

**Linear Cell Mass Increase during the
Cell Cycle of Escherichia coli.**

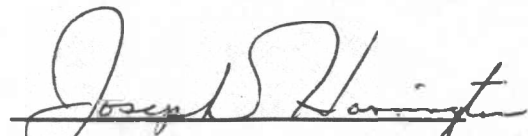
**Submitted in Partial Fulfillment of the Requirements for Graduation
with Honors to the Department of Biology at Carroll College, Helena,
Montana.**

**Patricia L. Winslow
April 1, 1986**

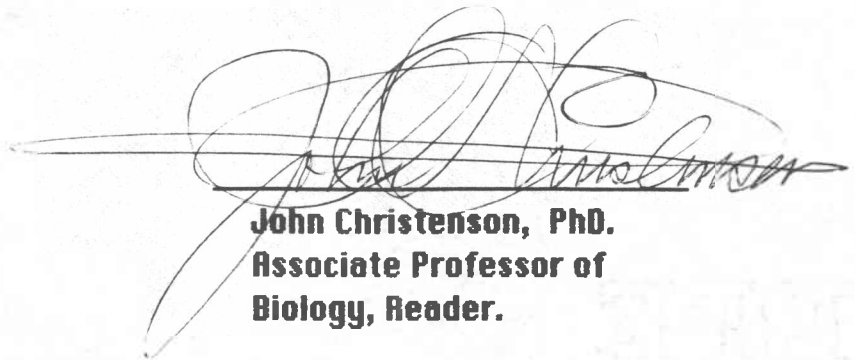


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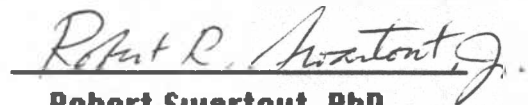
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April 1, 1986

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ABSTRACT

Linear increase of cell mass of undivided cells was determined during the division cycle of *Escherichia coli* B/rA. Synchronous growth was obtained by selection of cells from exponentially growing populations after velocity sedimentation in sucrose density gradients. Cell mass was calculated as a product of mean cell volume and buoyant density that were measured at intervals during synchronous growth. A linear increase in cell mass was observed in two different growth media which had doubling times of 40.0 and 30.1 minutes. The results support the theory of constant linear increase of cell mass throughout the division cycle.

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LITERATURE REVIEW

The bacterium Escherichia coli has been researched in a number and variety of studies of cell growth. These studies have included measurements of cell length of single cells by optical and electron microscopy and measurements of cell length and /or volume in synchronous cultures. However, none of these studies have measured a fundamental cell growth parameter, the increase of cell mass. Each of these approaches has observed different growth results of E.coli.

Single E.coli cells have been measured by different types of optical microscopy. Cullum and Vicente (1) used phase-contrast optics to determine linear length for exponential growing E.coli . Meijer, et al. (8) , using two substrains of B/rA E.coli , determined also a linear increase in cell length growth with light microscopy. Though these studies reveal substantial facts about cell length, the results are not accurate because of the poor resolving power of optical microscopy and the small size of E.coli cells (8). Also, cell diameter of these rod-shaped cells is assumed to be constant throughout the cell cycle.

Electron microscopy studies by Trueba, et al. (9) proved that the assumption of constant cell diameter was incorrect. Trueba discovered that cell diameters will decrease with increasing cell volume during most of the cell division cycle. However, electron microscopy is also not accurate because of the preparation of the cells for examination. Cells become flattened and shrink non-uniformly during preparation . A major problem with electron microscopy is that data involves unfounded assumptions for distributions of cell sizes at birth and at division. These assumptions

make growth rate predictions during cell cycle an estimate at best (6).

The most accurate estimate of cell length and volume is obtained through sizing by the Coulter Counter Multichannel Analyser developed by Kubitschek (3). Through modifications of the Coulter Counter, Kubitschek was able to count and determines the size of the rod-shaped bacterium with increased accuracy. Having established a method for accurately measuring volumes, Kubitschek, Baldwin and Graetzer (2) proved that cell buoyant densities are relatively unchanged before and during cell division. Their results, using equalibruim centrifugation and Percoll gradients, state that cell densities are unlikely to make any stepwise change of 0.1% during the cell cycle. Cell mass can now be accurately measured as a fundamental growth parameter. This has been done by using volume distributions of the Coulter Counter and cell buoyant densities obtained from equilibrium centrifugation.

METHODS AND MATERIALS

To maintain stock cultures of B/rA, the cells were grown on nutrient broth agar plates (23g/liter nutrient agar, 1 liter water). The plates were inoculated by streaking with the B/rA bacterial suspension and incubated at 37C for approximately 24 hrs. Once the cells had grown to visible colonies the plates were sealed and stored at 0C. Parent cultures were inoculated with a single colony from the nutrient agar plate, and cultured in a shaker waterbath at 37C in Erlenmeyer flasks (250 ml) containing 40 ml of MSGT medium (M9 salts, 1.2 ml 60% sucrose, 0.6 ml 20% glucose and 0.3 ml Hooglands' trace element salt solution) and in MSGT plus casamino acids (MSGT media plus 0.3 ml casamino acid). The cultures were grown overnight or until the stationary phase was reached.

To obtain cultures in the exponential growth phase the parental cultures were diluted 10^4 , 10^5 , and 10^6 . This range of dilutions allowed for variability in growth rate. Growth was measured as an increase in turbidity with a Klett-Summerson colorimeter. When cultures reached 10 units (10KU) on the colorimeter, samples were taken to establish synchronous cell cultures. Figure 1 (page 12) shows parental cultures grown in MSGT and MSGT+CAA, growing exponentially. The optical densities (KU) were plotted against time on semilogarithmic 2-cycle graph paper. The data were fitted with linear regression and the doubling time obtained. The actual doubling time for the cultures in MSGT and MSGT+CAA were 40.0 and 30.1 min, respectively. A constant doubling time of the culture is necessary for the credibility

of the culture : if the doubling time of the culture varied when we began synchronous growth, measurements of size and numbers would fluctuate through the division cycle of the synchronous cultures.

