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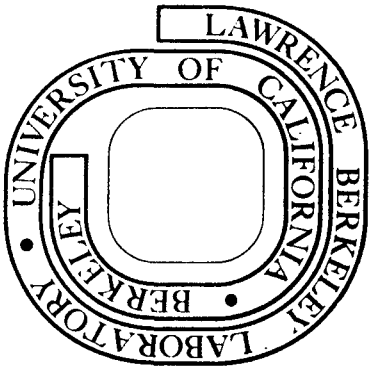

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STIMULATION OF WI-38 CELL CYCLE TRANSIT

Effect of Serum Concentration and Cell Density

James C. Bartholomew, Nicola T. Neff, and Priscilla A. Ross

Running Title: Stimulation of WI-38 Cell Cycle Transit

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ABSTRACT

Flow microfluorometry has been used to characterize the effects of serum concentration and cell density on the initiation of cell cycle transit of stationary phase (G_0) human diploid fibroblasts (strain WI-38). The concentration of serum used to stimulate these cultures had no effect on the time cells began appearing in S (the DNA synthetic period), nor on the synchrony with which they moved around the cell cycle. However, as the serum concentration increased, the fraction of the stationary phase population released from G_0 increased. Cell density modulated the ability of serum to stimulate cell cycle traverse. For example, at a cell density of 1.81×10^4 cells/cm^2, 78% of the population was sensitive to serum stimulation; whereas, when the density was increased to 7.25×10^4 cells/cm^2, only 27% of the population could be stimulated. This effect of cell density on the serum response is not simply the result of changing the ratio of serum concentration to cell density, but appears to reflect a true modulation of the population's sensitivity to serum stimulation. These results are consistent with the interpretation that the primary action of serum is to determine the probability of the transition of cells from a non-cycling G_0 state to a cycling state and that cell density determines the proportion of the population capable of undergoing this transition.

INTRODUCTION

The growth of human diploid fibroblasts in culture is characterized by a logarithmic growth phase followed by a stationary phase in which the cell number per culture remains constant. The mechanism(s) which establish this stationary phase are not known; however, it has been demonstrated that most of the cells in a stationary phase population have a DNA content equivalent to cells in the G_1 phase of the cell cycle (Wiebel and Baserga, '69; Macieira-Coelho and Berumen, '73). So far an adequate description of how stationary phase cells are distributed throughout G_1 has not been possible because of a lack of suitable markers for the various parts of G_1. Prescott (1968) has proposed that as a culture enters stationary phase, G_1 expands such that even though cells are still traversing the cell cycle they spend the majority of their cycle time in G_1. Smith and Martin (1973), on the other hand, have divided G_1 into two states, a "B-phase" in which the cells are progressing towards the initiation of DNA synthesis, and an "A-state" in which the cells can reside for an indeterminate period of time without progressing towards DNA synthesis or division. This "A-state" is analogous to the G_0 state proposed by other investigators (Rovera and Baserga, '73; Baserga et al., '73; Sander and Pardee, '72; Smets, '73; Augenlicht and Baserga, '74).

Whatever the nature of the stationary phase population, it has been observed with a number of cell culture systems that the addition of fresh serum to the medium of stationary phase cultures stimulates cells to synthesize DNA (Wiebel and Baserga, '69; Todaro et al., '65; Griffiths, '72; Temin, '71). Wiebel and Baserga (1969) observed this phenomenon with WI-38 cells and showed that addition of fresh serum to a stationary phase culture stimulated 11% of the population to

synthesize DNA within 36 hr. This stimulation of cell cycle traverse by serum addition suggests that the onset of stationary phase may be due to a reduction of the serum activity in the growing culture or a greater requirement for serum as the cell density increases. In the experiments reported here we have studied how serum and cell density interact to modulate the release of cells from stationary phase. The results of these experiments suggest that serum concentration affects the proportion of the population that can be stimulated from stationary phase, and that increasing cell density decreases the ability of serum to stimulate cell cycle traverse.

