Effects of Salts on the Halophilic Alga, Dunaliella viridis. II. Effects of Salts on Growth[1]

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Running title: Effects of salts on D. viridis

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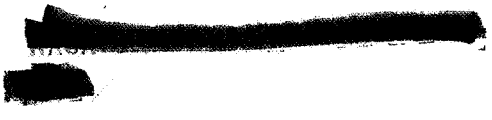
The range of NaCl concentrations permitting growth of Dunaliella viridis was found to be very broad and to be influenced to a slight, but significant, extent by the previous history of the cells. Growth in medium containing a high salt concentration (3.75 M) was preceded by a lag, whereas no lag was observed under the same conditions at optimal (1.28 M) salt levels. The amount of dilution tolerated by cultures before initiation of cell lysis was found to depend on the salt concentration of the growth medium. The NaCl requirement for growth could not be fulfilled by KCl, LiCl, or $MgCl_2$; in fact, KCl and LiCl inhibited growth when added at low concentrations to media containing adequate NaCl. The chloride requirement was found to be nonspecific; Na_2SO_4 or $NaNO_3$ could be substituted for NaCl.

In the preceding paper (2) we reported results of experiments with the halophilic green alga Dunaliella viridis which indicated that several enzymes from this organism (including those involved in carbon dioxide fixation and thus indispensable to the growth of this autotrophic cell) were inhibited by concentrations of salt far below that of the medium in which the cells were grown. This suggested that the cells may possess a mechanism for excluding salt, thereby maintaining an internal salt concentration lower than that of their environment. However, experiments performed with a closely related halophilic species, Dunaliella salina, have been reported (4) to indicate an internal salt concentration even higher than that of the highly concentrated saline medium in which the cells were grown.



In view of this discrepancy, we have undertaken a detailed study of the response of whole cells of our strain to NaCl and other salts.

MATERIALS AND METHODS

Isolation of culture. The axenic culture used in these experiments was isolated from a solar evaporation pond in San Francisco Bay by serial passage of the sample through the mineral medium containing 3.75 M NaCl (supplemented with penicillin and streptomycin to prevent bacterial growth).

The genus Dunaliella was first described by Teodoresco (8,9). He described two species, D. salina and D. viridis, differing mainly in that the former becomes red in media of high salt concentration whereas the latter remains green under all conditions. Lerche (3) recommended abandonment of the species D. viridis and described a number of new species of Dunaliella. The designation "viridis" has, however, continued in the literature and seems best to describe the culture used in these experiments since our cultures appeared green at all salt concentrations tested.

The cells used in these experiments are illustrated in the photomicrograph (Fig. 1). The organisms measure approximately 12 × 7 microns and contain a cup-shaped chloroplast, two anterior flagellae, and a red "eye-spot."

Growth of cultures. The mineral medium used for growth of the cultures was described previously (2). Other cultural conditions

were the same except that the 2,000 ml flasks contained only 250 ml of medium. The initial inoculum was 25,000 cells per ml. Cell counts were made with a hemocytometer on properly diluted or concentrated samples. Two to four samples from each duplicate culture were counted, and each experiment was repeated at least once.

RESULTS AND DISCUSSION

D. viridis was able to grow over a broad range of NaCl concentrations; but the medium in which the inoculum was grown had a slight but definite effect on both the range and the optimum (Fig. 2). When the inoculum was grown in 1.28 M NaCl, the range in which growth was observed varied between 0.2 and 3.8 M NaCl, with an optimum between 0.5 and 2.0 M NaCl. With an inoculum grown in 3.75 M NaCl, growth was observed between 0.5 and 4.8 M with an optimum between 0.8 and 2.0 M NaCl. The optimum salt levels reported here agree with those reported by Gibor (1) and Mil'ko (6).