Synchronous cell cultures were prepared by velocity sedimentation in sucrose gradients using selected cells from exponential phase cultures. When the exponential phase cultures reached 10 units on the colorimeter or approximately halfway into the exponential growth phase, 30 ml of the culture were filtered through a 0.45 μ m nitrocellulose membrane. Cells were then resuspended with a glass spreading rod into approximately 0.5 ml of unfiltered parental culture; 0.3 ml of this concentrate was pipetted onto a 6 ml, linear, 5-15 % sucrose gradient in the same growth medium. The gradient was formed in a 15 ml Corex glass centrifuge tube. The gradient tube was centrifuged at 3000 rpm for 45 to 75 sec in a swinging bucket roter (HB-4) of a Sorvall RC2-B centrifuge at 37C.

The result was a visible band of cells approximately half-way from the top of the gradient. Using a bent hypodermic needle, 0.3ml of cells of approximately the same size were then removed from the top 3-10% of the band and resuspended in 15 ml of the parental culture filtrate. The suspension was then returned to the shaker bath; 0.9 ml samples were periodically (~ 1 every 3 min) removed and mixed with 0.1 ml of 3.6% formaldehyde solution. The formaldehyde allowed us to fix the cells and stop growth of the synchronous cultures at the specific time intervals. The formaldehyde did not effect the bacterial cell volume according to previous experimentation (3). Samples were taken at intervals according to doubling time of bacteria. For example,

if the cells doubled approximately every 30 min, samples were taken every 3 minutes during synchronous growth. This procedure was to insure that sizing and counting of the samples would last at least one division. Cell count and volumes were obtained with a Coulter Counter Multichannel Analyzer system. Culture samples (300 λ ~ 0.3 ml) were added to 10 ml of 0.1 HCl. This procedure is recommended because the H ions of the HCl are highly motile and allow for rapid electrical response to passage of a cell. It also provides maximum ion concentration and minimum fluctuations that could disturb amplitudes of electrical pulses thereby increasing accuracy of sizing and detection of smaller cells (3). As the cells passed through the aperture (16 μ m diameter) they were counted and measured. The distributions were collected on an analyser scale of 128 channels. The data from these distributions were stored on an Apple II microcomputer for examination at a later date.

Figure 2 (page 13) shows representative results for Mean Channel Height vs Frequency as obtained by computer analysis. The quantitative result of the difference between the parental culture distribution (2a) and the initial distribution (2b) after filtration reflected a notable shift to the right. This shift was a signal that the initial synchronous cultures were growing. This procedure is necessary at this point to make sure that the cells are growing and to determine whether or not to continue the experiment. The last distribution (2c) demonstrated that the cells were in the division cycle. The newly divided smaller cells are to the left and the older undivided cells are to the right. Eventually, the older cells will divide

away and only younger cells will be visible on the graph.

Buoyant density determinations were accomplished by equilibrium centrifugation in Percoll gradients (Percoll and growth medium). Percoll is a colloidal silica covered with polyvinylpyrrolidone (PVP). Advantages of Percoll include its compatibility with living cells because it does not penetrate into or pass through biological membranes. Also, it does not disturb molarity of the media; and it is non-toxic to the cells. The remainder of each sample fixed with 3.7% formaldehyde were layered onto linear, 6 ml, 50-90% Percoll gradients in 15 ml Corex centrifuge tubes. The gradient tubes were then centrifuged 10 min at 12,000 rpm at 20C in angle-rotor head (SS-34) of the Sorvall RC2-B centrifuge. A cell band appeared two-thirds the way down and again using a bent hypodermic needle, 0.75 ml were taken from the upper and lower portions of each band. Cells were taken here to insure validity of results. The refractive index of each sample was measured with an Abbe refractometer. The buoyant densities were calculated from calibration equations shown below.

$$\rho = 6.458n - 7.622 \quad (1)$$

RESULTS

From distributions illustrated in Figure 2 (page 13), the relative Mean Channel Heights for MSGT and MSGT+CAA were obtained by estimation of the areas under the divided and undivided peaks. In Figure 4 (Page 15) these data were fitted with linear regression. The averages of the Mean Channel Volume of MSGT+CAA and MSGT were 1.78 u and 1.05 u, respectively. The discrepancy between volume averages for the two media may be due to the fact that rapidly growing cultures give larger cell volumes than more slowly growing ones.

Buoyant densities (Figure 5, page 16) were calculated from calibrations of the density vs index of refraction. The observed relationship was:

$$p=6.4568n-7.622 \quad (1)$$

This equation was legitimate for MSGT and MSGT+CAA media because the small amount of casamino acids did not alter buoyant densities of significantly.

These experiments showed that buoyant densities did not change significantly throughout the cell cycle. The slope and standard error of the slope were calculated for each buoyant density graph (Table 1, page 11). These data support the theory that there is no significant change of cell buoyant density. Also in both cases the standard errors of the slope were so large that there is no significant change in density throughout the synchronous growth cycle. These results agreed with previous studies which indicate that cell buoyant densities remain constant (2).

Once the above variables were obtained, the Mean Cell Mass could

(7)

be calculated as the product of volume and buoyant densities. The values (Figure 6, page 17) for Mean Cell Mass are fitted by linear regression and support the hypothesis of linear increase in cell mass. Further support of the hypothesis is illustrated by Figure 7 (page 18). This shows the same data for each medium plotted on semilogarithmic 2-cycle graph paper. The bending of the lines in these data show that the cell mass does not increase exponentially during the synchronous cell cycle. We can conclude from this that cell mass increases linearly with time or at a constant rate.