MATERIALS AND METHODS

Cell Culture Techniques. The WI-38 cells used in this study were obtained from Dr. Leonard Hayflick of Stanford University. They were generally received at population doubling levels between 15 and 19. The cells were carried in 100 mm plastic dishes (Falcon, Oxnard, Calif.) and incubated in a 10% CO_2 incubator at 37°. The cells were grown in Vogt and Dulbecco's modification of Eagle's medium (Vogt and Dulbecco, '63) containing 10% newborn calf serum (GIBCO, Grand Island, N.Y.). The cells were transferred when they reached their saturation density (1.3×10^5 cells/cm^2) by removal from the dishes with 0.05% trypsin (Difco, 1:250; Detroit, Mich.) in 25 mM Tris buffer, pH 7.4 containing 140 mM NaCl, 5 mM KCl, and 0.7 mM Na_2HPO_4 (isotonic Tris buffer). The reseeding density was 1/4th the saturation density. The cells used in these experiments were at passage level 20-25.

For stimulation of cell cycle transit, 10-day cultures of WI-38 cells were trypsinized as described above and reseeded in fresh dishes at the density to be tested and in media containing the required serum concentration. In previous experiments it was shown that this trypsinization procedure did not affect the stimulation of cells.

Flow Microfluorometry. The amount of DNA per individual cell was quantified by staining the cells with acriflavine (Trujillo and Van Dilla, '72) and measuring the amount of fluorescence per cell by flow microfluorometry (FMF). Thus, cells in G_1 have 2C DNA content and those in G_2 plus M (G_2+M) have a 4C DNA content. Cells in S have a DNA content distribution between 2C and 4C. These measurements were done in collaboration with Drs. Joe Gray and Marvin Van Dilla of the Lawrence Livermore Laboratory. The FMF technique has been described previously (Holm and Cram, '73). Briefly, the stained cells were passed individually through the beam of an argon-ion laser (Spectra-Physics, Mountain View, Calif.) tuned to 488 nm. The pulse of fluorescent light was filtered to reduce scattered exciting light and absorbed by a photomultiplier tube positioned at right angles to the laser beam. The resulting signal is amplified electronically and recorded in the memory of a pulse height analyzer (Northern Scientific, Middletown, Wisconsin). The data in the form of a DNA histogram was stored on magnetic tape and processed by a program written for a Sigma 2 computer (Xerox, Rochester, N.Y.). The proportion of the population in G_1, S, and G_2+M was determined by analyzing the data with a best fit computer program which assumes a Gaussian function under the G_1 and G_2+M peaks and a second order polynomial comprising the S continuum (Dean and Jett, '74). In fact, stain picked up by the cytoplasm alters the apparent distribution of cells in the G_1 and G_2+M from a true Gaussian peak. The magnitude of this effect was determined by measuring the peak shapes in populations containing no cells in S as judged by ^{3}H-thymidine incorporation and autoradiography. All subsequent calculations employed this correction for non-Gaussian distributions. Using this procedure, the determination of the percent of the population in S by FMF agreed to within 10% of values obtained by ^{3}H-thymidine incorporation and autoradiography.

RESULTS

Analysis of Cell Cycle Transit. The effect of serum concentration and cell density on the stimulation of cell cycle transit was monitored by measuring the increase in the amount of DNA per cell as a function of time after stimulation. Figure 1 shows the series of DNA histograms obtained when WI-38 cells at a density of 1.81×10^4 cells/cm^2 were stimulated with different concentrations of serum. Previous experiments had indicated that not much movement of cells out of G_1 and into S occurred before 18 hr, regardless of the serum concentration used. In addition, the exact time after stimulation that cells began entering S depended to some extent on the prior history of the stationary phase population. As reported by Augenlicht and Baserga (1974), the longer a culture was maintained in the stationary phase, the longer the lag time between stimulation and the initiation of DNA synthesis.