These cell counts represent total yields after 4 days of growth. The rates of growth at three of these salt concentrations are shown in Fig. 3. In 0.17 M NaCl (1%) there was an initial decrease due to cell lysis, followed by a short period of rapid growth. In medium with the same salt concentration as the inoculum (1.28 M) no lag was observed and the doubling period during the period of exponential growth was about 24 hours. In medium with a higher salt concentration (3.75 M) there was a lag of 2 days, followed by a

period of exponential growth at a slightly lower rate than that observed with the 1.28 M salt. When growth rates were measured with the inoculum grown in the higher salt concentration (3.75 M), the lag in growth in the high salt medium was again observed. Apparently some form of adaptation is necessitated by the high salt concentration.

The effect of dilution on cell lysis was studied further. The effect on the cell count of various degrees of dilution of the medium is shown in Fig. 4. Cells grown in 3.75 M NaCl tolerated dilution to 0.75 M; however, cells grown in 1.28 M NaCl could be diluted to 0.125 M without obvious lysis. The difference in tolerance suggests a difference in internal salt concentration, but the actual level at which the cells lysed probably reflects the limit of their adaptability to a hypotonic environment rather than being a direct reflection of the internal salt level. Similar results were obtained when the experiment was repeated with cells which had been centrifuged out of the medium and suspended in salt solution of the same concentration, thus indicating that it was the change in NaCl concentration, and not dilution of some other medium constituent, which was responsible for the effect.

We next attempted to determine whether the requirement of NaCl for growth was specific. In Table 1 are shown the results of an experiment in which various portions of the NaCl required for optimal growth were replaced by equimolar amounts of KCl. These results

show that KCl cannot replace NaCl, and is, in fact, quite toxic. Table 2 shows the results of adding low levels of KCl to a medium containing adequate levels of NaCl. After 1 day of growth, inhibition was observed in the medium containing 0.10 M KCl, and inhibition was observed even at 0.05 M KCl after 2 days growth. Levels of KCl higher than 0.1 M are extremely toxic and cause large decreases in cell number.

The results expressed in Table 3 indicate that LiCl and $MgCl_2$ are also unable to replace NaCl in the growth medium. No growth was observed in 1 M LiCl or 0.67 M $MgCl_2$, and LiCl was inhibitory at 0.2 M in the presence of adequate NaCl. A study of cation requirements in another species of Dunaliella, the less halophilic D. tertiolecta, revealed that other salts can substitute for sodium as osmoregulators as long as a minimum sodium concentration (0.01 M) is maintained (5).

The effect of replacing the chloride ion with other anions is shown in Table 4. The nitrate and sulfate ions supported growth at the same level as the chloride; the acetate ion did not. The chloride requirement is, therefore, not a specific one.

The enzymes studied in the previous paper (2) showed an equal inhibitory response to the chlorides of sodium, potassium, and lithium. The response of the whole cell, however, as measured by the effect on growth, is quite different. In this case, potassium and lithium are inhibitory, but sodium is not. The latter is, in

fact, required for growth. These results could be explained by postulating a specific mechanism for the exclusion of sodium from the cells. Other cations would be inhibitory because the mechanism is specific for sodium, and a salt tolerance in the enzymes would not be expected because this mechanism would result in a low internal salt concentration. Apparently, a similar situation occurs in another halophilic green alga, Chlamydomonas, in that the cells were found to be hypotonic with respect to the medium (7). Our results cannot, however, be reconciled with those obtained with D. salina (an organism closely related to D. viridis) which indicated a high internal salt concentration in this organism (4).

