JOURNAL OF BACTERIOLOGY, July 1969, p. 136-141

Vol. 99, No. 1
Printed in U.S.A.

Interaction of *Bdellovibrio bacteriovorus* and Host Bacteria

II. Intracellular Growth and Development of *Bdellovibrio bacteriovorus* in Liquid Cultures

MAZAL VARON AND M. SHILO

Department of Microbiological Chemistry, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

Received for publication 28 February 1969

The intracellular life cycle of *Bdellovibrio bacteriovorus* 109 growing on *Escherichia coli* in a dilute nutrient medium exhibits a period of constant infective titer while the parasite grows and elongates inside the host cell. This period is terminated after 2 to 4 hr, and the number of the plaque-forming units in the culture rises rapidly to as much as six times the initial titer. The growth pattern of *Bdellovibrio* is similar with actively growing or resting host cells, or with host cells killed by ultraviolet irradiation or by heating at 70 C. The yield of *B. bacteriovorus* strain 109 in two-membered cultures with *E. coli* B depends on the host concentration and may reach 7.5×10^{10} cells per ml. Penicillin, which has no effect on the attachment and penetration of *Bdellovibrio*, inhibits its multiplication.

During the past few years, the intracellular life cycle of *Bdellovibrio bacteriovorus* has been studied in several laboratories by phase-contrast (4, 9) and electron microscopy (2, 3, 7). It was found that the *Bdellovibrio* cell, having attached to the surface of the bacterial host cell, penetrates through the cell wall and starts to grow in the periplasm of the host. During the invasion process, or soon after, the host cells change from rods to spheroplasts. Inside these spheroplasts, the *Bdellovibrio* cells increase in size until they divide and leave the host cells.

In a previous paper (9), we described the conditions for *Bdellovibrio* attachment and penetration into the host cells. We have now turned to the next stages of its development, namely, intracellular growth and multiplication. The understanding of these stages gained in this study makes it possible to obtain high yields of active *Bdellovibrio* cultures and could serve as the basis for future studies of the effects of *Bdellovibrio* on the host during intracellular development.

MATERIALS AND METHODS

Media. Dilute Nutrient Broth (DNB medium) had the following composition (mg/liter): Nutrient Broth, 800; Casamino Acids, 500; Difco yeast extract, 100; $Ca(NO_3)_2$, 200; $FeSO_4$, 10; and $MnSO_4$, 10. The *p*H was adjusted to 7.2 with 2.5 N NaOH. Non-nutrient TM solution consisted of 0.002 M tris(hydroxymethyl)aminomethane (Fluka, Buchs, SG Switzerland), and $Ca(NO_3)_2$, $FeSO_4$, and $MnSO_4$ at the same concentrations as in DNB medium. The *p*H of TM medium was adjusted to 7.8 with 1 N NaOH.

Bacterial strains and cultivation. The strains used were *B. bacteriovorus* 109 and *Escherichia coli* B-2262. Starter cultures of *Bdellovibrio* were usually prepared in 20 ml of DNB medium by use of an inoculum of 10^8 to 2×10^8 plaque-forming units (PFU) and 10^9 to 2×10^9 host cells. Growth was completed after 16 to 18 hr of incubation at 30 C in a New Brunswick rotatory shaker. For experiments with resting or killed host cells, the starter was prepared in 20 ml of TM medium, and the inoculum consisted of 10^9 to 2×10^9 PFU and 10^{10} to 2×10^{10} host cells. *Bdellovibrio* grown under such conditions attached to *E. coli* host cells with an efficiency of 90% within 20 min of incubation in DNB medium at 30 C. *E. coli* B was grown in Difco Nutrient Broth at 37 C in a New Brunswick rotatory shaker. The cells were centrifuged at 12,000 × *g* for 5 min and washed. For the study of *Bdellovibrio* growth in growing host cells, *Bdellovibrio* cells were suspended in DNB medium; for the study of *Bdellovibrio* growth in resting or nonviable host cells, *Bdellovibrio* cells were suspended in non-nutrient TM solution. Cell numbers were adjusted by use of a Klett-Summerson colorimeter (filter no. 42).

Plaque assays were carried out in DNB medium by the double-layer technique as described elsewhere (9).

