phase cells, should be a hetero-

TABLE 1

COMPARISON OF RELATIVE FREQUENCIES OF MARKERS IN THE EXPONENTIAL PHASE DNA TO THE STATIONARY PHASE DNA OF W168 AND W23 STRAINS

Markers	Normalized marker ratio of W168 against W168 stationary DNA	Normalized marker ratio of W23 against W23 stationary DNA
ade/met	1.28	1.91
thr/met	1.29	1.66
leu/met	1.22	1.30
ileu/met	0.91	1.03

Each value is an average of more than five experiments, and fluctuation of the ratios is usually within 10%. Recipient strains used for this experiment are: *leu-met-ade* (Mu8u5u6), *leu-met-thr* (Mu8u5u5), *leu-met-ileu* (Mu8u5u1).

TABLE 2

COMPARISON OF RELATIVE FREQUENCIES OF MARKERS IN EXPONENTIAL AND STATIONARY PHASE DNA TO SPORE DNA

Markers	Normalized marker ratio of W168 against W168 spore DNA		Normalized marker ratio of W23 against W23 spore DNA	
	Exponential DNA	Stationary DNA	Exponential DNA	Stationary DNA
ade/met	2.01	1.65*	1.97	1.06*
thr/met	1.66	1.23*	1.67	—
leu/met	1.37	1.05*	1.37	1.14*

Each value is an average of more than four experiments, and fluctuation of the ratios is usually within 10%. The values with asterisks (*) are based on single experiments. Recipient strains used for this experiment are: *leu-met-ade* (Mu8u5u6), *leu-met-thr* (Mu8u5u5).

geneous population with respect to the replication stages of their chromosomes. The other model is that randomization of the starting point occurs at the initiation of each new cycle of chromosome replication.[3, 7] Definite support can now be given to the first model because of the following two experiments.

If there is a phase of the life cycle of strain W168 in which the chromosomes are in the completed form, we could use the DNA from cells in such a phase as the standard DNA for marker frequency analysis. Then by using the new standard we should be able to find the same polarity in W168 as in W23 for both exponential and stationary phase cells of strain W168, provided that the first model is correct. DNA isolated from the spore of strain W168 was used to serve as a standard DNA, and the results are given in Table 2 together with comparable data for strain W23. The differences of marker frequencies in strain W168 are now evident, and the polarity is similar to that of strain W23. Moreover, the W168 DNA from stationary phase cells shows basically the same differences of marker frequencies, although the extent of the difference seems to be less than in DNA from exponential phase cells, indicating that there is some tendency to accumulate completed chromosomes. Thus, replication of the W168 strain chromosome is seen to be exactly the same as that of strain W23 when spore DNA of W168 is used as the standard. The only difference between the two strains appears in the stationary phase; in strain W23 chromosomes accumulate in the completed form, while in strain W168 chromosomes accumulate at various stages of replication.

In Meselson and Stahl's transfer experiment from N^{15} to N^{14} medium with *E. coli*,[8] DNA isolated from cells transferred to the N^{14} medium and allowed to grow for one generation showed only a hybrid DNA band in CsCl density gradient centrifugation. However, if randomization of the starting point takes place, one could predict that in such a transfer experiment there should be significant amounts of both heavy and light DNA in addition to the hybrid DNA molecule. A theoretical calculation for a random starting model demands 11 per cent heavy, 78 per cent hybrid, and 11 per cent light DNA at one generation after transfer.[9] To examine this point, $N^{15} \rightarrow N^{14}$ transfer experiments were made for both strains W23 and W168. The results are shown in Figure 1. In these experiments, we should be aware of complications caused by heterogeneity of the generation time in a population which would result in the presence of parental and light DNA molecules as