ACKNOWLEDGMENTS

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TABLE 1. Effect of KCl on the growth of D. viridis

Concentration of NaCl	Concentration of KCl	Inoculum	1 day	3 days
M	M		cells/ml	
1.25		25,000	60,000	160,000
1.00	0.25	25,000	9,800	2,500
0.75	0.50	25,000	3,300	0
0.50	0.75	25,000	0	0
0.25	1.00	25,000	0	0
	1.25	25,000	0	0

TABLE 2. Effect of KCl on the growth of D. viridis

Concentration of NaCl	Concentration of KCl	Inoculum	1 day	2 days
M	M	cells/ml		
1.00		25,000	55,000	94,000
1.00	0.05	25,000	54,000	66,000
1.00	0.10	25,000	17,000	1,800
1.00	0.20	25,000	1,200	0
1.00	0.30	25,000	1,000	0
1.30		25,000	61,000	95,000

TABLE 3. Effect of LiCl and $MgCl_2$ on the growth of D. viridis

Medium	Inoculum	1 day	2 days
		cells/ml	
1 M NaCl	25,000	63,000	105,000
1 M LiCl	25,000	22,000	17,000
1 M NaCl + 0.2 M LiCl	25,000	41,000	58,000
1 M NaCl + 0.4 M LiCl	25,000	36,000	43,000
1.4 M NaCl	25,000	59,000	94,000
0.67 M $MgCl_2$	25,000	21,000	10,000

TABLE 4. Effect of various anions on the growth of D. viridis

Medium	Inoculum	1 day	2 days
		cells/ml	
1.0 M NaCl	25,000	45,000	70,000
1.0 M $NaNO_3$	25,000	49,000	69,000
0.5 M Na_2SO_4	25,000	43,000	68,000
1.0 M CH_3COONa	25,000	15,000	9,000

FIGURE LEGENDS

Fig. 1. Dunaliella viridis. Magnification ; phase contrast optics. Cells grown in medium containing 3.75 M NaCl.

Fig. 2. Effect of NaCl concentration on the growth of D. viridis.

Fig. 3. Growth of D. viridis in media of various salt concentrations. The inoculum was grown in medium containing 1.28 M NaCl.

Fig. 4. The effect of dilution of the medium on cells of D. viridis. The cultures were diluted in a single step from the initial salt level (1.28 or 3.75 M) to the level indicated by each point. Counts were made 15 min after the dilution.

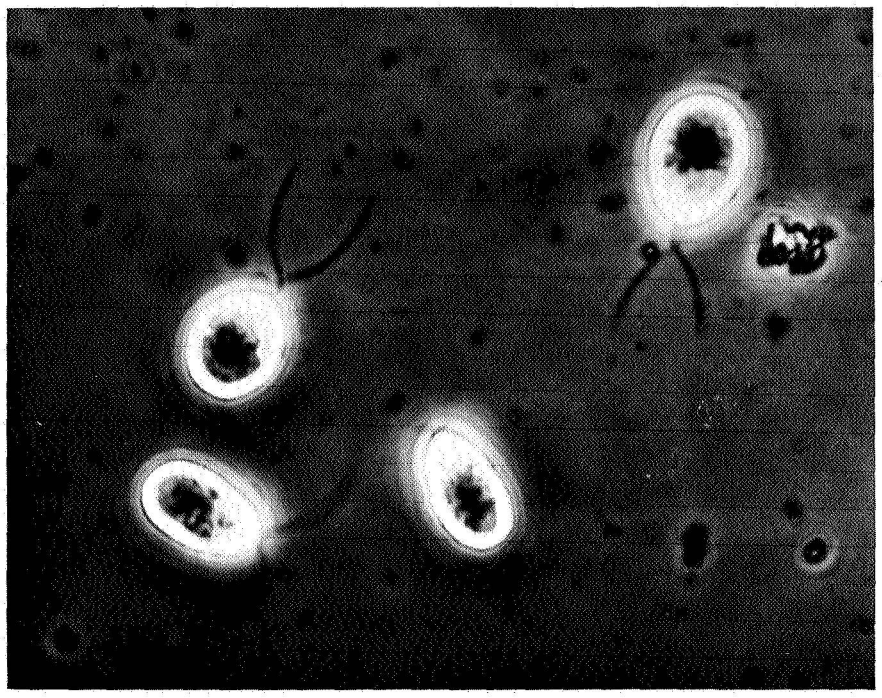

Figure 1.