RESULTS

Intracellular growth of B. bacteriovorus in two-membered cultures with E. coli. When *B. bacteriovorus* strain 109 and *E. coli* B were mixed in DNB or TM medium at a multiplicity of infec-

tion (MOI) of 0.1 in a shaking water bath at 30 C, the number of PFU remained constant for the first few hours, after which a rapid increase in PFU was observed (Fig. 1). This growth pattern is similar to bacteriophage growth, but, whereas the "latent period" in bacteriophage infection is terminated within minutes and the "burst size" is several hundreds (8), the period of constant infective titer in *Bdellovibrio* infection continued for 3.5 hr and the number of progeny per infected host cell was 4.6 in DNB medium and 3.3 in TM solution. In different experiments, the number of progeny *Bdellovibrio* cells released varied from four to six in DNB medium and from three to five in TM solution. The rise in the number of PFU was followed by another period of constant infective titer which was terminated within 1.5 hr. When host cells were no longer visible microscopically, the number of PFU remained constant for several hours (Fig. 1) before declining (9).

A small increase in the number of PFU is often observed during the first hour after host and parasite are mixed (Fig. 1, 2, and 6). This increase was found to occur within the first 20 min of incubation in liquid medium and only in the presence of the host. The number of PFU remained constant when the *Bdellovibrio* inoculum was diluted in fresh medium containing no host cells; thus, this increase does not seem to be a result of separation of chains or clumps.

Observations with a phase-contrast microscope showed that during the period of constant infective titer the parasite penetrates the host-cell wall, inhabits the periplasm, and grows there until it surrounds the host protoplast. At the end of this period, the long *Bdellovibrio* cell divides into four to six daughter cells which finally leave the ghost of the host cell. The new generation of *Bdellovibrio* cells move actively and are capable of immediately infecting other host cells.

In additional experiments carried out under the same conditions, the duration of the first growth cycle was found to vary from 2 to 4 hr. A possible explanation for this variability could be the different physiological state of the *Bdellovibrio* inoculum. Starr and Baigent (7) concluded, on the basis of microscopic observations, that *Bdellovibrio* cells which had been free-living for several days might take a much longer period to complete their parasitic cycle than cells taken immediately after release from the host. We therefore examined young *Bdellovibrio* cells from a 16-hr culture and older cells taken from a 48-hr culture. In the first case, the first growth cycle was completed in 2 hr, whereas in the second case a period of 4 hr was required to produce the first burst (Fig. 2). The second growth cycle was shorter than the first one and was nearly the same (1.5 to 2 hr) in all experiments. It seemed to be independent of the physiological age of the *Bdellovibrio* inoculum.

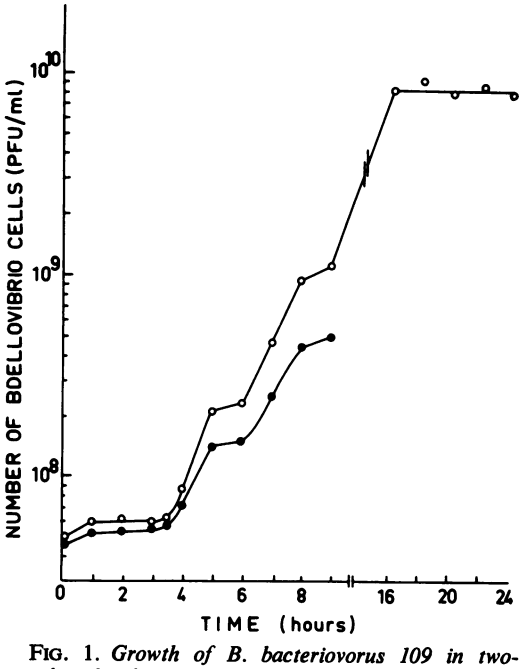

FIG. 1. *Growth of B. bacteriovorus 109 in two-membered cultures with E. coli B in DNB and TM media. A 1-ml amount of 18-hr Bdellovibrio culture was added to 19 ml of DNB (○) or TM (●) medium containing 10^9 E. coli cells/ml. The cultures were incubated in a shaker at 30 C, and 0.5-ml portions were taken for plaque assays.*

