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MASTER

TITLE:

FLOW MICROFLUOROMETRIC AND SPECTROPHOTOFLUOROMETRIC ANALYSIS OF DNA STAINING IN MAMMALIAN CELLS

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FLOW MICROFLUOROMETRIC AND SPECTROPHOTOFLUOROMETRIC ANALYSIS OF DNA STAINING IN MAMMALIAN CELLS

Running title: DNA Staining in Mammalian Cells

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ABSTRACT

The effects of various fixative agents, pH, ionic strength, stain concentration, and magnesium concentration on DNA staining with the antibiotics olivomycin, chromomycin A3, and mithramycin were examined with DNA in solution and in mammalian cells. Ethanol-fixed Chinese hamster cell populations (line CHO) stained with mithramycin and analyzed by flow microfluorometry (FMF) provided DNA distribution patterns with a high degree of resolution. Glutaraldehyde-fixed cells exhibited about one-half the fluorescence intensity of ethanol-fixed cells and yielded DNA distributions with G_1 peaks having coefficients of variation (CVs) one and one-half times as large as for ethanol-fixed cells. However, the percentages of cells in G_1, S, and G_2 + M were quite comparable. DNA distributions obtained for formalin-fixed cells were unacceptable for computer analysis.

Similar values were obtained by FMF for the fluorescence intensities of CHO cells stained over a pH range of 5.0 to 9.0. Below pH 5.0, there was a large decrease in fluorescence intensity and, from pH 9.0 to 10.0, a significant increase in intensity of stained cells. However, values for the distribution of cells in various phases of the cell cycle obtained by computer analysis remained essentially the same over the pH range 3.0 to 10.0. The fluorescence intensity of cells stained in mithramycin solutions containing 0 to 1.75 NaCl increased gradually up to about 1 M NaCl. The intensity of cells stained in 1 M NaCl was one and one-half times greater than cells stained in the absence of NaCl. However, the CV of the G_1 peaks changed only slightly from 0.15 to 1 M NaCl. Cells stained in 1.75 M NaCl yielded poor DNA distributions, with a large CV compared to cells stained in 0.15 M NaCl.

Cells stained with increasing concentrations of mithramycin up to 250 µg/ml showed a sharp increase in fluorescence intensity, judged by FMF, up to 50 µg/ml

and then a more gradual increase up to 100 µg/ml. The relative intensity of stained cells was changed very little at dye concentrations above 100 µg/ml. DNA distributions for cells stained at all concentrations yielded virtually the same percent of cells in the various phases of the cell cycle. Cells stained with mithramycin solutions containing 0 to 70 mM $MgCl_2$ showed a gradual increase in fluorescence intensity as a function of increasing $MgCl_2$ concentration. At a concentration of 70 mM $MgCl_2$, cells exhibited a 23% increase in intensity above cells stained in 15 mM $MgCl_2$; however, no improvement in resolution of the DNA distribution patterns was noted. Cell staining in the absence of $MgCl_2$ was extremely poor. Other metal ions tested included the chlorides (15 mM) of cadmium, calcium, cobalt, copper, lead, manganese, mercury, and zinc. None of these divalent metal ions proved superior to magnesium, and only cells stained in the presence of zinc, lead, and cadmium yielded bimodal DNA distributions. Of these, only zinc could be recommended as an alternative for magnesium; however, cells stained in 15 mM $ZnCl_2$ yielded only about half the intensity of cells stained in 15 mM $MgCl_2$.

Spectrophotofluorometric analysis of olivomycin, chromomycin A3, and mithramycin yielded spectral patterns which were quite similar but not identical in shape. Addition of magnesium to the solutions induced a spectral shift in the emission peaks toward the longer wavelengths by about 25 nm and also produced a 10-fold decrease in the fluorescence intensity of the dye. Addition of DNA in solution to the mithramycin-magnesium complex increased the fluorescence 10 times but did not produce any major spectral shift. Increasing the ionic strength of the mithramycin solution did not change the spectral patterns of either the mithramycin-magnesium or mithramycin-magnesium-DNA complex, nor did it produce a change in fluorescence intensity of these

complexes. These results and those obtained by FMF analysis indicate that the increase in fluorescence intensity of stained cells as a function of increasing ionic strength is due to changes in chromatin structure, providing a greater number of binding sites for the dye complex.

