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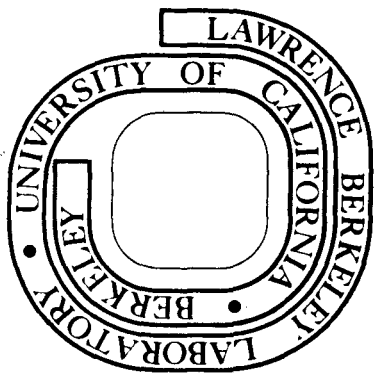

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STIMULATION OF WI-38 CELL CYCLE TRANSIT - Effect of Serum Concentration and Cell Density

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Running Title: Stimulation of WI-38 Cell Cycle Transit

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SUMMARY

Flow microfluorimetry has been used to characterize the effects of serum concentration and cell density on the initiation of cell cycle transit of stationary phase human diploid fibroblasts (strain WI-38). The concentration of serum in the medium had no effect on the kinetics of release of cells from stationary phase (G_0) at any cell density tested. However, as the serum concentration in the medium increased, the proportion of the cell population released and the synchrony of the released population increased. Cell density also affected the proportion stimulated: 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. The effect of cell density on the serum response is not simply the result of changing the ratio of serum concentration to cell density. It appears to reflect a true modulation by cell density of cell sensitivity to stimulation. These results are consistent with the interpretation that the primary action of serum is to determine the rate of transition from a non-cycling G_0 state to a cycling G_1 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 population are in the G_1 phase of the cell cycle [1, 2]. Prescott [3] has proposed that as the culture enters stationary phase the length of G_1 expands such that, even though cells may still be traversing the cell cycle, the majority of their transit time is spent in G_1. Smith and Martin [4], on the other hand, have proposed that the actual time spent traversing G_1 remains constant, but that the probability of G_1 transit in stationary phase is very low. The Smith and Martin model supposes that cells in G_1 can exist in two states. In one state, the cell is committed to replicate its DNA and divide. In the second state, the cell is not progressing towards DNA replication and mitosis. There have been a number of reports that cells in stationary phase are in this non-cycling or G_0 state [5-9].

Whatever the nature of the stationary phase population, Wiebel and Baserga [1] demonstrated that the addition of fresh serum to human diploid fibroblasts would stimulate some of the cells to leave G_1 (or G_0) and traverse the cell cycle. 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. With this idea in mind and in order to characterize more fully the stationary population, we have studied the effect of serum and cell density on the stimulation of cell cycle traverse. Our experiments indicate that the concentration of serum in the medium and the cell density interact to determine the fraction of the population of WI-38 cells that can be stimulated to traverse the cell cycle.

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 [10] containing 10% newborn calf serum (GICBO, Grand Island, N.Y.). The cells were transferred when they reached their saturation density 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.

Autoradiography

To determine the percentage of the population synthesizing DNA at each time point, the cultures were pulse labeled for 15 min with 1 μCi/ml ^{3}H-thymidine (20.1 C/mM; New England Nuclear, Boston, Mass.). At the end of the pulse the cells were washed on the dishes 2 times in isotonic Tris buffer, swollen with 0.075 M KCl and fixed with methanol-acetic acid (3:1) [11]. After air drying the dishes were coated with Kodak Nuclear Track Emulsion NTB-2 (Eastman Kodak, Rochester, N.Y.), exposed in the dark at room temperature for 14 days, developed, and fixed. The cells were stained with crystal violet and the percentage of cells with labeled nuclei was determined by counting a total of 2000 cells for each time point.

Flow Microfluorimetry

The amount of DNA per individual cell was quantified by staining the cells with acriflavine [12] and measuring the amount of fluorescence per cell by flow microfluorimetry (FMF). 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 [13]. 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.) [14]. The proportion of the population in G_1, S, and G_2 plus M (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 [15]. 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 shape 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 % of the population in S by FMF agreed to within 10% of values obtained by ^{3}H-thymidine incorporation and autoradiography.