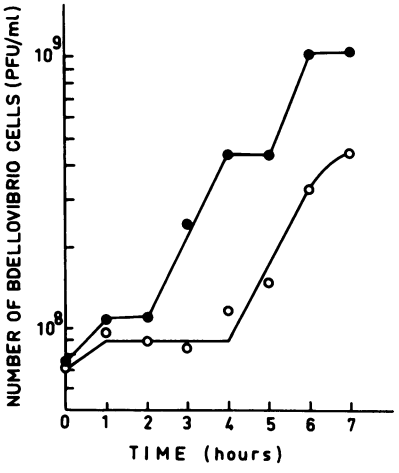

FIG. 2. *Effect of the age of Bdellovibrio inoculum on its growth. The parasite cells were taken from a 16-hr (●) and a 48-hr (○) culture. Incubation as in Fig. 1.*

Effect of host and parasite concentrations on Bdellovibrio growth rate and yield. At an MOI of 0.1 or less, the *Bdellovibrio* growth rate was not significantly affected by the inoculum size (Fig. 3 and 4) or by the initial host concentration (Fig. 4). The final yield was also unaffected by the inoculum size (Fig. 3), but was markedly influenced by the initial concentration of host cells in the medium (Fig. 5). Figure 5 shows that, within the range tested, a linear relationship exists between the host concentration and the *Bdellovibrio* yield as measured by the optical density of the cultures after complete disappearance of the host. The curve describing the viability of *Bdellovibrio* (as expressed in PFU) is more complex, because part of the parasite population had perished by time of sampling. In TM medium, lysis of up to 5×10^{10} host cells per ml was completed within 24 hr by a *Bdellovibrio* inoculum of 10^7 PFU/ml. With a smaller parasite inoculum (10^5 to 10^6 PFU/ml), host digestion was completed within 48 hr. The highest yields obtained under these conditions were 6×10^{10} PFU/ml in TM medium and 7.5×10^{10} PFU/ml in DNB medium.

Physiological condition of host as requirement

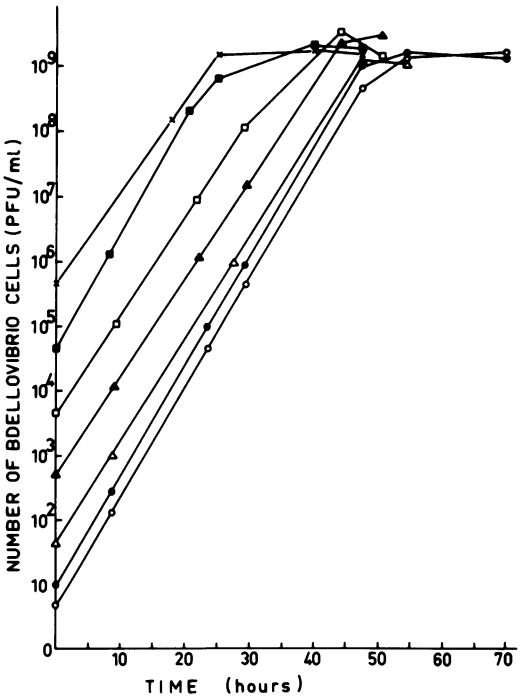

FIG. 3. *Effect of the inoculum size on growth of B. bacteriovorus 109. Tenfold dilutions in DNB medium were prepared from an 18-hr Bdellovibrio culture; 1 ml of each dilution was added to 19 ml of DNB medium containing 10^9 E. coli cells/ml. Incubation as in Fig. 1.*

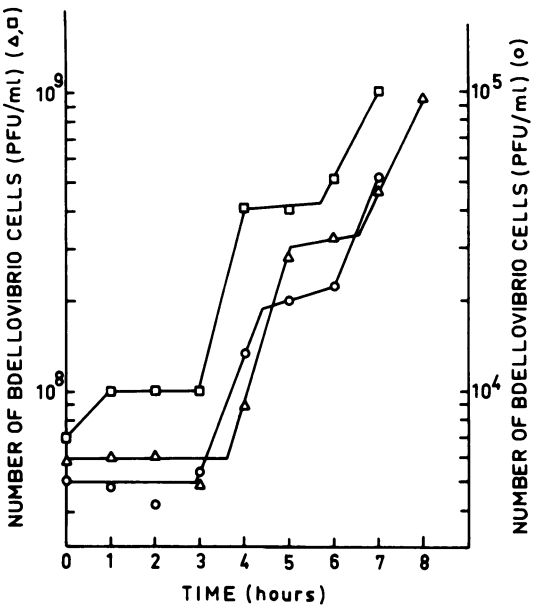

FIG. 4. *Effect of the initial concentration of host and parasite on the growth pattern of B. bacteriovorus 109. A 1-ml amount of an 18-hr culture of Bdellovibrio or of a 10^{-4} dilution of the same culture was added to 9 ml of DNB medium containing 10^9 (□, ○) or 10^{11} (△) E. coli cells/ml. Incubation as in Fig. 1.*