INTRODUCTION

Cell staining techniques currently employed for single-cell analysis of DNA in flow systems have been developed primarily for use in cell-cycle analysis studies where the fractions of cells in G_1, S, and G_2 + M can be obtained rapidly from the DNA distribution patterns. For such studies, the staining technique is specifically designed to provide accuracy in quantitation of cellular DNA content, with the desire for obtaining a high degree of resolution in the DNA profiles. Although these types of analyses have been extremely fruitful in numerous studies involving cell-cycle kinetics (1,2), determinations of ploidy levels in cells (3,4), and even chromosome analysis (5,6), the staining methods employed have provided little information with regard to revealing those changes in chromatin structure which are known, from other biochemical studies, to occur under various experimental conditions. It is hoped that staining protocols could be devised to provide analytical determinations for assessment of changes in chromatin structure which would be useful for monitoring the functional activity of cells.

Three antitumor antibiotics have been examined recently and found useful for staining and analyzing DNA in flow systems (7). These compounds, mithramycin, chromomycin A3, and olivomycin, are highly specific for DNA but not for RNA. Furthermore, cell staining with these agents does not necessitate manipulations which would adversely affect chromatin structure. The studies described in this report were performed to determine the effects of various fixative agents, pH, ionic strength, stain concentration, and magnesium concentration on the DNA-dye complex formed with mithramycin, chromomycin A3, and olivomycin and DNA in solution and in mammalian cells. Efforts have been made not only to optimize the conditions for staining DNA but also to design approaches which would provide fluorometric assessment for changes in chromatin structure.

MATERIALS AND METHODS

Spectrophotofluorometric Assay

The excitation and emission spectra for mithramycin (Pfizer), chromomycin A3 (Calbiochem), and olivomycin (DCT/NCI) in (a) aqueous solution (100 µg/ml), (b) complexed with magnesium (15 mM $MgCl_2$), and (c) complexed with magnesium plus calf thymus DNA (50 µg/ml saline) were obtained using the automatic recording Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Springs, Md., USA). Illumination from a xenon tube was used for excitation, and a R466 (Hamamatsu Corporation) photomultiplier tube was used to monitor the fluorescence emission. Data presented represent the uncorrected spectra.

Cell Fixation

Chinese hamster cells (line CHO) maintained in suspension culture were fixed (a) in 70% ethanol as previously described (8), (b) with 1% glutaraldehyde in balanced salt solution (pH 7.4), or (c) in 4% formalin (pH 7.4) for at least 18 hr prior to staining.

Cells fixed in each of the above reagents were stained with an aqueous mithramycin solution (100 µg/ml) containing 15 mM $MgCl_2$ and 0.15 M NaCl. Ethanol-fixed cells were also stained with mithramycin solutions buffered in the pH range 3.0 to 10.0 (pH 3.0 to 6.0 acetic acid-acetate buffer; pH 7.0 to 8.0 Tris-HCl buffer; and pH 9.0 to 10.0 carbonate-bicarbonate buffer). Chromomycin A3 and olivomycin solutions (100 µg/ml in 0.15 M NaCl containing 15 mM $MgCl_2$) were also used to stain ethanol-fixed cells.

To determine the effects of ionic strength on cell staining, ethanol-fixed cells were stained in mithramycin solutions (100 µg/ml in 15 mM $MgCl_2$) containing

0 to 1.75 M NaCl (saline). Ethanol-fixed cells were also stained with mithramycin solutions containing 10 to 250 μg/ml of mithramycin. In other experiments, ethanol-fixed cells were stained with mithramycin solutions containing 0 to 1 M $MgCl_2$ or with one of the following chlorides (15 mM) of other divalent metal ions including cadmium, calcium, cobalt, copper, lead, manganese, mercury, and zinc.