RESULTS

Stimulation of Cell Cycle Transit

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 [1,16-19]. Wiebel and Baserga [1] 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. In

their experiments it was observed that the proportion of the population stimulated could be increased to 27% by changing the medium as well as adding fresh serum. In order to study the effect of serum concentration and cell density on the proportion of the population stimulated to traverse the cell cycle we first repeated the experiments of Wiebel and Baserga to establish the proper conditions for maximum stimulation of cell cycle traverse. The cells used in these experiments were grown to their saturation density (1.3×10^5 cells/cm^2) in medium containing 10% serum. Previous experiments had indicated that under these conditions the cells reach stationary phase in 7 days [20]. In stationary phase approximately 96% of the population has a DNA content equivalent to G_1 cells and 4% has the DNA content of G_2/M cells [2, 20]. The medium used in these stimulations was either fresh medium, or medium that had supported the growth of WI-38 cells to stationary phase ("old medium"). In all cases, colchicine (final concentration 0.2 ug/ml) was added 17 hr after stimulation to trap cycling cells in mitosis. The colchicine was added at 17 hr to minimize cytotoxic effects [21] and to preceed the entrance of cells into G_2/M. The cells were harvested 40 hr after stimulation and the samples were analyzed by FMF. By 40 hr all the cells in the population that were stimulated to traverse the cell cycle had left S and were in G_2/M. The resulting DNA histograms contained two discrete peaks, one peak representing cells having a DNA content equivalent to G_1 cells and the second representing cells having a DNA content equivalent to G_2/M cells.

The data in Table 1, section A confirm the findings of Wiebel and Baserga [1] and suggest that when the cell layer is undisturbed serum addition can stimulate a small fraction of the population to traverse the cell cycle, and that this proportion can be increased by adding fresh medium as well as serum. When no serum was added to the fresh medium no stimulation of cell cycle transit was observed. It should be noted that the unstimulated population treated with colchicine, as described above, gave the same

population distribution as untreated stationary phase cells [2, 20]. The lack of an effect of colchicine on the size of the G_2/M population implies that the stationary phase population is truly arrested in G_0 and is not slowly traversing the cell cycle. In these experiments, the designation G_1 has been used to include all cells having a DNA content of post-mitotic but pre-DNA replicating cells. Since the FMF technique only measures DNA content, it cannot distinguish cells that are progressing towards the initiation of DNA synthesis from cells that are arrested (G_0 cells). According to this definition, G_0 cells are in a subclass of G_1 and, using FMF, cannot be identified.

When WI-38 cells were trypsinized and reseeded onto new dishes, the degree of stimulation with a particular treatment depended upon the density at which the cells were seeded. If the seeding density was high (7.25×10^4 cells/cm^2) such that every cell appeared by microscopic examination to be in contact on all sides with other cells, then the degree of stimulation (Table 1, section B) was almost identical to that seen with undisturbed cell layers. The similarity in these two groups of data suggests that the trypsinization and reseeding procedures by themselves do not stimulate cell cycle traverse. At a density of 1.81×10^4 cells/cm^2 the cell-cell contact was considerably reduced and the amount of stimulation seen with each treatment was significantly increased (Table 1, section C).

Effect of Serum Concentration on G_1 to G_2/M Transit

Having found that the maximum stimulation of G_1 to G_2/M transit occurred at low cell density with fresh medium and serum, the effect of serum concentration on the time necessary for the stimulated population to reach S was investigated. FMF is particularly suited for these measurements because of the ability of the technique to determine whether individual cells of a population are in G_1, S, or G_2/M by measuring the DNA content per cell. By taking FMF points at various times after stimulation, a dynamic picture of how the population

moves out of G_1 through S and into G_2/M can be obtained. In addition, FMF has the advantage that it can easily analyze large numbers of cells.