for Bdellovibrio growth. The question was raised whether *Bdellovibrio* required living host cells for reproduction. *E. coli* cells killed by ultraviolet irradiation or heating were tested for their competency to serve as hosts. It was found that *E. coli* irradiated prior to infection by an ultraviolet dose which killed 99.999% of the cells was capable of supporting normal *Bdellovibrio* growth (Fig. 6, Table 1). Heating the host for 15 min at 70 C prolonged the time required for completing the growth cycle and somewhat diminished the average number of progeny *Bdellovibrio* cells released from a single infected host cell, but the final *Bdellovibrio* yield was almost the same as with an untreated host (Table 1). Heating at 98 or 120 C rendered *E. coli* incapable of serving as host for *Bdellovibrio* growth. Since 0.001% of the *E. coli* cells remained viable after heating at 70 C for 15 min, there was a possibilitv that the difference between the treatment at this and higher temperatures depended upon the infection of this small viable fraction. To test this, *Bdellovibrio* cells were mixed with host cells killed at 120 C to which were added numbers of living host cells approximately equal to the number in the fraction surviving heating at 70 C. Although *Bdellovibrio* showed some growth, the addition of living cells did not result in the same extent of

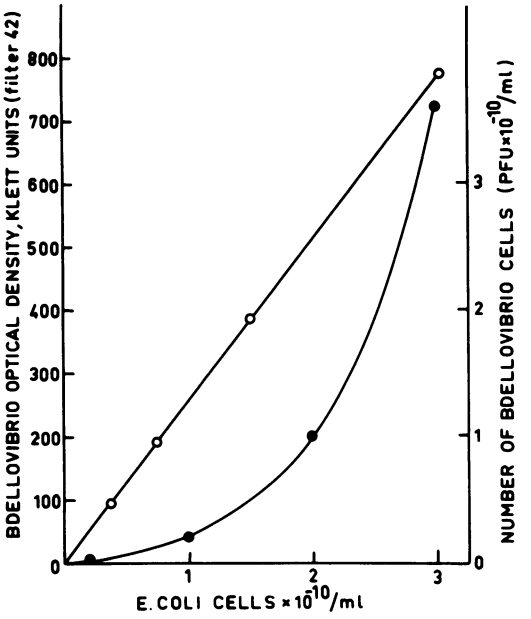

FIG. 5. *Relationship between the host concentration and the yield of B. bacteriovorus 109. A 1-ml amount of a Bdellovibrio suspension containing 10^9 PFU/ml was added to 9 ml of TM medium containing various concentrations of E. coli cells. The two-membered cultures were incubated as in Fig. 1. The number of PFU (●) and the optical density (○) in all the samples were determined after 24 hr of incubation (when all host cells had disappeared from the most concentrated culture).*

growth as occurred with host cells heated at 70 C (Table 1).

Penicillin inhibition of Bdellovibrio growth. The early stages of *Bdellovibrio* infection, namely, attachment and penetration into the host cells, are not affected by penicillin (9). It was observed by phase-contrast microscopy that the intracellular development of *B. bacteriovorus* 109 in the presence of penicillin was irregular and the parasite became spherical. Penicillin (G, sodium salt, Rafa Laboratories, Jerusalem, Israel) was introduced into TM medium to a final concentration of 100 units per ml, and viability counts of *Bdellovibrio* were made at hourly intervals. The results showed a constant number of PFU for 6 hr. Penicillin concentrations higher than 100 units per ml had a bactericidal effect on the parasite, and the number of PFU rapidly decreased.