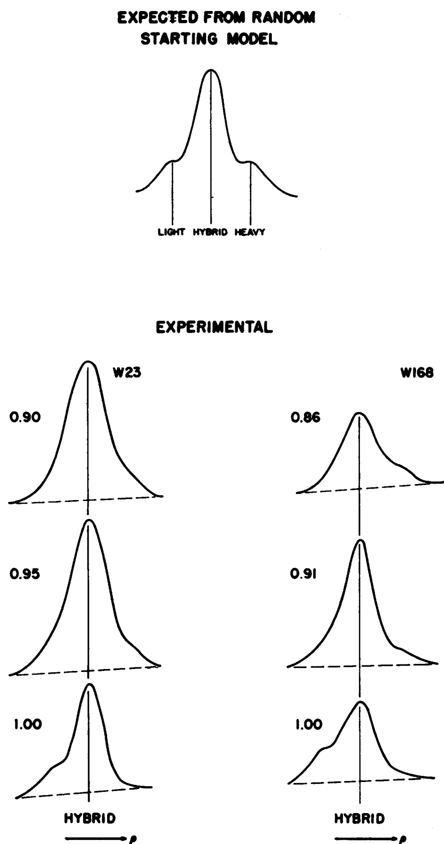

FIG. 1.—$N^{15} \rightarrow N^{14}$ transfer experiments in W23 and W168. Overnight cultures (16–17 hr) of *B. subtilis* strains W23 and W168 in a supplemented N^{15}-medium were diluted 20-fold into growth flasks containing the same medium. The supplemented N^{15}-medium contained 0.1 gm $N^{15}H_4Cl$, 1.4 gm K_2HPO_4, 0.6 gm KH_2PO_4, 0.1 gm Na_3-citrate·2 H_2O, 0.02 gm $MgSO_4 \cdot 7 H_2O$, 0.5 gm glucose, and 0.05 gm of dried N^{15}-*B. subtilis* hydrolysate in 100 ml H_2O (pH 7.0). The N^{15}-*B. subtilis* hydrolysate was prepared by growing W23 cells in a N^{15}-medium C (nitrogen-free medium C plus 1 gm/l $N^{15}H_4Cl$) for 42 hr. The cells were collected in 5-ml tubes, suspended in distilled water, and an equal volume of 12 *N* HCl was added. The tubes were sealed under vacuum and heated for 22 hr at 110°C. The hydrolysate was dried *in vacuo* over KOH, and the carbon residue was extracted 3 times with distilled water and discarded. The extract was dried in a lyophylizer, yielding 265 mg dry weight from one liter of culture. The N^{14}-*B. subtilis* hydrolysate was likewise prepared using W23 cells grown in N^{14}-medium C. The cell growth was measured by optical density in Klett units. At Klett unit 60 (exponential phase) the cells in supplemented N^{15}-medium were filtered on a Millipore filter (pore size, 0.45 μ) and washed once with N^{14}-medium C. The cells were resuspended in 2 vol of a supplemented N^{14}-medium C which contained 1 gm $N^{14}H_4Cl$ instead of 0.2 gm $(NH_4)_2SO_4$, 50 mg of dried N^{14}-*B. subtilis* hydrolysate, and 10 μg/ml each of guanine, adenine, cytosine, and thymine. At 0.86, 0.91, and 1.0 generation (measured by optical density of the culture) after transfer of strain W168 to supplemented N^{14}-medium C, 5-ml aliquots of cells were removed, killed by heating at 60°C for 10 min, centrifuged and resuspended in 0.25 ml versene-saline, lysed with lysozyme (1 mg/ml, 15 min at 37°C) and duponol (2.5% final concentration for 10 min at 37°C). The total volume of lysate was 0.3 ml. At 0.90, 0.95, and 1.0 generation after transfer to supplemented N^{14}-medium C, 5-ml aliquots of W23 cells were removed, killed, and lysed in the same way. The generation time in the supplemented N^{15}-medium was about 40 min for both W23 and W168, and in the N^{14}-medium 45 min and 50 min for W23 and W168, respectively. 0.2 ml of each lysate was centrifuged in a Spinco model E ultracentrifuge with 7.7 molal CsCl for 20 hr at 44,770 rpm at 25°C using N^{15}-*Pseudomonas aeruginosa* DNA (ρ = 1.743 as density reference).

well as hybrid molecules at one generation after transfer.[3, 9, 10] Since this complication causes an increase in the relative amounts of heavy and light DNA, the results shown in Figure 1 are taken as evidence against the second model, in which randomization of the starting point after each replication cycle is assumed.