Stationary phase WI-38 cells at low cell density which were stimulated with fresh medium and serum, began to initiate DNA synthesis between 18 and 22 hr after stimulation. As reported by Augenlicht and Baserga [9], the exact time of initiation depended to some extent on the prior history of the G_1 population (unpublished observation). In the experiment described in Figure 1, some cells began to synthesize DNA by 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 the data in Figure 1 and gives a measure of how the stimulated population moves into S and G_2/M. These values are reported in Table 2 and indicate that serum has no effect on the time it takes 50% of the stimulated population to move from G_1 to S or from S to G_2/M. From these measurements the length of S was calculated by subtracting $T^{S}_{1/2}$ from $T^{G_2}_{1/2}$ (see Table 2). The value of 5.0 $\pm$ 0.2 hr reported in Table 2 for S compares favorably with that obtained by Macieira-Coelho et al. [22].

One effect of serum on the stimulation of cell cycle transit was on the synchrony of the stimulated population. Synchrony is defined here as the rate at which the stimulated portion of the population moves across the boundary between one phase of the cell cycle and the next. These rates were determined by measuring the slopes of the curves in Figure 1 at $T_{1/2}$ and are reported in Table 2 as percentages of the stimulated portion of the population crossing the boundary per hour. When the serum concentration in the medium was increased 10-fold the rate of entry from G_1 to S was increased by a factor of 4. This effect was also seen on the rate of entry from S to G_2/M indicating that the population does not lose synchrony as it passes through S at any of the serum concentrations tested.

Factors Influencing the Proportion of G_1 Cells Stimulated

A second effect of serum concentration on cell cycle transit is on the proportion of the population stimulated to leave G_1. By comparing the areas under the various curves in Figure 1 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 x 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, as described above, 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 concentr tions of 8% and above (Figure 2). When the stimulation experiment was repeated at a 4-fold higher cell density (7.25 x 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 (date not presented).

The cell density had no effect on the time required for the stimulated population to enter S or G_2/M. 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 (Figure 2), the populations at lower density are always stimulated to a higher extent. For example, a population at 1.81×10^4 cells/cm^2 stimulated with medium containing 1% serum stimulated 21.4% of the population to move from G_1 to G_2/M. Whereas, at a four fold higher cell density (7.25×10^4 cells/cm^2) and with medium containing 4% serum 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.

DISCUSSION

The events leading to the onset of stationary phase in cultures of WI-38 human diploid fibroblasts are not well understood. In another report, we 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 [20]. Presumably, the action of serum is to supply some growth promoting activity [23-25]. Serum is also necessary for the stimulation of stationary phase WI-38 cells to traverse the cell cycle [1]. As seen in Table 1, the absence of serum in the medium resulted in little if any stimulation regardless of the cell density.

The data in Table 1 also suggest that the growth of a culture of WI-38 cells reduces the ability of the medium to stimulate cell cycle traverse. Under none of the conditions tested was the old medium as stimulatory as fresh medium plus serum. This loss of activity cannot be entirely attributed to the serum components in the medium since the addition of fresh serum to old medium (final concentration of 20%) did not completely replenish the lost activity. It appears that the growth of cells in medium either depletes the serum and non-serum components of the medium or adds toxic materials.

The amount of stimulatory activity measured in the old medium was dependent on the density of the cells being stimulated. As seen in Table 1 section C, at a density of 1.81×10^4 cells/cm^2, 28% of the population was stimulated by old medium to move from G_1 to G_2/M. At a density four times greater (7.25×10^4 cells/cm^2), only 4% of the population was stimulated. To estimate the activity remaining in old medium, we used the dose-response relationship at 1.81×10^4 cells/cm^2 shown in Figure 2. At this cell density 28% of the population was released from G_1 by old medium which was originally 10% in fresh serum. This amount of stimulation corresponds to that obtained with fresh medium containing 3% serum (Figure 2). From this analysis, the growth of WI-38 cells in medium reduced its stimulatory activity by at least 2/3.

These observations are consistent with the interpretation that, as a culture of WI-38 cells grows, increasing cell density results in a lower sensitivity to the growth promoting activity of medium as well as a decreased total activity in the medium. Stationary phase would then result from both a reduction of the amount of growth promoting activity in the medium and a density dependent decrease in the sensitivity of the population to that activity.