DISCUSSION

Under suitable conditions, the attachment of young and active *Bdellovibrio* cells to the host cells is a rapid process which is completed within a few minutes after host and parasite are mixed. Since attachment of such *Bdellovibrio* cells involves almost the whole parasite population (≧90% in DNB medium, and only slightly less in TM medium) and is irreversible, and because the time required is relatively short compared with the complete life cycle of the parasite, it was possible to study the rate of *Bdellovibrio* intra-

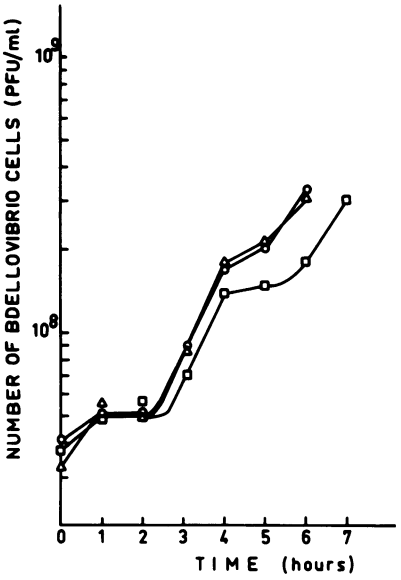

FIG. 6. *Growth of B. bacteriovorus 109 on heat-killed and ultraviolet-irradiated host cells. A 1-ml amount of a 16-hr culture of Bdellovibrio grown in TM medium on viable host cells was added to 19 ml of TM medium containing 10^9 E. coli cells/ml. The host cells were all viable (○), heated at 70 C for 15 minutes (□), or irradiated with a Hanovia 1.25-w ultraviolet lamp for 2 min at a distance of 20 cm (△). Incubation as in Fig. 1.*

TABLE 1. *Growth of B. bacteriovorus 109 on killed E. coli B cells in TM medium*[a]

Treatment	No. of viable host cells/ml of two-membered culture	*Bdellovibrio* PFU/ml after 24 hr of incubation at 30 C
None	2×10^9	3×10^9
Ultraviolet irradiation	2×10^4	4×10^9
70 C, 15 min	4×10^4	2×10^9
98 C, 15 min	0	5×10^6
120 C, 15 min	0	4×10^6
120 C, 15 min	2×10^{4b}	2×10^7

[a] A 1-ml amount of a *Bdellovibrio* suspension containing 3×10^6 PFU was added to 19 ml of TM medium containing viable or treated host cells at a total concentration of 2×10^9. Irradiation was carried out as in Fig. 6.

[b] Added after heating.

cellular development without first removing the remaining small fraction of free parasites.

Like the latent period in bacteriophage development (8), the period of *Bdellovibrio* intracellular growth and multiplication is expressed by a constant number of PFU. This period ends with the burst of new, small, and very motile *Bdellovibrio* cells, which are capable of immediately infecting other host cells.

The duration of the first growth cycle under standard environmental conditions varies from 2 to 4 hr. The following cycles are always shorter and less variable than the first one (1.5 to 2 hr under the experimental conditions described). This difference between the length of the first growth cycle and subsequent growth cycles seems to be due to physiological differences between *Bdellovibrio* cells just liberated from the host and *Bdellovibrio* cells which have been deprived of a host for several hours. This assumption is supported by the fact that *Bdellovibrio* cells taken from cultures many hours after the host cells have disappeared showed a much slower growth cycle on reinoculation than did cells inoculated shortly after release from the host. Recently, Seidler and Starr (4) reported similar results in one-step growth experiments.

A similar "nascent" activity was described for bacteriophage by Wollman and Stent (10). They showed that the T4 phage, which usually requires tryptophan for adsorption, is able to adsorb to bacteria without added cofactors if it is taken immediately after release from the host; this ability is thereafter gradually lost.

Since the period of constant infective titer in our experiments includes the period of attachment and penetration of *Bdellovibrio*, it is evident that any factor affecting either of these processes would also influence the period of constant infective titer. This may be the reason for the apparently slower growth with host cells treated for 15 min at 70 C. Attachment within 20 min of mixing with such heat-treated hosts has already been shown to be inefficient compared with the rate of attachment with untreated or ultraviolet-irradiated cells (9). Slow intracellular development may retard the completion of the lysis of the host suspension by a few days and result in the development of a heterogeneous *Bdellovibrio* population composed of cells released from the host at different times. Usually, only part of such a population is active (e.g., a small percentage of the total *Bdellovibrio* cells attach within the first 30 min as compared with over 90% of fully active populations); *Bdellovibrio* grown under such conditions could give ambiguous results in any physiological study. To obtain a large percentage of highly active *Bdellovibrio* cells, it is essential to use a "young," physiologically active parasite inoculum, the size of which is of little consequence. Using such parasite inocula, high yields of *Bdellovibrio* can be obtained provided that high host concentrations are supplied.