The DNA histograms were analyzed as described in MATERIALS AND METHODS to determine the fraction of the population in the various cell cycle phases. As seen in Figure 2, cells began to synthesize DNA between 20 and 22 hr at all concentrations of serum tested. The time necessary for 50% of the stimulated population ($T_{1/2}$) to move from one cell cycle phase to the next was computed from this data and gives a measure of how the stimulated population moves through the cell cycle. These values are reported in Table 1. Reliable values for $T_{1/2}$ were obtained for all serum concentrations except for 1%. With this concentration of serum, the proportion of the population stimulated was so small that reliable estimates for the $T_{1/2}$ of the S to G_2+M transit could not be obtained. The values obtained for the other serum concentrations indicate that the concentration of serum used to stimulate the cultures had no effect on the time it took the stimulated population to move from G_1 to S,

or from S to G_2+M. These values were also used to calculate the length of S by subtracting $T^{S}_{1/2}$ from $T^{G_2}_{1/2}$ (see Table 1). Serum concentration had little effect on the length of S and the average value of 6.7 $\pm$ 0.2 hr reported in Table 1 for S compares favorably with that obtained by Macieira-Coelho et al. (1966).

To measure the effect of serum on the synchrony of the stimulated population, the width of the peaks in Figure 2 were calculated at $T_{1/2}$. These values represent the spread of a stimulated population in a particular cell cycle phase. When the peak widths are compared from populations stimulated with different concentrations of serum (Table 1), it is evident that serum does not affect the synchrony of the stimulated population. The average peak width of the population in G_2+M was not significantly different from that of the population in S, suggesting that there is no significant loss of synchrony as the populations move through S and into G_2+M.

Factors Influencing the Proportion of G_1 Cells Stimulated. By comparing the areas under the various curves in Figure 2, it was observed that as the serum concentration decreased, so did the proportion of the population stimulated. To examine this effect more thoroughly, stationary phase WI-38 cells were seeded at 1.81×10^4 cells/cm^2 in medium containing different concentrations of serum, and the proportion of the population stimulated to traverse the cell cycle was determined. The stimulated cells were trapped in mitosis by addition of colchicine (0.2 µg/ml), and after 36 hr the fraction of the population that had moved from G_1 to G_2+M was measured by FMF. Under these conditions, the fraction of the population stimulated increased as a function of serum concentration, reaching a maximum of 78% of the population stimulated with serum concentrations of 8% and above (Figure 3). When the stimulation experiment was repeated at a 4-fold higher cell density (7.25×10^4 cells/cm^2), the proportion

of the population in G_2+M after 36 hr reached a maximum of only 27% of the population stimulated at serum concentrations of 16% or greater. Above 20% serum, an apparent toxic effect was seen at both cell densities, resulting in a decrease in the amount of stimulation (data not presented).

DISCUSSION

The events leading to the onset of stationary phase in cultures of WI-38 human diploid fibroblasts are not well understood. Ryan et al. (1975) have shown that the cell density at which this transition occurs can be regulated, within limits, by the concentration of serum in the growth medium. Presumably, the action of serum is to supply some growth promoting activity (Holley and Kiernan, '71; Temin, '68; Houck and Cheng, '73).

Although it has been demonstrated that the majority of stationary phase cells are in G_1 (Wiebel and Baserga, '69; Macieira-Coelho and Berumen, '73; Todaro et al., '65; Griffiths, '72; Temin, '71), little is known about how the population is distributed in G_1. For example, it is not known whether the population is blocked at one point in G_1 (G_0) or spread uniformly throughout the phase. The model proposed by Smith and Martin (1973) suggests that cells can exist in two states, a "B-phase" in which the cell is committed to growth and cell dividion and an "A-state" corresponding to G_0 which is indeterminate. Factors that would affect the growth of a population would primarily affect the ratio of cells in the two states. According to this model a population in the stationary phase contains mostly cells that are in the "A-state" and stimulation would simply involve increasing the probability of transition from the "A-state" to the "B-phase". Smith and Martin (1974) argue that the concentration of serum in the medium used to stimulate

stationary phase cells determines the number of cells making the transition from the "A-state" to the "B-phase" per unit time. We observed that the concentration of serum had no effect on the time it took the stimulated population to reach or G_2+M, nor on the synchrony of the released population. Thus, within the limits of the concentrations tested, the effect of serum on the growth of WI-38 cells in culture cannot be explained on the basis of an effect on the rate of transition of cells from a non-cycling to a cycling state.