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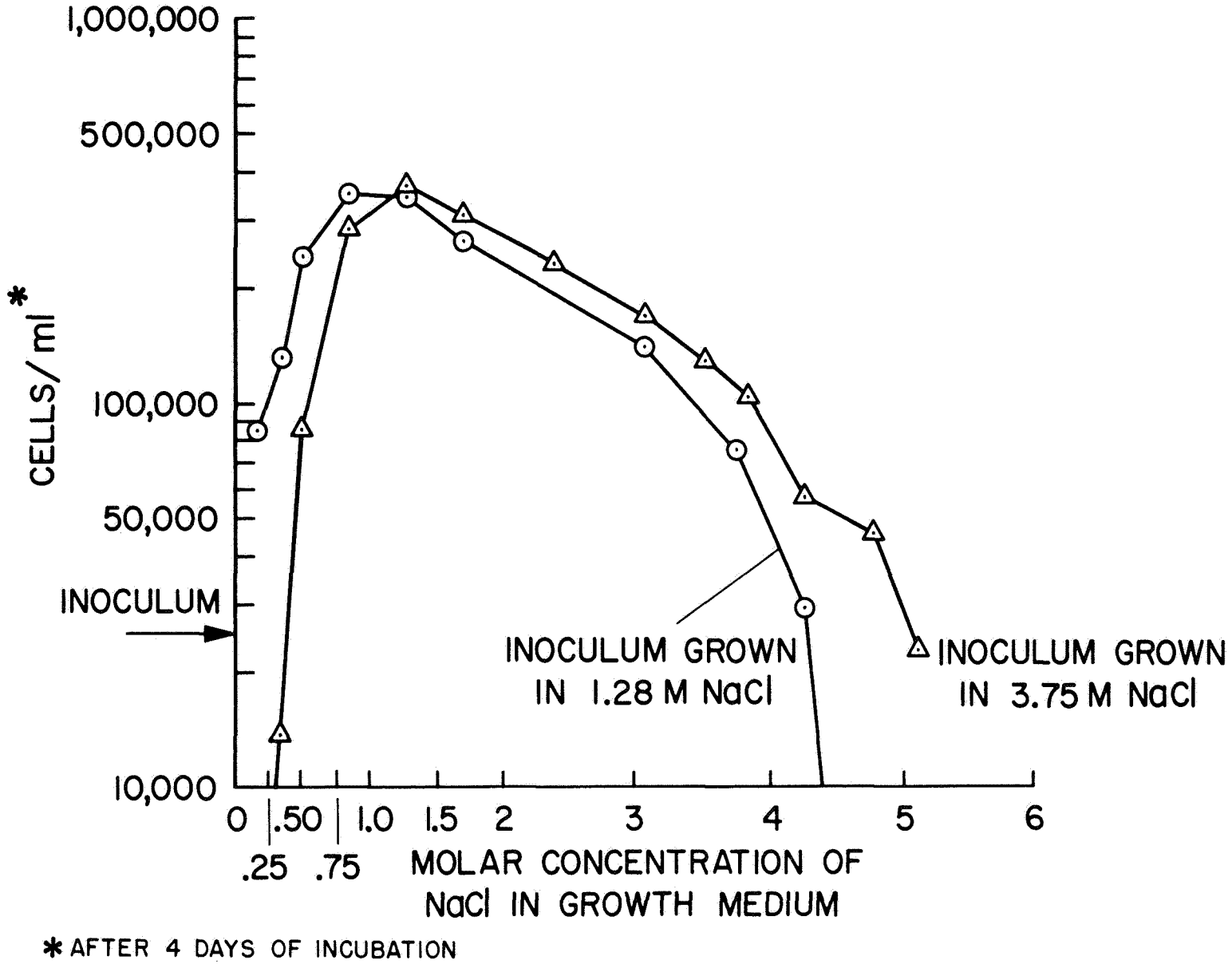

Figure 2.