Reinitiation of chromosome replication: The process of spore germination in Penassay medium was analyzed by measuring changes in optical density at 650 mμ, DNA synthesis, and marker ratios.[11] The results are shown in Figure 2. Surprisingly, the *ade/met* ratio reached *four*. Obviously, we cannot accommodate this phenomenon in our replication model with one replication point per chromosome. However, both the turbidity of the culture and the DNA have an unusually short doubling time—a minimum of 20 min compared with the ordinary 35–40-min generation time in the standard medium[1] (medium C[4] plus 0.05% casein hydrolysate plus 50 μg/ml of L-tryptophan). Figure 2*B* also shows that the rise of the ratio *ade/met* may have a step in the middle. Moreover, this unusual ratio

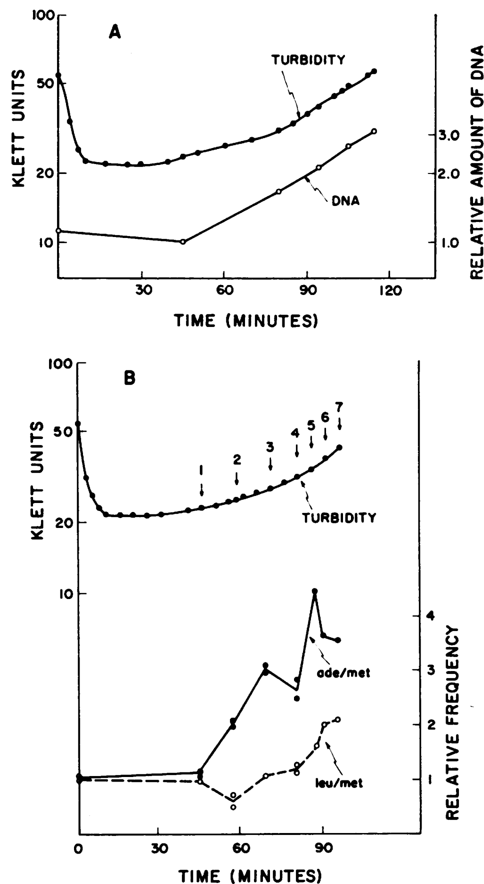

FIG. 2.—Change of marker frequencies during germination of spores of strain W23. The germination mixture consisted of 1.9 ml purified spore suspension in H_2O (OD_{650} = 12.17), 19 ml H_2O, and 40 ml Difco Penassay medium (1.75%). The mixture was incubated at 37°C in a growth flask with constant shaking. (*A*) DNA was assayed by the indole method.[17] Five-ml samples from the culture were heated at 60°C for 10 min and centrifuged. Cells were suspended in 1 ml cold 5% trichloroacetic (TCA), kept in an ice bath for 30 min, centrifuged down, resuspended in 1 ml 5% TCA, incubated at 100°C for 15 min, and centrifuged. The supernatant (1 ml in 5% TCA) was mixed with 0.5 ml 12 *N* HCl and 0.5 ml 0.06% indole, incubated at 100°C for 10 min, and assayed for DNA according to Keck.[17] (*B*) Eight-ml samples were taken at various times, and DNA was isolated according to the method previously described.[2] Marker ratios were obtained relative to those of DNA from the stationary culture of strain W23.[1]

of *ade/met* is not limited to the case of spore germination. The ratio of *four* was also observed in exponential phase cells growing in Penassay medium in which the generation time is shorter than usual (Fig. 3). Thus it appears that in rapidly growing cells the chromosome has two new replication points, starting at the origin before the first reaches the terminus. In general, the situation in which more than one replication point (or fork) exists in one chromosome may be called *multifork replication*. The value *four* for the *ade/met* ratio and the stepwise rise of the *ade/met* ratio during the germination of spores may suggest that there is a certain regularity in the positions of replication points. The schematic picture of the idealized situation is given in Figure 4 and is called *dichotomous replication*.