Flow Analysis

Stained cells were analyzed as previously described in the Los Alamos flow microfluorometer (FMF II) (9,10) using the 457-nm line (150 mW) of an argon-ion laser. Except where indicated, all cells were analyzed in the dye solution. The coefficients of variation (CVs) and percentages of cells in G_1, S, and G_2 + M were derived from the DNA distribution patterns using the Dean and Jett (11) computer program.

RESULTS

Spectrophotofluorometric Analysis

The antibiotics mithramycin, chromomycin A3, and olivomycin are closely related compounds, differing only slightly in their chromophore structures and sugar components (12). These agents have a high specificity for helical DNA but not for RNA, and they all resemble actinomycin in their requirement for the 2-amino group of guanine for DNA-complex formation. However, unlike actinomycin, they do not cause uncoiling of helical DNA, suggesting that these compounds do not intercalate (13).

The excitation and emission spectra for the three antibiotics complexed with magnesium are quite similar (Fig. 1) whether free or bound to DNA in solutions containing $MgCl_2$. The major excitation and emission peaks for

unbound mithramycin and chromomycin are approximately 425 and 575 nm, respectively. In the absence of $MgCl_2$, the excitation peak (not shown) for mithramycin lies at 400 nm and the emission peak at 550 nm. Olivomycin (unbound) differs somewhat, having an excitation peak at about 418 nm and an emission peak at 550 nm. Upon complexing with DNA, the excitation peak shifts 5 to 10 nm toward the red region and the emission peak 5 to 10 nm toward the blue region of the spectrum. Similar spectral shifts are noted for chromomycin and mithramycin when bound to DNA. Addition of magnesium (15 mM) to aqueous mithramycin (3.3 µg/ml) quenches the fluorescence of the dye about 10-fold. However, addition of DNA (1 µg/ml final concentration) to the mithramycin-magnesium complex enhances the fluorescence about 10 times. Addition of DNA to mithramycin solutions lacking magnesium causes only a slight shift in the emission peak (5 nm) toward the blue region of the spectrum (maximum 545).

These data support the speculations of Ward et al. (12) and imply that there is first an interaction between the magnesium and the antibiotic, causing a spectral shift in emission of about 25 nm (red) and that it is the antibiotic-magnesium complex which then interacts with the DNA. This interesting observation would permit the spectrophotofluorometric detection of the dye-magnesium-DNA complex by monitoring spectral shifts as well as fluorescence enhancement.

Comparison of DNA Distributions

The DNA distributions for cells stained with olivomycin, chromomycin A3, and mithramycin (100 µg/ml in 0.15 M NaCl containing 15 mM $MgCl_2$) (Fig. 2) are all quite comparable, and computer analysis of the distributions yielded essentially equivalent results for the percentages of cells in G_1, S, and G_2 + M. Cells stained with olivomycin have a slightly greater fluorescence intensity than chromomycin-stained or mithramycin-stained cells; however, the CVs of the

G_1 peaks are similar (i.e., olivomycin, 4.3%; chromomycin, 4.7%; mithramycin, 4.5%).

Effects of Fixative Agents

In our original protocol (14), ethanol was added to the mithramycin staining solution in order that adequate perforation of the plasma membrane could be achieved to permit the large mithramycin molecule (molecular weight 1085) to penetrate the cell. Cells may also be fixed in 70% ethanol, as in the present study, and then stained in mithramycin solutions containing no alcohol. Other reagents such as glutaraldehyde and formalin have fixative properties for cell membranes which are more favorable than ethanol for some studies, particularly where Coulter volume measurements are to be performed in addition to DNA analysis.