DISCUSSION AND CONCLUSION

The results of the experiment (Figures 5-7) provide evidence that cell mass increase was linear or close to linear during the division cycle at doubling times of 40.0 and 30.1 min. These results support earlier findings of Kubitschek, Baldwin and Greatzer (2) using the E.coli cells and the same method.

In the past most experiments of cell growth involved increase of cell volume and length (1,7,8). However, these measurements were not accurate indications of cell growth; first, the width of the cell was incorrectly assumed constant during division and cell length was measured by optical microscopy which has limited resolution power. The combination of inaccurate measurement of cell lengths and assuming the width of the cell remains constant during division does not suggest linear volume increase during the cell cycle. The results presented here do suggest linear cell volume increase in agreement with Kubitschek's earlier findings (4).

The use of stable conditions during synchronous growth contribute to the reliability of the methods used in this study. Previous experiments did not protect cells from the shock of changing osmotic pressure and / or temperature fluctuations. In this work, osmotic pressure fluctuations were reduced through the use of 2% sucrose in all growth media and in all gradients. Cultures were always grown in the same media, and after filtration, cells were placed back into the same media

from which they were grown. The temperature was a constant 37°C in the shaking waterbath, in establishing gradients, and during centrifugation.

The determination of linear increase in cell mass is only one independent method of determining growth kinetics in synchronous cultures. According to Kubitschek's research (6), another method is the use of cell fractions for determinations of protein, RNA, and lipid content. The use of specific radioactive precursors was useful in these determinations. Protein and RNA precursor pools may also be determined. Still another approach to determine growth kinetics is the measurement of rates of turnover of protein, RNA, and lipid. The pattern of cell growth can be approximated from rates of uptake of precursors for the three macromolecules. A combination of these results should reveal major differences in controls for each of the macromolecules accumulated.

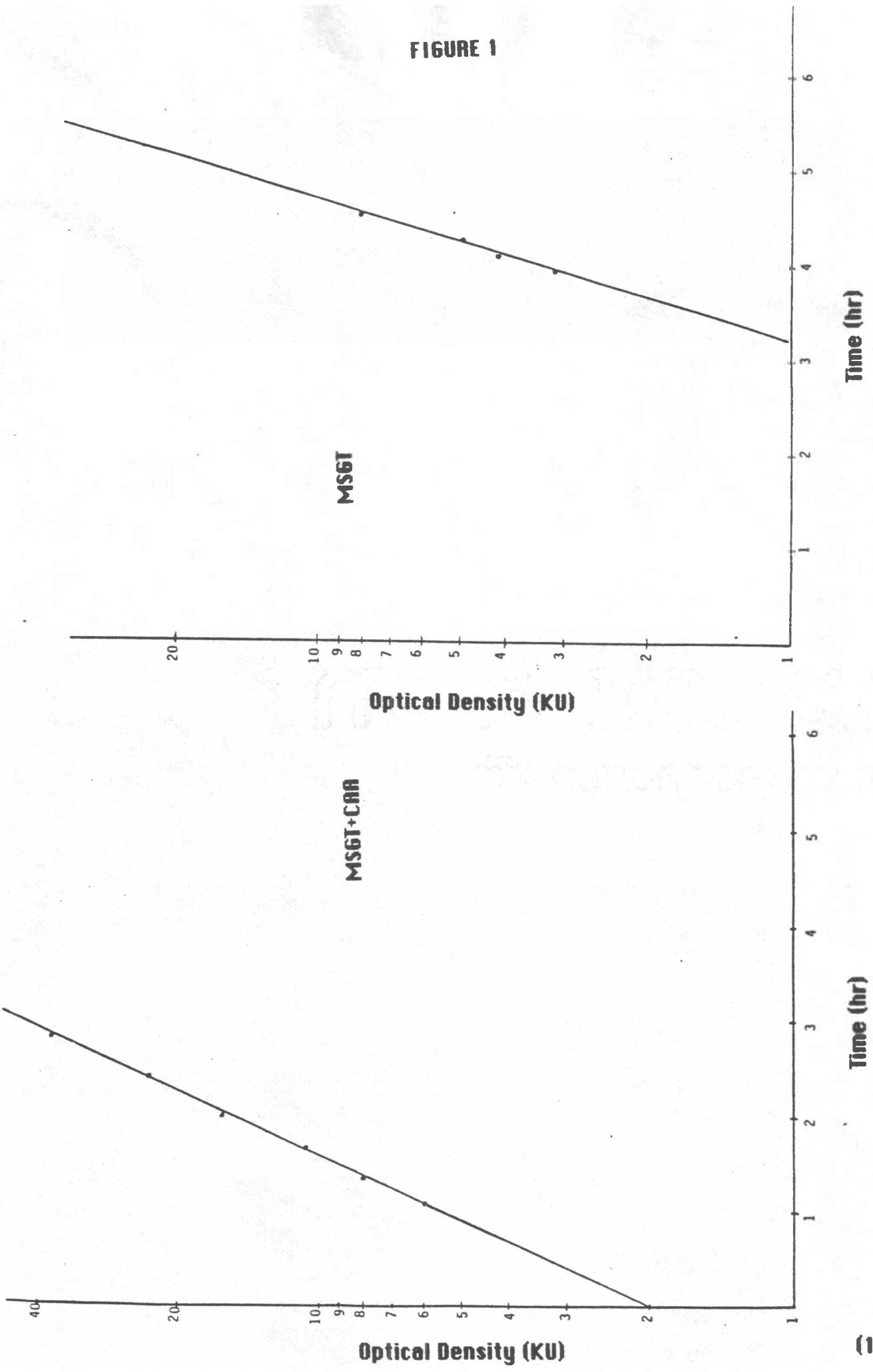
By uncovering cellular growth kinetics and the control of their physiological and biosynthetic mechanisms, researchers will be able to make important advances in biology and medicine. Understanding how a normal cell controls its growth will help in carcinogenesis research and embryonic differentiation studies. Medical treatments could be targeted toward the controls of the cell. Antibiotics and tumor inhibitors could act as chemical intermediates that restore or modify the control of cell growth. Cell growth kinetics and its controls therefore play a highly significant role in biology and medicine.