Although it has been demonstrated that the majority of stationary phase cells are in G_1 [1,2,16-20], 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 [4] suggests that cells can exist in two states, a "B-phase" in which the cell is committed to growth and cell division 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". Our data is consistent with this model if it is assumed that the concentration of serum in the medium used to stimulate stationary phase WI-38 cells determines the rate of transition from the "A-state" to the "B-phase". We observed that the concentration of serum had no effect on the time it took the stimulated population to reach S or G_2/M, but had a considerable effect 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 length of individual cell cycle phases, but is more consistent with an effect on the rate of transition of cells from a non-cycling to a cycling state.

The level of serum in the medium not only affects the synchrony of the released population, but also affects the proportion of the population 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 2, 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.

In summary, our experiments indicate that the density of cultures of WI-38 cells determines the proportion of the population sensitive to medium stimulation of cell cycle transit. The concentration of serum in the medium can, within limits set by cell density, regulate the proportion of the population stimulated. Serum concentration has the additional effect of modulating the synchrony of the released population in a manner consistent with the model proposed by Smith and Martin [4] for regulation of cell cycle transit.

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TABLE 1

STIMULATION OF CELL CYCLE TRANSIT

TREATMENT	% STIMULATION
A. CELL LAYER UNDISTURBED	
1. NO STIMULANT ADDED	4.0
2. FRESH MEDIUM MINUS SERUM	4.0
3. OLD MEDIUM* + SERUM TO 20%	9.6
4. FRESH MEDIUM 20% IN SERUM	35.9
B. CELL LAYER TRANSFERRED AND SEEDED AT 7.25×10^4 CELLS/CM^2	
1. OLD MEDIUM*	4.0
2. FRESH MEDIUM MINUS SERUM	4.0
3. OLD MEDIUM* + SERUM TO 20%	15.0
4. FRESH MEDIUM 20% IN SERUM	35.6
C. CELL LAYER TRANSFERRED AND SEEDED AT 1.81×10^4 CELLS/CM^2	
1. OLD MEDIUM*	28.0
2. FRESH MEDIUM MINUS SERUM	7.0
3. OLD MEDIUM* + SERUM TO 20%	51.9
4. FRESH MEDIUM 20% IN SERUM	71.5

*Old medium refers to medium 10% in serum that has supported the growth of WI-38 cells to saturation density.

TABLE 2. KINETIC PARAMETERS OF SERUM STIMULATION OF WI-38 CELLS

	$G_1 \rightarrow S$		$S \rightarrow G_2/M$		Length of S
% Serum	$T^S_{1/2}$ (hr)$^\$$	$S_{T_{1/2}}$ (%/hr)*	$T^{G_2}_{1/2}$ (hr)$^\$$	$S_{T_{1/2}}$ (%/hr)*	$T^{G_2}_{1/2} - T^S_{1/2}$ (hr)
1	23.3	2.03	28.6	1.70	5.3
2	24.6	3.90	29.4	3.60	4.8
4	22.9	5.23	28.1	5.15	5.2
10	22.9	7.80	27.7	7.10	4.8
					Avg. 5.0 ± 0.2

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

* $S_{T_{1/2}}$ refers to the slope of each curve in Figure 1 at $T_{1/2}$, and is given in % of the population per hour.

FIGURE CAPTIONS

Fig. 1. Abscissa: time after stimulation (hours); ordinate: (A) % of the population in S + G_2/M; and (B) % of the population in G_2/M. The cells were stimulated by medium containing: (●), 1%; (○), 2%; (□), 4%; (△), 10% serum.

Stimulation of stationary phase WI-38 cells by serum. At 0 time WI-38 cells were seeded at 1.81×10^4 cells/cm^2 in medium containing the above serum concentrations. Samples were withdrawn at the indicated times and prepared for FMF. The DNA histograms were analyzed as described in MATERIALS AND METHODS. From 30,000 to 100,000 cells were analyzed for each sample.

Fig. 2. Abscissa: % serum; ordinate: % of the population stimulated.