The DNA distributions of ethanol-fixed and glutaraldehyde-fixed cells stained with mithramycin (Fig. 3) are not only similar in shape but, upon computer analysis, also yield essentially the same values for the percentages of cells in G_1, S, and G_2 + M. However, using FMF analysis methods, glutaraldehyde-fixed cells exhibit only about one-half the fluorescence intensity of ethanol-fixed cells and yield DNA distributions with G_1 peaks having a CV nearly one and one-half times larger than ethanol-fixed cells. The G_1 and G_2 + M peaks in DNA profiles of formalin-fixed cells are extremely broad, and these distributions have proved unacceptable for computer analysis. In addition, formalin-fixed cells stain only very lightly. The fluorescence intensity of glutaraldehyde-fixed and formalin-fixed cells did not increase significantly even after 24 hr in the stain solution based on FMF analysis.

It was found extremely important that ampoules of glutaraldehyde obtained under nitrogen (electron microscope-grade) be used to prepare the fixative solution

immediately prior to use. Lower grades of glutaraldehyde imparted an undesirable fluorescence to the cells and, in some instances, also caused significant cell clumping. This problem was not encountered when using the formalin or ethanol fixative reagents.

Effects of pH

Ethanol-fixed cells stained with mithramycin solutions in the pH range 3.0 to 10.0 and analyzed by FMF showed a 4-fold increase in fluorescence intensity between pH 3.0 to 5.0 (Fig. 4). From pH 5.0 to 9.0, there was little change in fluorescence; however, between pH 9.0 and 10.0, there was an additional 13% increase in fluorescence intensity. Above pH 10.0, reproducible results were difficult to obtain at this time since, on many occasions, precipitates formed in the staining solutions and/or cell clumping became excessive at elevated pH ranges. Efforts are being continued to alleviate these problems in order to examine the accessibility of dye-binding sites when the DNA is undergoing mild and gradual alkaline hydrolysis.

Effects of Ionic Strength

The fluorescence intensity of cells stained in solutions of mithramycin (Fig. 5), chromomycin, or olivomycin (100 µg/ml in 15 mM $MgCl_2$) containing 0 to 1.75 M NaCl increased significantly from 0 to 1 M MaCl but then changed only slightly from 1 to 1.75 M NaCl. The data for olivomycin and chromomycin are quite similar to that obtained for mithramycin shown in Fig. 5 except that the increase in fluorescence intensity of cells determined by FMF between the range of 0.15 M to 1 M NaCl was about 32% and 51%, respectively, following chromomycin and olivomycin staining, compared to about 40% following mithramycin staining.

Cells treated for 1 hr in 1 M NaCl prior to staining with mithramycin in 0.15 M NaCl showed no increase in fluorescence intensity over untreated cells; however, cells initially stained in mithramycin solutions containing 1 M NaCl, then restained with mithramycin in 0.15 M NaCl, and analyzed by FMF retained the fluorescence intensity observed following staining in 1 M NaCl. These results indicate that irreversible dye-DNA complexes form at elevated ionic strength and that the effects of ionic strength alone on the DNA are only transient and produce no permanent observable changes in chromatin structure or stainability of the DNA in the absence of the dye.

Spectral analysis of the mithramycin-magnesium complex in solutions over a range of 0 to 1.75 M NaCl revealed no significant change in fluorescence intensity of the antibiotic as a function of increasing saline concentration. Addition of calf thymus DNA to these solutions produced only the fluorescence enhancement noted in Fig. 1; however, there was no significant difference in fluorescence intensity of the solution lacking NaCl, compared to the 0.75 M NaCl solution. These results indicate that, although changes in ionic strength do not affect the intensity of free mithramycin or mithramycin bound to DNA in solution, such changes do exert a pronounced effect on chromatin structure in cells which is observable only in the presence of mithramycin.

Effects of Stain Concentration

Cells stained for at least 1 hr with varying concentrations of mithramycin (data not shown) up to 250 µg/ml in 15 mM $MgCl_2$ and 0.15 M NaCl showed a sharp increase in fluorescence intensity, judged by FMF, up to 50 µg/ml and then a more gradual increase up to 100 µg/ml. The relative intensity of stained cells changed very little above 100 µg/ml. DNA distributions for cell populations stained at the various mithramycin concentrations yielded virtually the same

results for the percentages of cells in the various phases of the cell cycle; however, the CVs of the G_1 peaks were somewhat greater at stain concentrations below 50 μg/ml.