**Slope and Standard Error of the Slope of Buoyant Densities
during Synchronous Growth**

Table 1

Medium	Slope	Standard Error of the Slope
MSGT	8.33×10^{-5}	2.34×10^{-4}
MSGT+CRR	3.13×10^{-4}	-3.45×10^{-4}

FIGURE 1

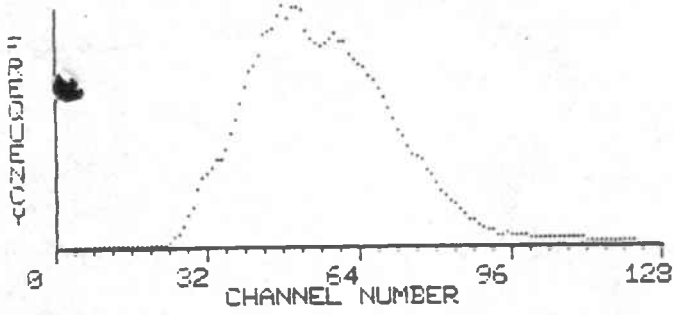


OPTICAL DENSITIES DURING EXPONENTIAL GROWTH

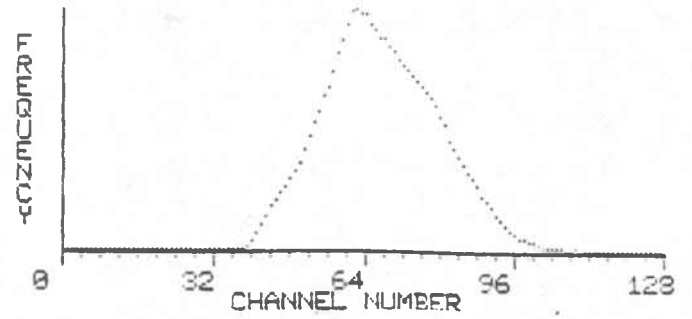
FIGURE 2

MSGT

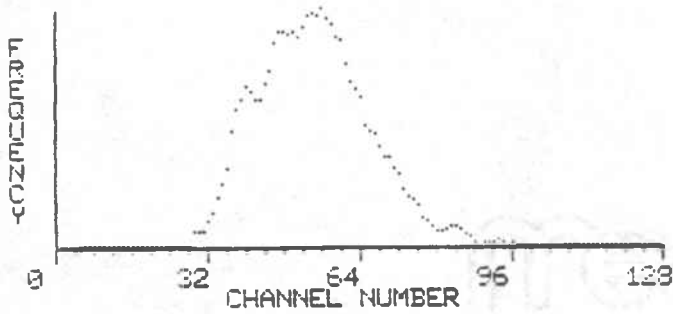
MSGT+CRA



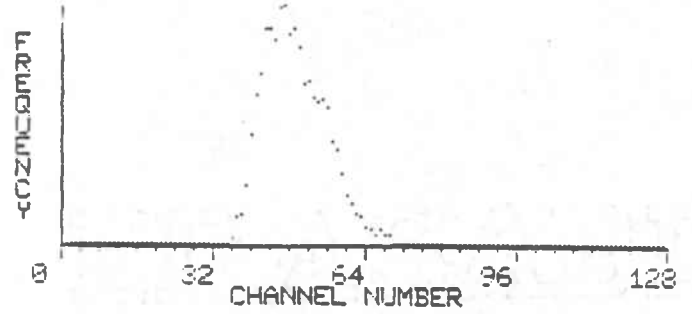
(a) Parental Distribution



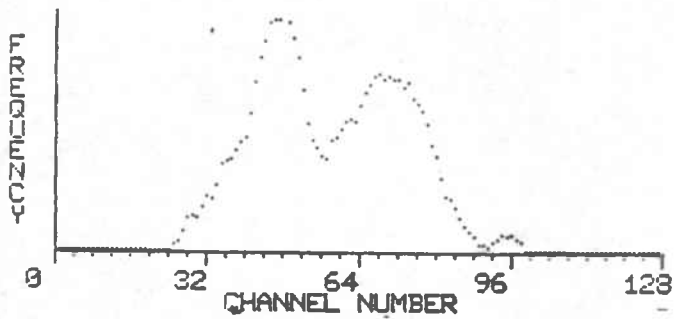
(a) Parental Distribution



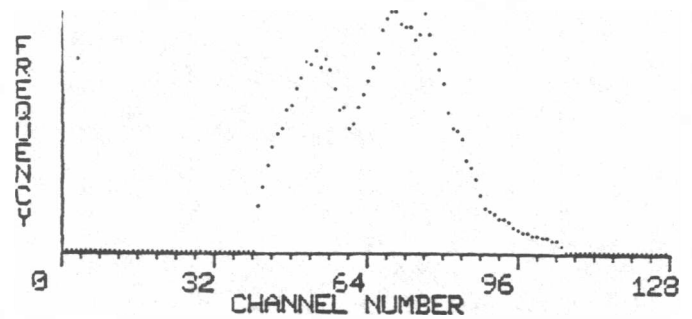
(b) Initial Distribution of Synchronous Culture after Filtration