The level of serum in the medium does affect the proportion of the population of WI-38 cells that can be released from G_1. This sensitivity to serum stimulation is modulated by the cell density at which the cultures are stimulated. For example, as seen in Figure 3, at a density of 1.81×10^4 cells/cm^2, the maximum percentage of the population stimulated was 78% whereas, at a density of 7.25×10^4 cells/cm^2, the maximum percentage decreased such that only 27% of the population was stimulated.

The cell density had no effect on the time required for the stimulated population to enter S or G_2+M (unpublished data). However, because of the small proportion of the population stimulated by serum at high cell densities, it was not possible to accurately measure the effect of serum concentration on the synchrony of those populations. The decreased stimulation of cell cycle transit seen at high density is not due simply to a decrease in the ratio of serum concentration to cell density. If the proportions of the population stimulated are compared at equivalent ratios of serum concentration to cell density, the populations at lower density are stimulated to a higher extent. For example, as seen in Figure 2 when a population at 1.81×10^4 cells/cm^2 was stimulated with medium containing 1% serum, 21.4% of the population moved from G_1 to G_2+M whereas, at an equivalent serum to cell density ratio but a four-fold higher cell density (7.25×10^4 cells/cm^2) and serum concentration, only 8.7% of the

population was stimulated. Similar results are obtained if other ratios of serum concentration to cell density are compared. This observation indicates that cell density by itself can regulate the proportion of the population capable of being stimulated to traverse the cell cycle.

In summary, our experiments indicate that the density of cultures of WI-38 cells determines the proportion of the population sensitive to serum stimulation of cell cycle transit. The concentration of serum in the medium can, within limits associated with cell density, regulate the proportion of the population stimulated. However, the synchrony of the released population is not affected by serum concentration, suggesting that WI-38 cells do not regulate cell cycle transit in a manner consistent with the model proposed by Smith and Martin (1974) for regulation of cell cycle transit.

Our data is more consistent with the model proposed by Prescott in which stationary phase cells are distributed throughout G_1. Stimulation of cells with serum involves a lag time not affected by serum concentration, and causes a fraction of the population to move into S with a distribution which reflects the asynchrony of the population in stationary phase.

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Table 1. Kinetic Parameters of Serum Stimulation of WI-38 Cells

	$G_1 \rightarrow S$		$S \rightarrow G_2+M$		
% Serum	$T^S_{1/2}$ (hr)‡	Peak* width (hr)	$T^{G_2}_{1/2}$	Peak* width (hr)	$T^S_{1/2} - T^{G_2}_{1/2}$
1	24.0	5.6	--	--	--
2	23.0	6.4	30.0	6.0	7.0
4	22.0	6.4	28.5	7.6	6.5
10	22.0	6.0	28.5	6.8	6.5
		Avg. 6.1 ± 0.3		Avg. 6.8 ± 0.8	Avg. 6.7 ± 0.2

‡ $T_{1/2}$ refers to the time it takes 50% of the stimulated populstion to move from one cell cycle phase to the next. The superscripts refer to the phase the population is entering.

* Width of the peak measured at $T_{1/2}$.

FIGURE CAPTIONS

Fig. 1. DNA Histograms of Serum Stimulated WI-38.

At 0 time WI-38 cells were seeded at 1.81×10^4 cells/cm^2 in medium containing the indicated serum concentrations. Samples were withdrawn at the indicated times and prepared for FMF. From 30,000 to 100,000 cells were analyzed for each sample.

Fig. 2. Release of WI-38 Cells from Stationary Phase.

The DNA histograms from Fig. 1 were analyzed as described in MATERIALS AND METHODS. The symbols refer to the serum concentration: (o), 1%; (●), 2%; (□), 4%; (■), 10%.

Fig. 3. Effect of serum on the stimulation of cell cycle transit.

At 0 time WI-38 cells were seeded at 1.81×10^4 cells/cm^2 (o) and 7.25×10^4 cells/cm^2 (●) in medium containing different concentrations of serum. Colchicine (0.2 µg/ml) was added at 17 hr and the samples were harvested at 36 hr and analyzed by FMF. From 30,000 to 100,000 cells were analyzed for each sample.