Effects of Magnesium Concentration and Other Metal Ions

Cells stained with mithramycin solutions containing 0 to 70 mM $MgCl_2$ showed a gradual increase in fluorescence intensity as a function of increasing $MgCl_2$ concentration (Fig. 6). At a $MgCl_2$ concentration of 70 mM, cells exhibited a 23% increase in intensity above cells stained in 15 mM $MgCl_2$; however, no improvement in resolution of the DNA distribution patterns was noted. Cell staining in the absence of $MgCl_2$ was extremely poor. Other metal ions tested included the chlorides (15 mM) of cadmium, calcium, cobalt, copper, lead, manganese, mercury, and zinc. None of these divalent metal ions proved superior to magnesium, and only cells stained in the presence of zinc, lead, and cadmium yielded bimodal DNA distributions. Of these, only zinc could be recommended as an alternative for magnesium; however, cells stained in 15 mM $ZnCl_2$ yielded only about half the intensity of cells stained in 15 mM $MgCl_2$.

The fluorescence intensity of CHO cells stained with mithramycin solutions containing no magnesium was decreased by 90% over control cells. However, results for the cell-cycle distribution of cells were quite comparable, irrespective of the presence of magnesium in the stain solution. The CVs of the G_1 peaks were about 2.5 times larger for cell populations stained in the absence of $MgCl_2$. Cells pretreated with 10 mM EDTA prior to staining in solutions lacking magnesium likewise showed a 90% decrease in fluorescence intensity compared to controls.

Cells stained with mithramycin and then resuspended and analyzed in 0.15 M NaCl and 15 mM $MgCl_2$ showed a 60% decrease in fluorescence compared to cells

analyzed (FMF) in the stain solution. When cells were resuspended in only 0.15 M NaCl, there was a 75% decrease in fluorescence intensity compared to controls. The DNA distributions were quite comparable, irrespective of the resuspending solutions used for analysis; however, the G_1 peaks of cell populations analyzed in solutions lacking mithramycin were about 2 to 3 times larger than for control cells.

These results indicate that magnesium does enhance and improve mithramycin staining but that it is not an obligatory factor for binding of the dye to chromatin in cells. As noted in this study, nearly 7% of the fluorescence obtainable with control cells was obtained with staining in the absence of $MgCl_2$. However, cells stained in the presence of $MgCl_2$ did retain 40% of their fluorescence when resuspended in 15 mM $MgCl_2$ and analyzed by FMF. Increasing the magnesium concentration did increase the fluorescence intensity of cells but did not significantly improve the resolution obtained for the DNA distributions above a concentration of 15 mM.

DISCUSSION

The three antibiotics, olivomycin, chromomycin A3, and mithramycin, exhibit similar spectral patterns when analyzed as (a) the free dyes; (b) complexed with magnesium ions; or (c) complexed with both magnesium and DNA in solution. Likewise, cells stained and analyzed by FMF provide comparable DNA distribution profiles which, upon computer analysis, yield essentially the same results for the percentages of cells in the various phases of the cell cycle. Although results of experiments designed to determine the effects of fixation and various environmental conditions on cell staining with olivomycin and chromomycin are not available at this time, preliminary results to date indicate that these compounds probably react to conditions of varying pH, salinity, magnesium

concentration, etc., in a manner quite similar to mithramycin.

Based upon conditions used in the present study, it appears that ethanol (70%) is superior to glutaraldehyde (1%) for fixation prior to mithramycin staining and that formalin (4%) is not a desirable fixative for use in such studies. However, glutaraldehyde can be used as an alternative for ethanol at the expense of some broadening of the G_1 and G_2 + M peaks in the DNA distribution. Results obtained with the various fixative agents must reflect differences in their mode of action on the chromatin structure and, therefore, the manner in which they affect the dye-binding sites on the DNA. Glutaraldehyde fixation does appear to decrease mithramycin staining and/or intensity of cells by one-half; however, based on these results alone, it could be erroneous to imply that glutaraldehyde obstructs mithramycin binding at one-half of the available sites, since the apparent changes in quantum efficiency which occur when the dye binds to the DNA make it difficult to interpret changes in fluorescence intensity directly.