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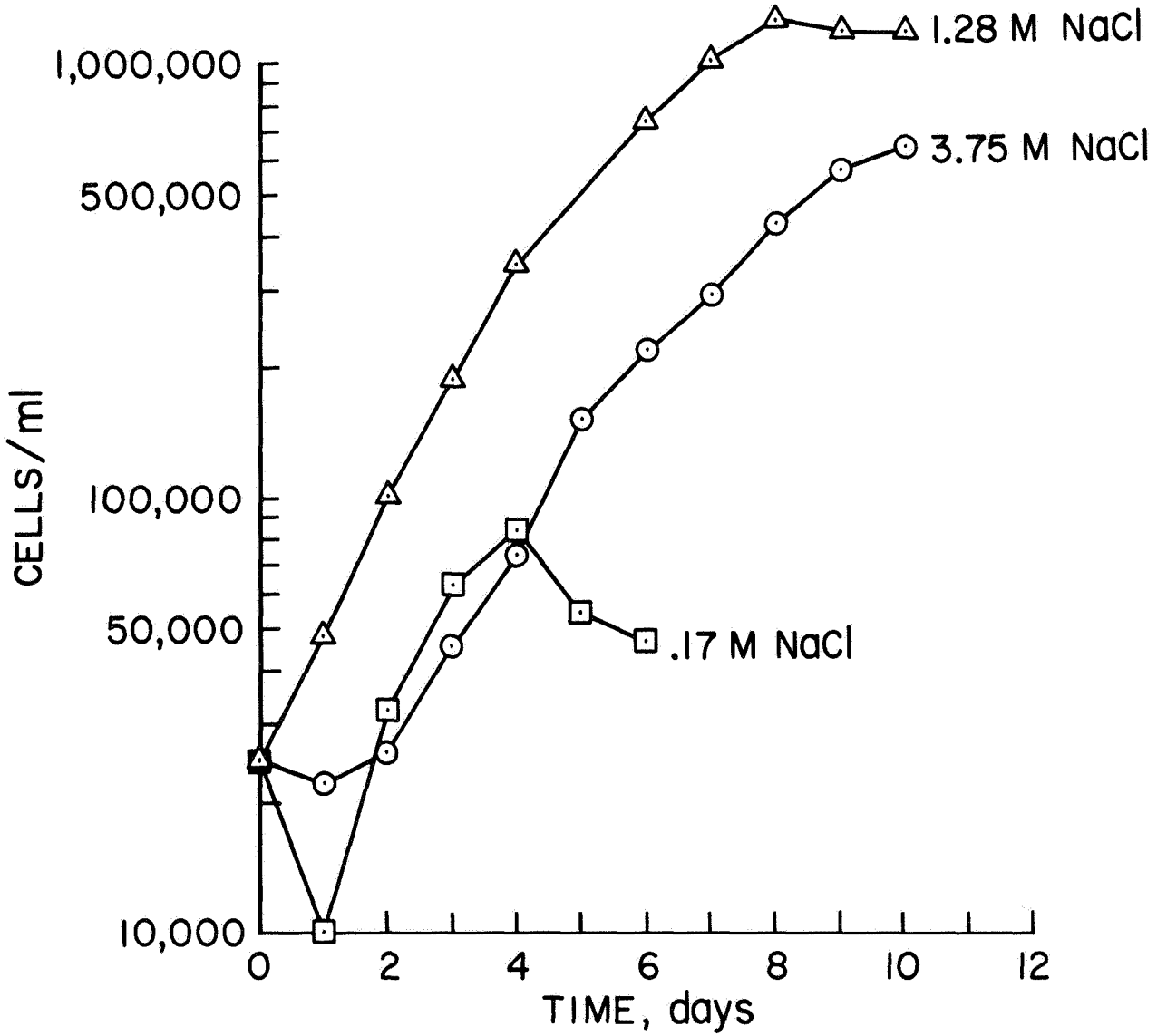

Figure 3.