(b) Initial Distribution of Synchronous Culture after Filtration



(c) Division of Cells



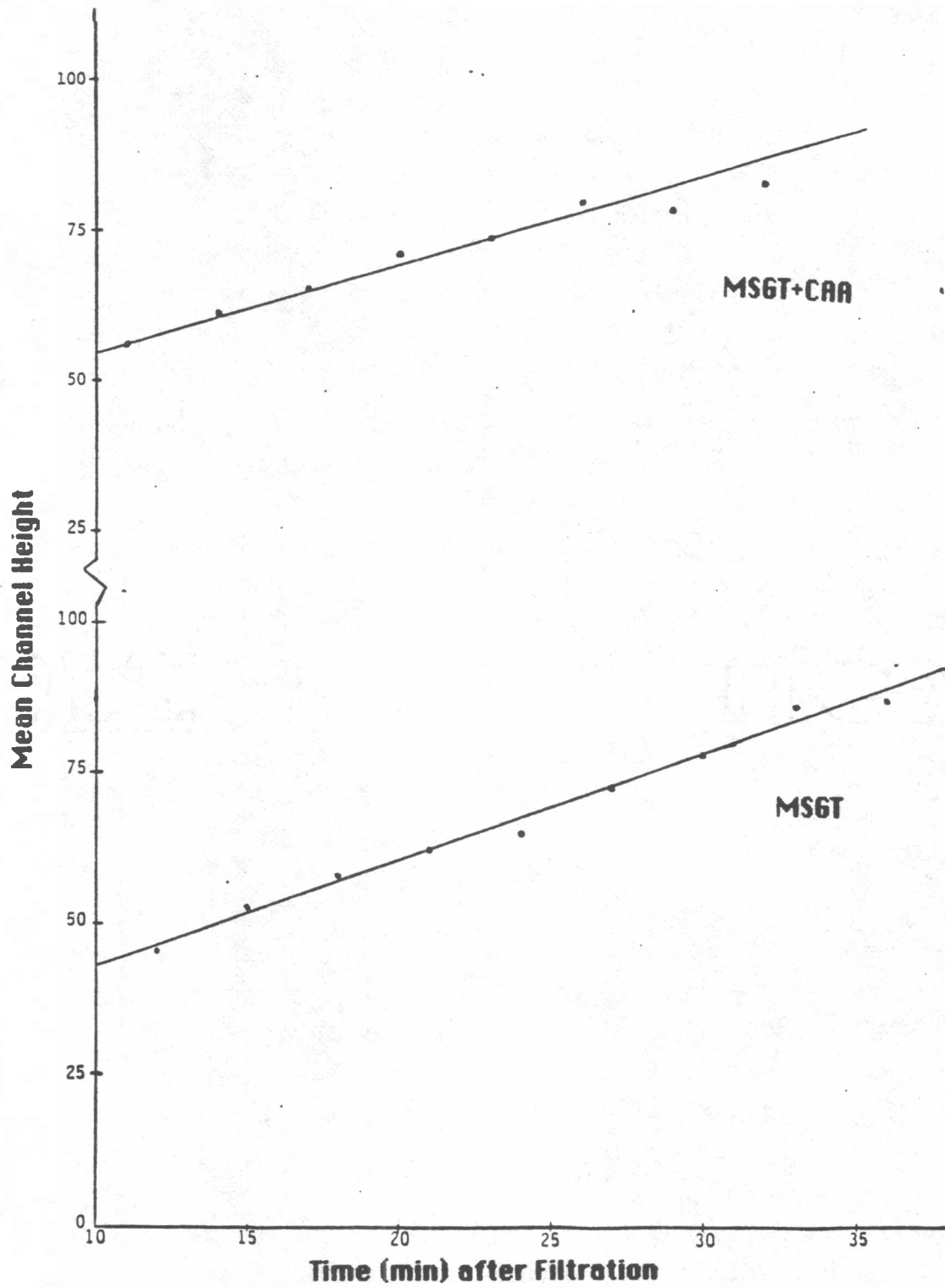
(c) Division of Cells