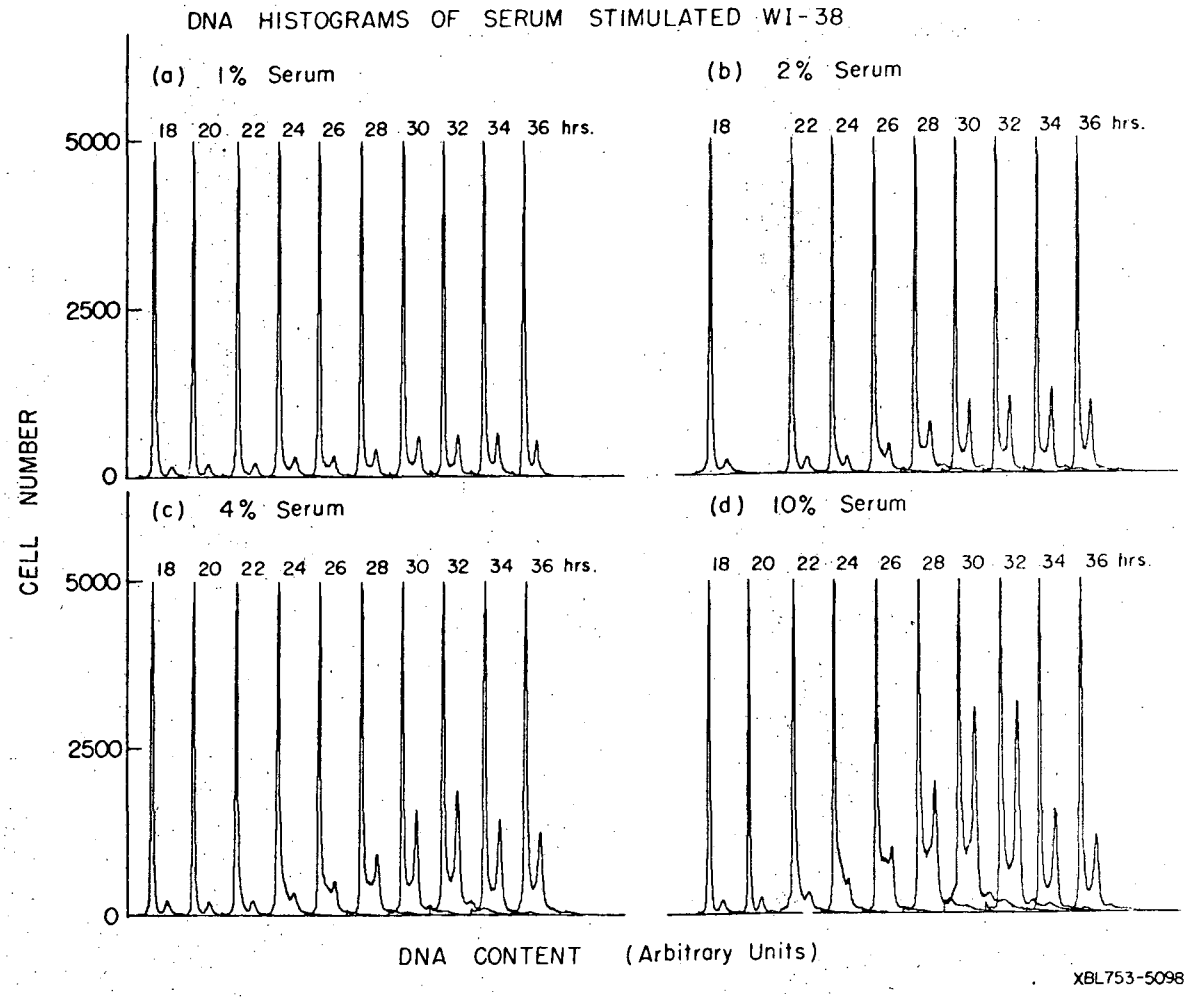
DNA HISTOGRAMS OF SERUM STIMULATED WI-38
(a) 1% Serum
(b) 2% Serum
(c) 4% Serum
(d) 10% Serum
18 20 22 24 26 28 30 32 34 36 hrs.
18 22 24 26 28 30 32 34 36 hrs.
5000
2500
0
CELL NUMBER
DNA CONTENT (Arbitrary Units)
XBL753-5098