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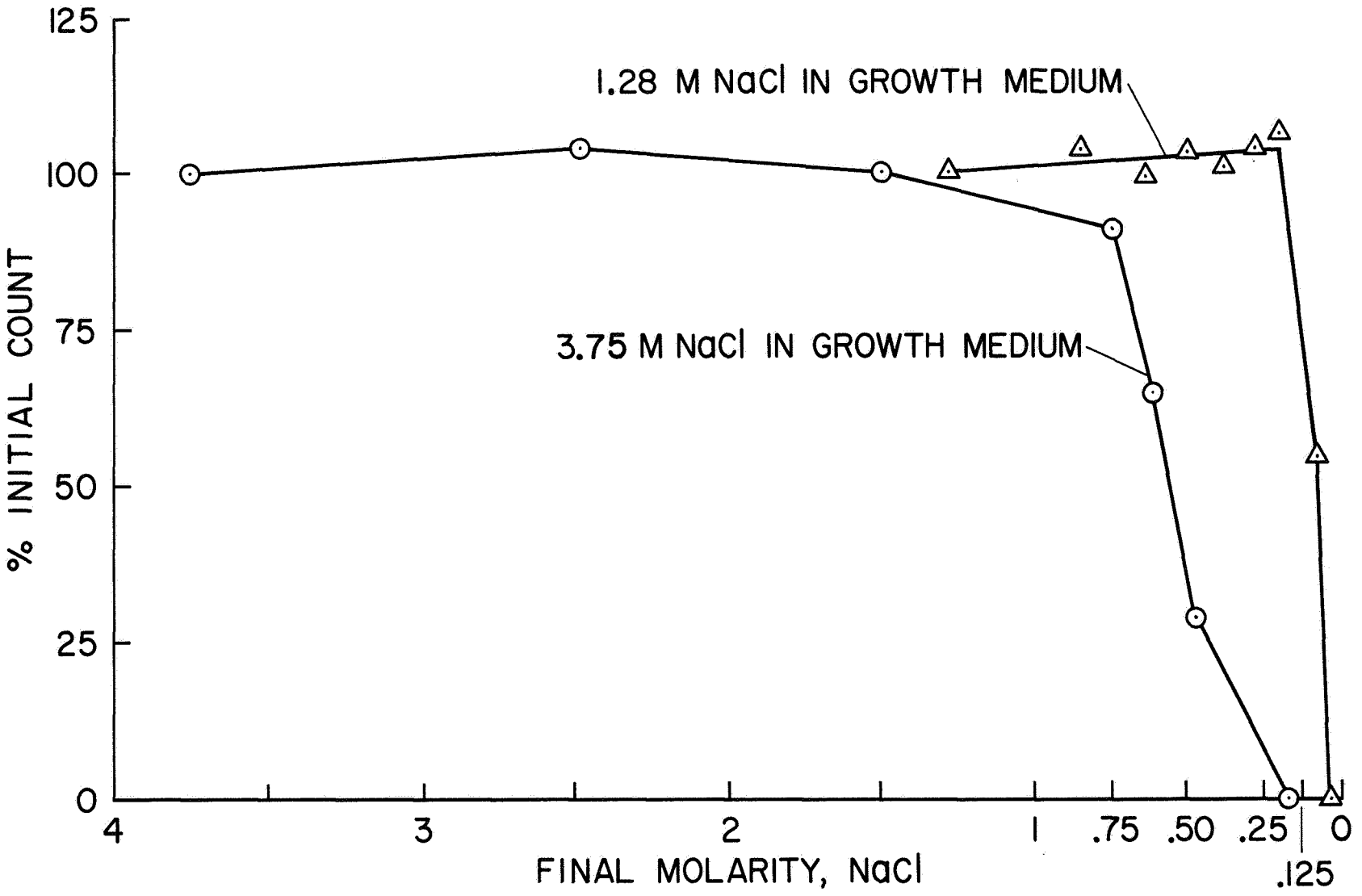

Figure 4.

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