MEAN CHANNEL NUMBER

VS

FREQUENCY

FIGURE 3

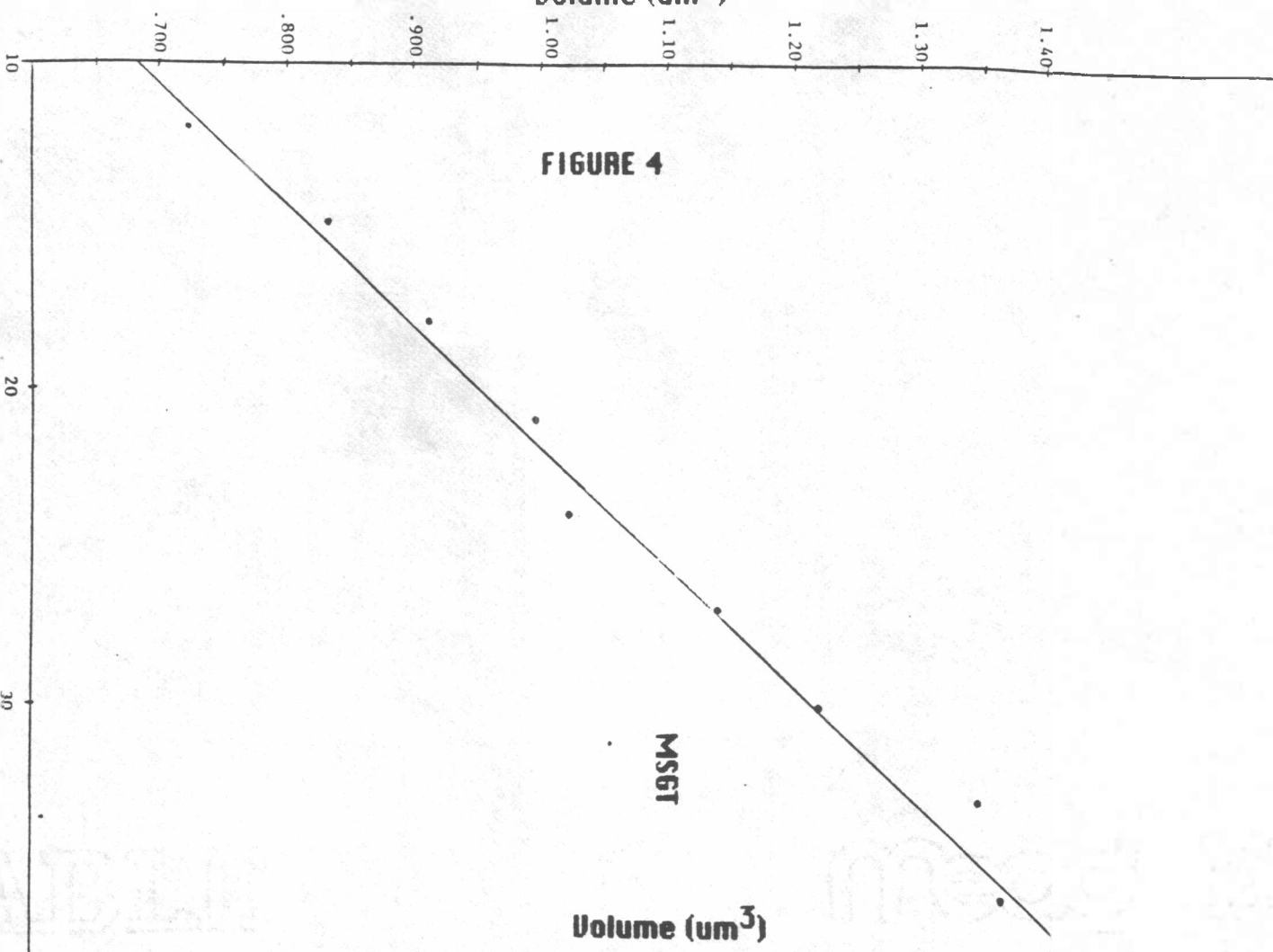


DURING SYNCHRONOUS GROWTH MEAN CHANNEL HIEGHT

FIGURE 4

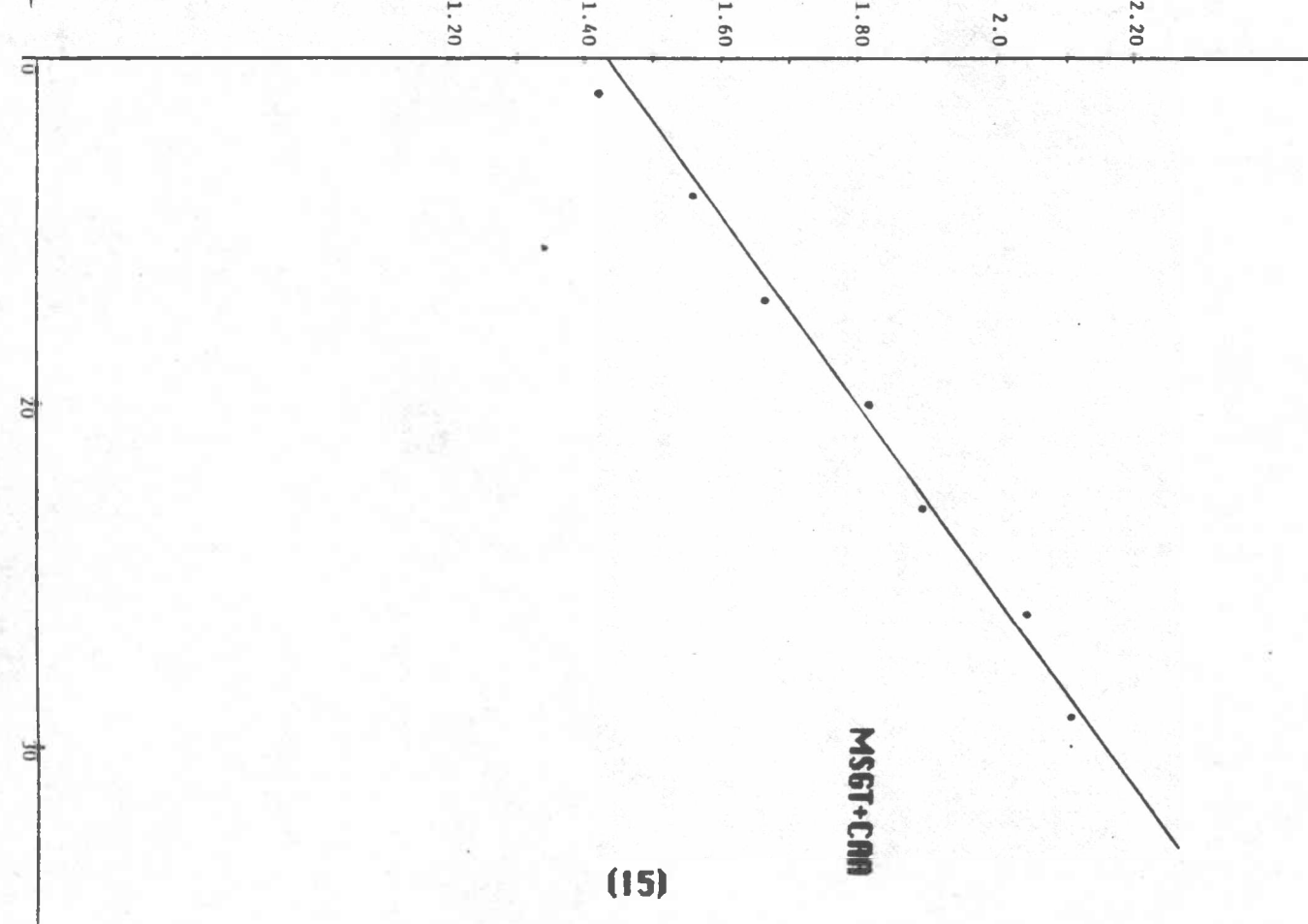