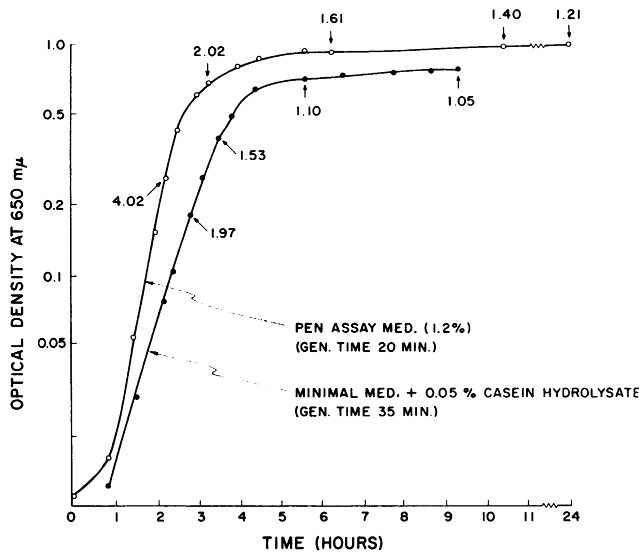

FIG. 3.—The growth rate and *ade/met* ratio of strain W23 in standard medium (medium C plus 0.05% casein hydrolysate plus 50 μg/ml of L-tryptophan) and Penassay medium (1.2%) at 37°C were compared. The *ade/met* ratios were obtained by normalizing against a standard stationary DNA of strain W23. Figures on the growth curve represent the normalized *ade/met* ratios.

Note that in dichotomous replication, the ratio *four* of *ade/met* is demanded. A theoretical analysis of this model in relation to marker frequencies, growth rate, and the DNA amount per chromosome will be presented elsewhere.[12]

Discussion.—The evidence presented above indicates that regulation of chromosome replication is under both genetic and physiological controls. Thus the difference between polar and apparently nonpolar replication of *B. subtilis* is seen to be strain-specific. A similar situation has been reported in *Escherichia coli*, where Hfr strains are polar but F$^-$ strains are not (Nagata, 1963, ref. 13). The present results show that nonpolarity, at least for strain W168 of *B. subtilis*, does not come from randomization of the replication origin, but apparently from the absence of regulation of the initiation. Thus, the basic features of chromosome replication (origin and direction of replication) are not different between the polar (W23) and the apparent nonpolar (W168) strains. An obvious prediction from the results is that the synchronous replication of chromosomes observed in strain W23[2] when the stationary cells are transferred to a fresh medium should not occur in

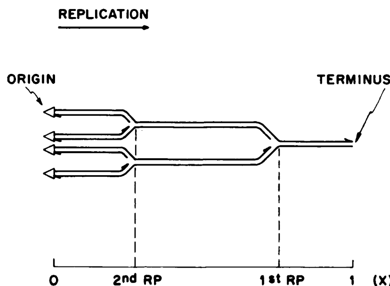

FIG. 4.—A schematic representation of dichotomous replication of the chromosome. Before the first replication position (*RP*) reaches the terminus, the second *RP* starts from the origin. In an exponentially growing population, the simplest steady state can be set if the distance between the first and second *RP*'s occupies half of the chromosome, and each replication point proceeds at a constant rate. In reality, the two replication points at the second *RP* may not necessarily be at the same position.

strain W168. Such an experiment has been carried out, and the result was just as predicted.[3]

Physiological control has been suggested from the results in *E. coli* (Maaløe, 1961, ref. 14) which indicated the necessity for protein synthesis for the initiation of chromosome replication. The finding of multifork replication in *B. subtilis* gives another clue to the regulation mechanism, for in this case the repression of the reinitiation during chromosome replication can be broken in rapidly growing cells. Nevertheless, the timing or rhythm of initiation seems to be well regulated, and its mechanism should be an interesting object for future study. In this connection, a hypothetical model connecting the regulation of initiation of chromosome replication to some specific structure of the cell surface[15] is appealing. Obviously, dichotomous replication is advantageous for bacterial growth because the bacteria can replicate chromosomes faster than by having one replication point. With one replication point, the replication time, and consequently the cell generation time, would be limited by the maximal rate of DNA polymerase.