Bdellovibrio growth on heat-killed cells was recently demonstrated by Burger et al. (1). These authors showed that *Rhodospirillum rubrum* heated at 80 C was susceptible to *Bdellovibrio*, but the plaque-forming efficiency on lawns of such cells was very low. In our experiments with *E. coli* killed by heating at 70 C or by ultraviolet irradiation, *Bdellovibrio* growth was similar in rate and yield to the growth with untreated host cells. This shows that the parasite does not depend on host viability for its reproduction.

The ability of *Bdellovibrio* to grow on host cells suspended in a non-nutrient medium, and the relationship existing between the host concentration and the parasite yield, point to the conclusion that *Bdellovibrio* derives the materials for its cell components from the host cells. However, it is now clear that *B. bacteriovorus* strain 109 has certain biosynthetic activities which are host-independent: it elongates after its release from the host (9); it is capable of amino acid incorporation into acid-insoluble material; and, when deflagellated by a mechanical treatment, it regenerates its flagella (Varon, *unpublished observations*). It might be that this extracellular growth of *Bdellovibrio* is an unbalanced type of growth. The inability of *Bdellovibrio* cells to divide outside of the host cells may be due to the lack of a single or a few limiting factors which have to be supplied by the host, whereas most of its components can be synthesized autonomously. The occurrence of host-independent mutants, which may be obtained with high frequency in certain *Bdellovibrio* strains (5), indicates that the requirements of the parasite supplied by its host may be few. This is in accord with the study of Simpson and Robinson (6), which showed that the parasitic *B. bacteriovorus* 6-5-S possesses mechanisms for obtaining energy suitable to support an independent existence.

ACKNOWLEDGMENTS

Our thanks are due to S. C. Rittenberg for critical reading of this manuscript and to B. Golek for editing.

LITERATURE CITED

1. Burger, A., G. Drews, and R. Ladwig. 1968. Wirtskreis und Infektionscyclus eines neu isolierten *Bdellovibrio bacteriovorus*-stammes. Arch. Mikrobiol. 61:261–279.
2. Lépine, P., A. Guélin, J. Sisman, and D. Lamblin. 1967. Etude au microscope électrionique de la lyse de *Salmonella* par *Bdellovibrio bacteriovorus*. Compt. Rend. 264:2957–2960.
3. Scherff, R. H., J. E. DeVay, and T. W. Carroll. 1966. Ultra-

structure of host-parasite relationships involving reproduction of *Bdellovibrio bacteriovorus* in host bacteria. Phytopathology 56:627–632.

4. Seidler, R. J., and M. P. Starr. 1969. Factors affecting the intracellular parasitic growth of *Bdellovibrio bacteriovorus* developing within *Escherichia coli*. J. Bacteriol. 97:912–923.
5. Shilo, M., and B. Bruff. 1965. Lysis of gram-negative bacteria by host-independent ectoparasitic *Bdellovibrio bacteriovorus* isolates. J. Gen. Microbiol. 40:317–328.
6. Simpson, F. J., and J. Robinson. 1968. Some energy producing systems in *Bdellovibrio bacteriovorus* strain 6-5-S. Can. J. Biochem. 46:865–873.
7. Starr, M. P., and N. L. Baigent. 1966. Parasitic interaction of *Bdellovibrio bacteriovorus* with other bacteria. J. Bacteriol. 91:2006–2017.
8. Stent, G. S. 1963. Molecular biology of bacterial viruses. W. H. Freeman and Co., San Francisco.
9. Varon, M., and M. Shilo. 1968. Interaction of *Bdellovibrio bacteriovorus* and host bacteria. I. Kinetic studies of attachment and invasion of *Escherichia coli* B by *Bdellovibrio bacteriovorus*. J. Bacteriol 95:744–753.
10. Wollman, E. L., and G. S. Stent. 1952. Studies on activation of T4 bacteriophage by cofactor. IV. Nascent activity. Biochim. Biophys. Acta 9:538–550.