Time (min) after Filtration

MEAN CHANNEL VOLUME DURING SYNCHRONOUS GROWTH



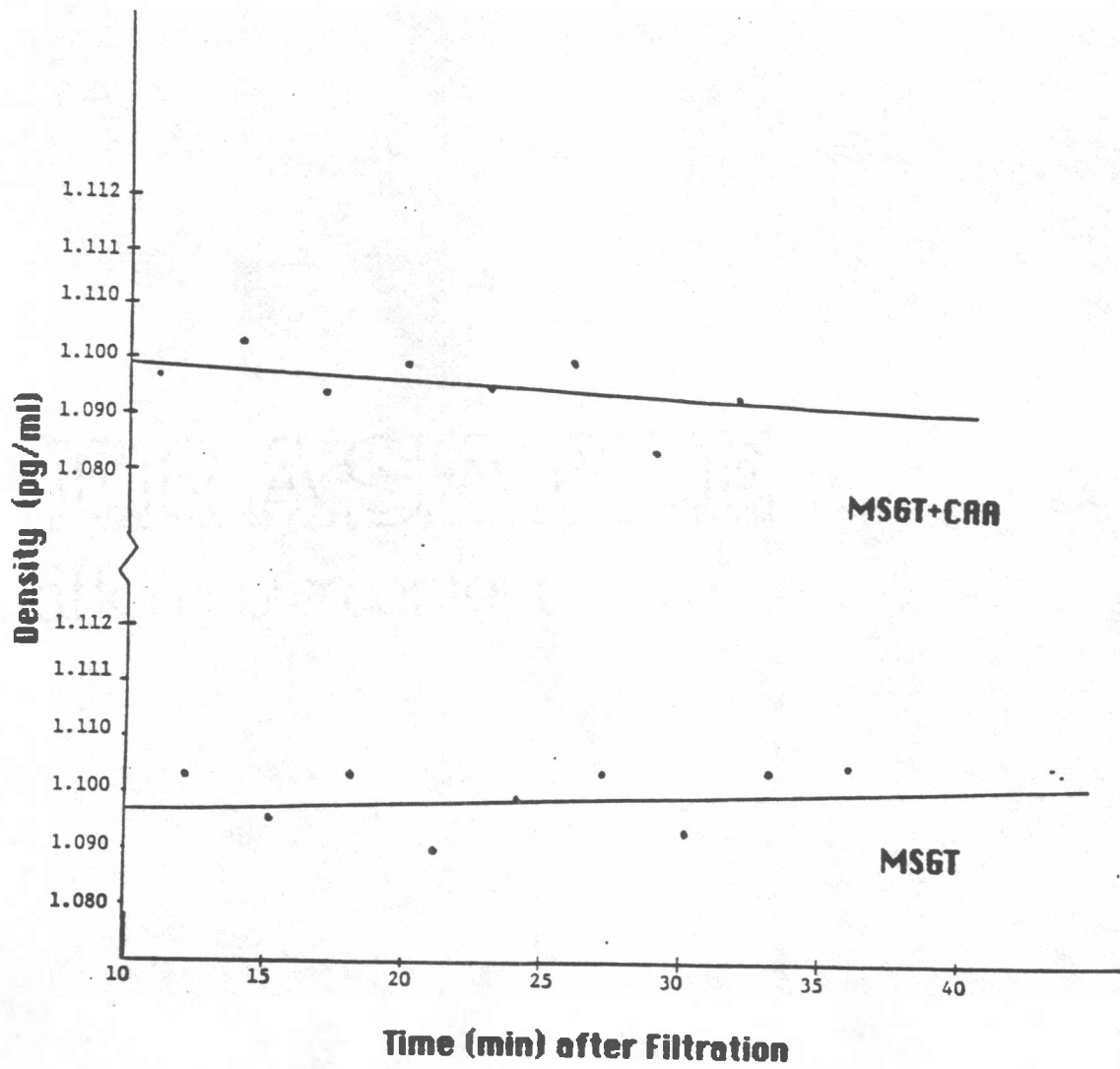
MSGT

Volume (μm^3)