Summary.—In contrast to strain W23 of *Bacillus subtilis*, strain W168 does not show polarity of chromosome replication when the marker frequencies are compared in DNA from exponential phase cells and in DNA from stationary phase cells. This apparent nonpolar behavior of strain W168 arises from a less rigid regulation of chromosome replication rather than from a difference in the mode of replication (origin and direction of replication). When the spore DNA of W168 is used as the standard, exactly the same polarity as in strain W23 is observed. These results indicate that strain W168 does not accumulate completed chromosomes in the stationary phase, while in the spores chromosomes are in the completed form. In addition, evidence for multifork replication of chromosomes occurring in rapidly growing cells is presented.

Note added in proof: Recently, the dichotomous replication has been unequivocally demonstrated by germinating heavy (D_2O) spores to light medium and analyzing marker distributions in heavy, hybrid, and light DNA molecules (Oishi, M., H. Yoshikawa, and N. Sueoka, in preparation).

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[11] During spore germination, the frequency of various markers increases in the same sequential

order shown in Fig. 1, details of which will be reported elsewhere.[16] R. G. Wake [*Biochem. Biophys. Res. Commun.*, **13**, 67 (1963)] reported evidence of the sequential replication of indole and methionine markers during spore germination.

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*ON THE ASSIMILATION OF ENERGY FROM INORGANIC SOURCES IN AUTOTROPHIC FORMS OF LIFE**

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The biological assimilation of carbon dioxide would be thermodynamically impossible without a simultaneous—or preceding—assimilation of energy: in green cells electromagnetic energy and, in chemosynthetic organisms, inorganic chemical energy is converted into the energy of organic chemical compounds. This fundamental process cannot be considered clarified until it is resolved as a sequence of thermodynamically feasible interactions of well-defined participants.

Energy assimilation may be studied on green cells or on chemosynthetic bacteria. The latter offers one decisive advantage: in chemoautotrophs the assimilation of energy is inseparably linked to a transformation of chemical individuals. Events of this nature are accessible to chemical analysis and to equilibrium considerations.

In view of these advantages we studied *Nitrobacter winogradskyi* Buch. The source of energy in this species is the oxidation of nitrite to nitrate. In a previous paper from our laboratory,[1] this reaction had been demonstrated to be coupled with a reduction of DPN. The addition of DPNH and ATP to cell-free preparations of Nitrobacter results in the immediate assimilation of $C^{14}O_2$ as demonstrated here in recent experiments (unpublished), and shown independently by Aleem and Nason.[2] Therefore, the assimilation of carbon may be considered separately as a second sequence of chemical reactions in which DPNH and ATP are utilized. This paper is concerned only with the first sequence, the assimilation of energy.

Methods.—Cultivation: *Nitrobacter winogradskyi* Buch was grown at 35°C in a purely inorganic medium with addition of the required trace elements as described elsewhere.[3] The culture flasks were aerated with 10 vol % CO_2 in air.

Preparation of homogenates and particles: The cells were harvested from batch cultures by centrifugation in continuous flow, and freed of remaining salts by repeated washing. They were finally resuspended in a medium containing: 300 mmoles sucrose, 1 mmole EDTA·Na_2, and 1 mmole glutathione (·SH) per 1000 ml of water. Samples of 15 ml of this cell suspension, containing 1–2 ml of packed cells, were homogenized with 30 gm #13 glass beads (English Glass Co.) in a chilled 50-ml chamber of a Servall-omnimixer for 4 min. The glass beads were separated from the homogenate by filtration through a coarse fritted glass filter funnel. Intact cells were removed by centrifugation at 7000 × *g* for 10 min. All active particle preparations used in these experi-