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 (○) and 7.25×10^4 cells/cm^2 (●) in medium containing different concentrations of serum. Colchicine (0.2 ug/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.

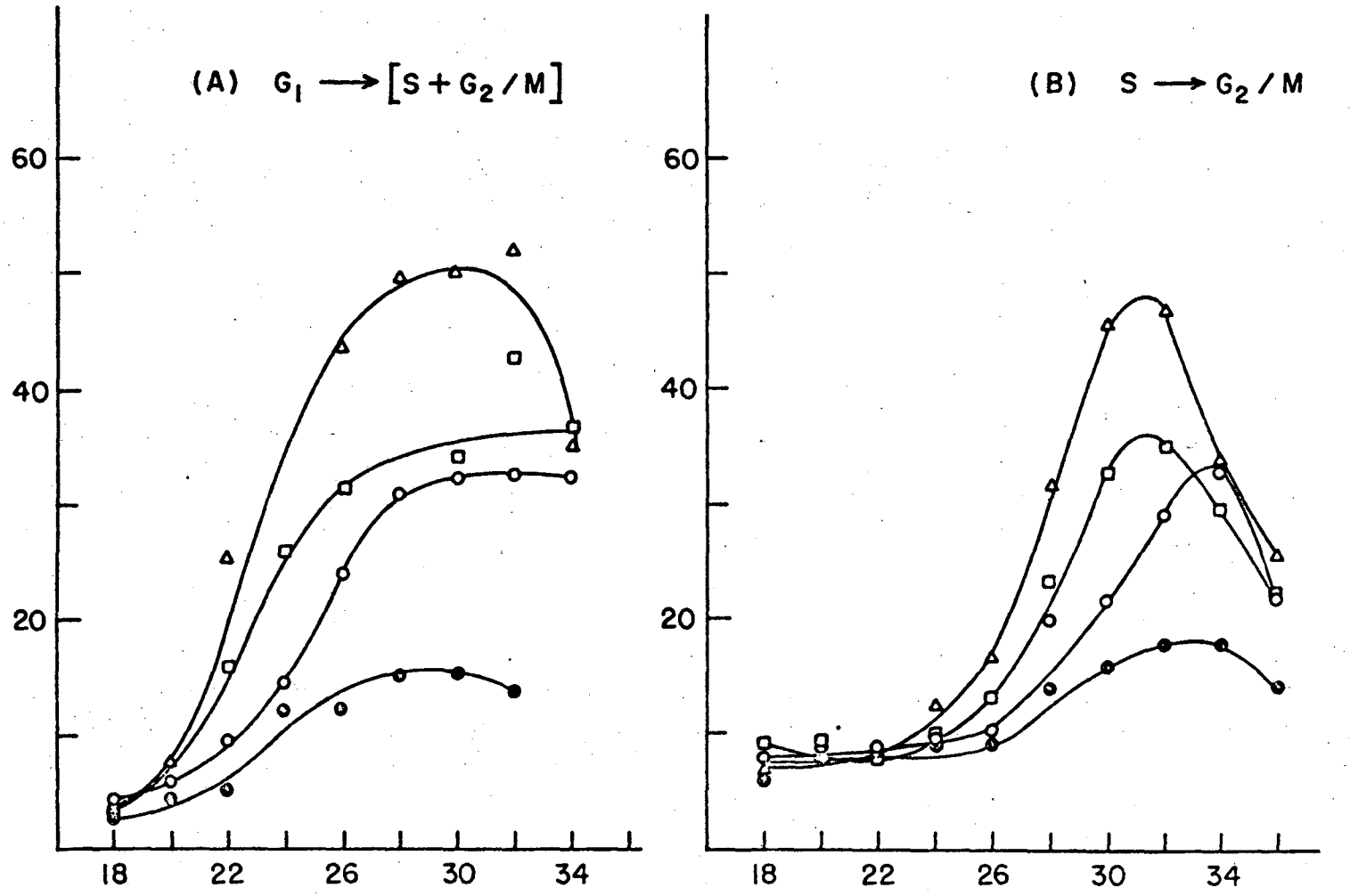

Fig. 1

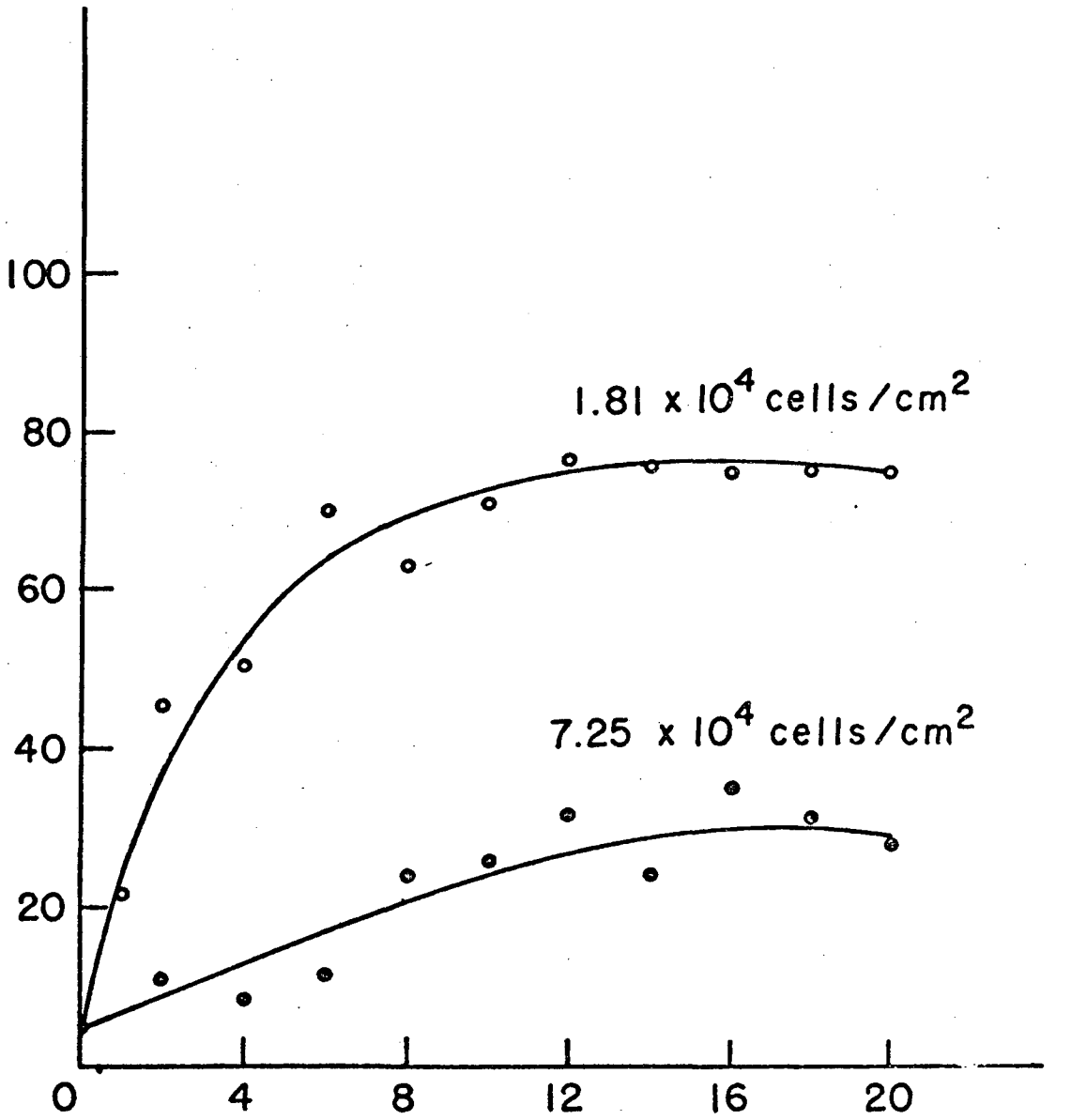

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Fig. 2

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