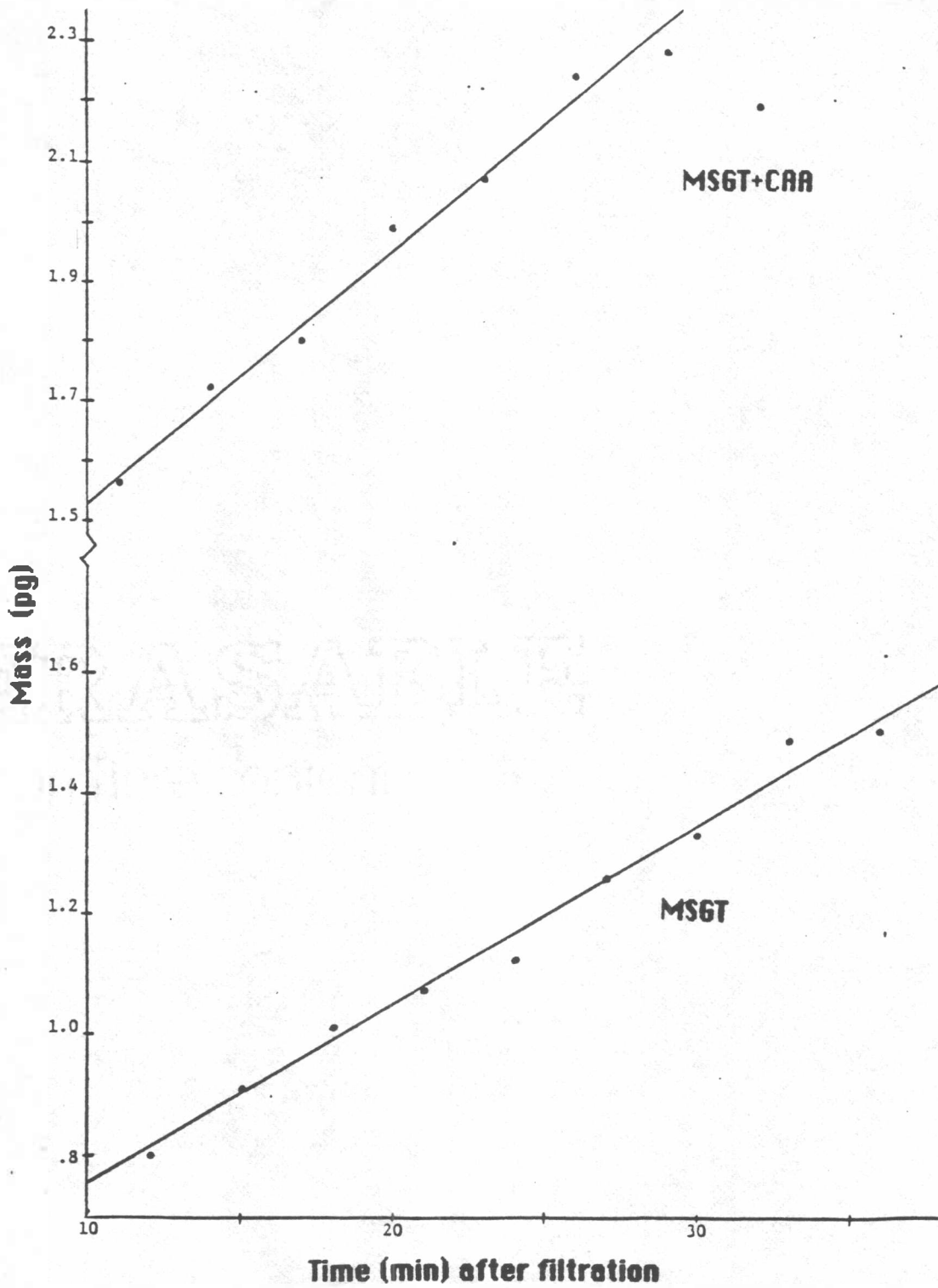
MSGT+CMN

FIGURE 5



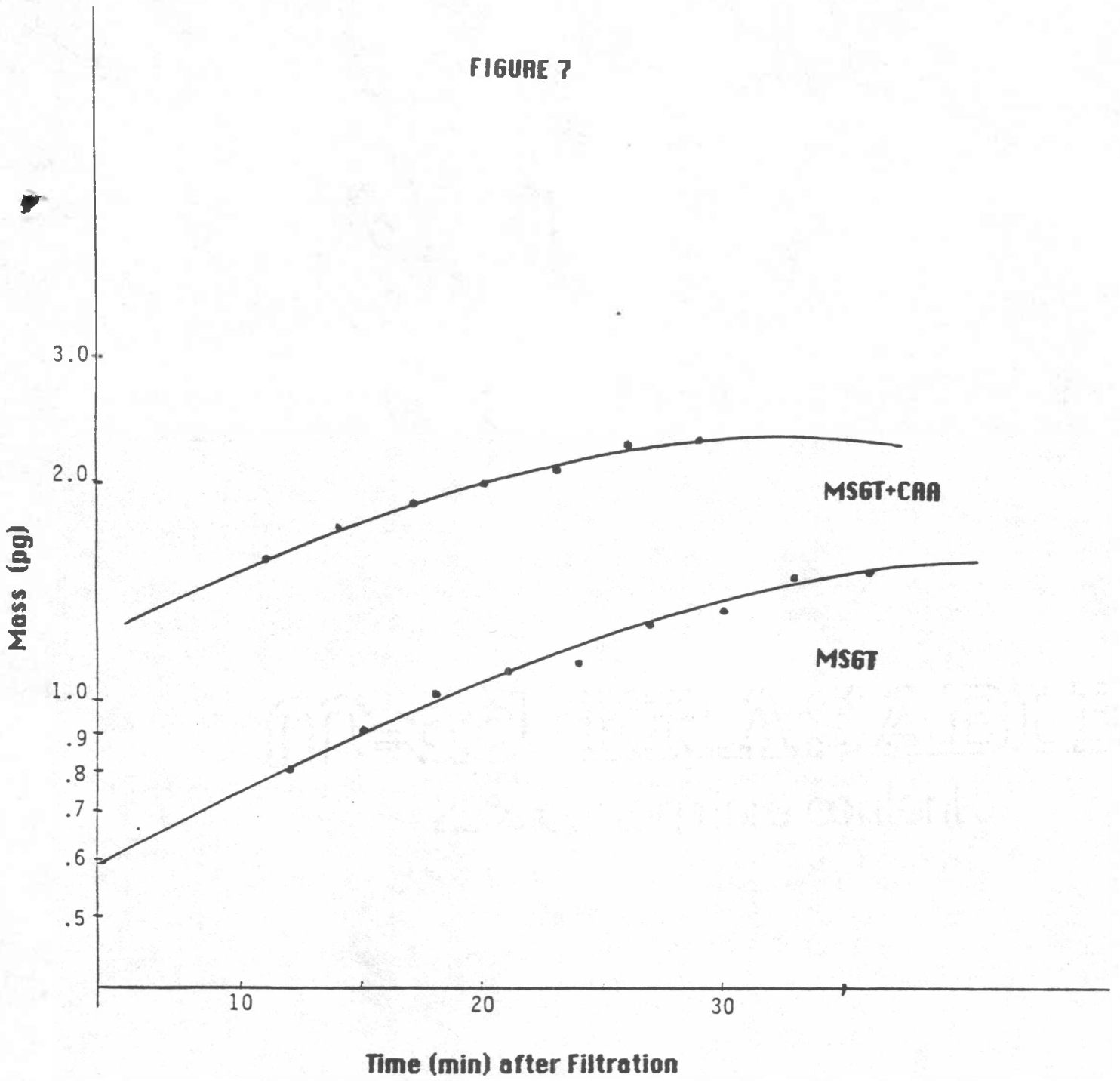
BUOYANT DENSITIES DURING SYNCHRONOUS GROWTH

FIGURE 6



MEAN CHANNEL MASS DURING SYNCHRONOUS GROWTH

FIGURE 7



MASS DURING SYNCHRONOUS GROWTH (SEMILOGRITHMIC SCALE)

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