Fig. 1

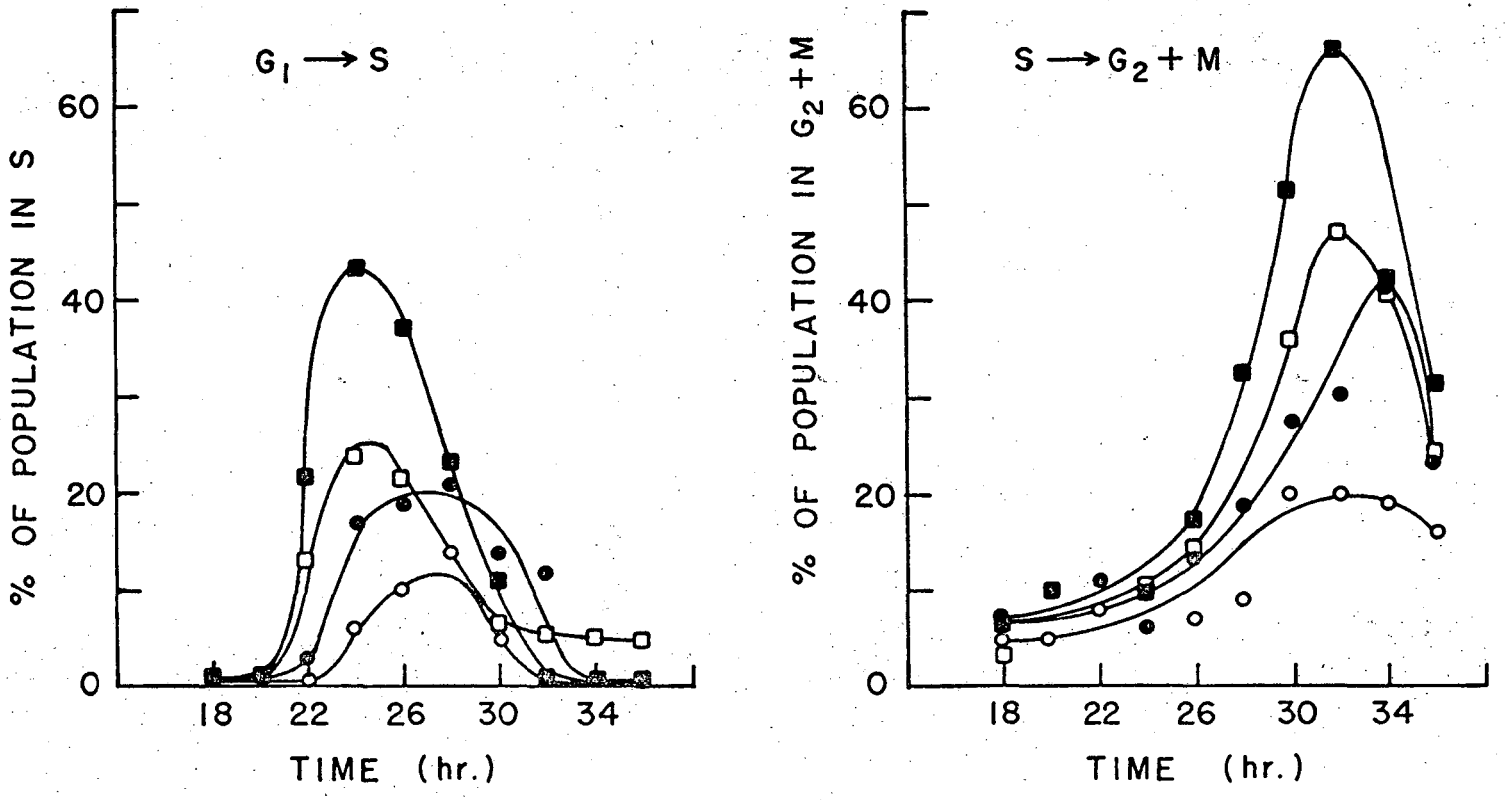

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Fig. 2

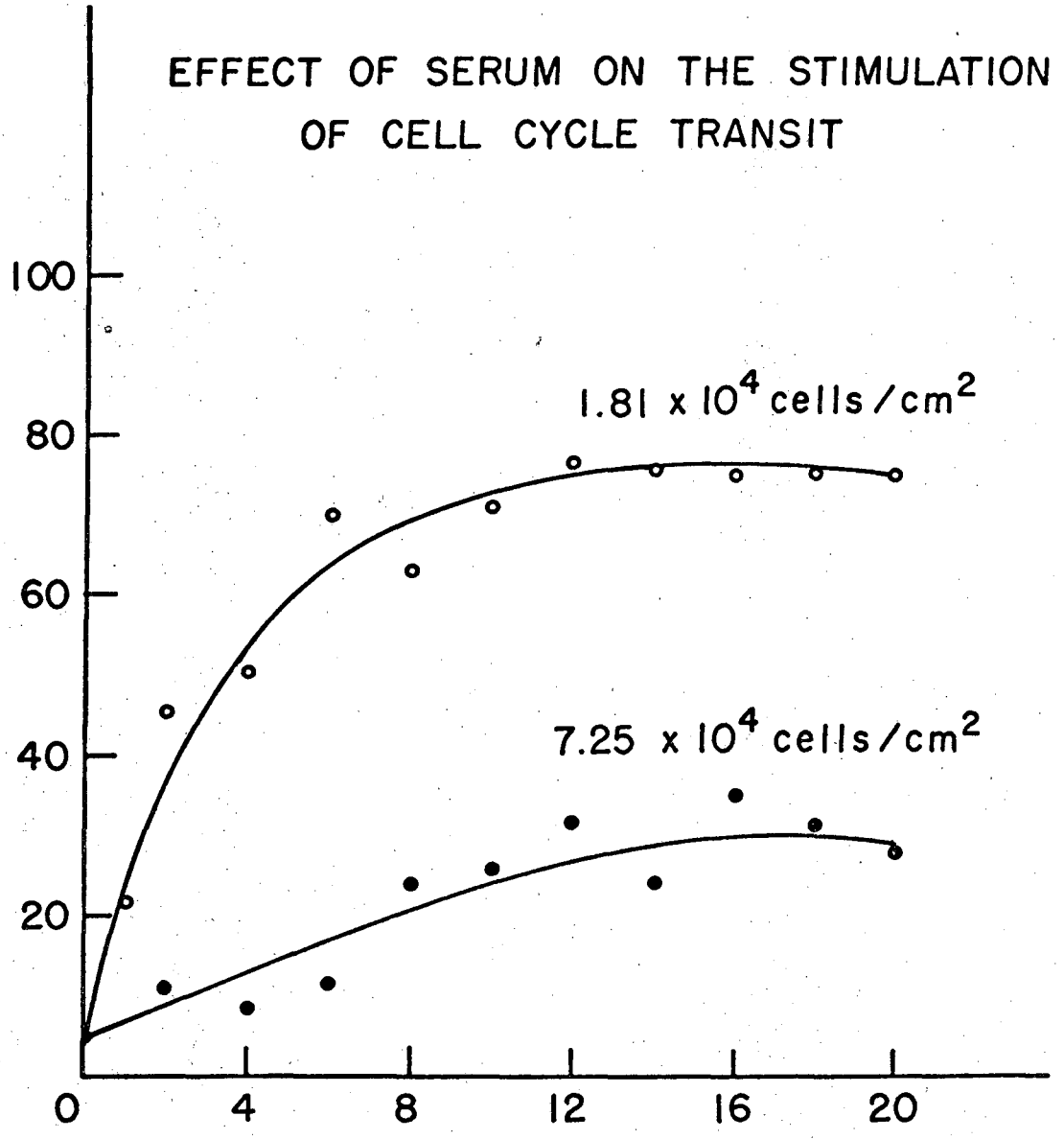
EFFECT OF SERUM ON THE STIMULATION
OF CELL CYCLE TRANSIT
100
80
60
40
20
0
4
8
12
16
20
1.81 x 10^4 cells/cm^2
7.25 x 10^4 cells/cm^2
XBL753-5097

Fig. 3

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