Optimal staining with mithramycin can be achieved in the pH range 5.0 to 9.0. At present, conditions above pH 10.0 cannot be recommended until the technical difficulties of salt precipitation and cell clumping can be alleviated. Spectral studies do indicate that the fluorescence intensity of the antibiotics is diminished at low pH (i.e., 3.0 to 4.0); however, the significant increase in fluorescence intensity in stained cells between pH 9.0 and 10.0, as noted by FMF, could not be explained on the basis of an increase in dye intensity. Mild alkaline hydrolysis of the DNA most likely is providing more dye-binding sites at pH 10.0.

Variations in ionic strength had a dramatic effect on intensity of stained cells but no noticeable effect on intensity of the DNA-dye complex in solution. Furthermore, staining at elevated NaCl concentrations (i.e., 1 to 1.75 M)

increased the fluorescence intensity of cells and produced irreversible effects on dye-chromatin interaction as based on FMF analysis. At 1.75 M NaCl, the G_1 peaks began to broaden significantly. Investigations are presently in progress to use these phenomena for studying and correlating chromatin stainability with chromatin structure.

Cell staining for about 1 hr in solutions varying in their mithramycin concentration increased the fluorescence intensity of stained cells up to 100 µg/ml but did not significantly improve the resolution in the DNA distributions above 50 µg/ml of the antibiotic. In a previous study (7), we demonstrated that cells stained for 24 hr with concentrations of mithramycin as low as 10 µg/ml could provide DNA distributions which were quite comparable to cells stained in 100 µg/ml. This study varied from the present investigation in that the staining solution contained 20% ethanol and no NaCl; however, ethanol appears to decrease mithramycin fluorescence (Crissman et al., unpublished results) so that the results would be expected to be somewhat improved with analysis in the absence of ethanol.

Magnesium was found to quench and, at the same time, to produce spectral shifts in all antibiotics examined in this study. Magnesium also significantly affected cell staining and analysis when present in the stain solution and/or the carrier medium for flow analysis. The presence of magnesium ions appears to stabilize a portion of the dye-chromatin complex even when the free dye is removed. However, best results were obtainable when flow analysis was performed on cells suspended in the dye solution containing magnesium. At present, no other divalent metals have proven superior to magnesium for cell staining.

In the present study, we have attempted to determine the effects of various fixative agents on subsequent cell staining with mithramycin and also to note the effects of variation of the staining environment on DNA in solution

and in mammalian cells for mithramycin, chromomycin A3, and olivomycin. In general, these three antibiotics appear spectrally similar and are equally efficient for staining and analyzing DNA in flow systems. It is hoped that other antibiotics will become available which may even supersede the use of these compounds and possibly provide useful methods also for staining RNA in cells.

It appears from the present study that at least variations in both pH and ionic strength can have significant effects on the stainability of the chromatin in cells, although, except for low pH, these variations do not exert any major effect on fluorescence of the antibiotic or its complex with DNA in solution. These lend promise to the possibility of studying changes in chromatin structure by FMF. Interestingly enough, Swartzendruber (15) has recently shown that the incorporation of bromodeoxyuridine into growing cells during S phase can significantly increase the fluorescence of mithramycin-stained cells, enabling the distinction between traversing and nontraversing cells by FMF analysis. Since the use of these antibiotics is a relatively new approach for cell staining, possibly other such benefits of these novel methods will be recognized in the future.

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Fig. 1. Fluorescence excitation and emission spectra for olivomycin, chromomycin, and mithramycin unbound (———) and bound (-----) to calf thymus DNA. The antibiotic concentration was 3.3 μg/ml in 0.15 M NaCl and 15 mM $MgCl_2$, and the final DNA concentration was 1 mg/ml. Spectra for the unbound dye were expanded vertically 10 times for comparative purposes.

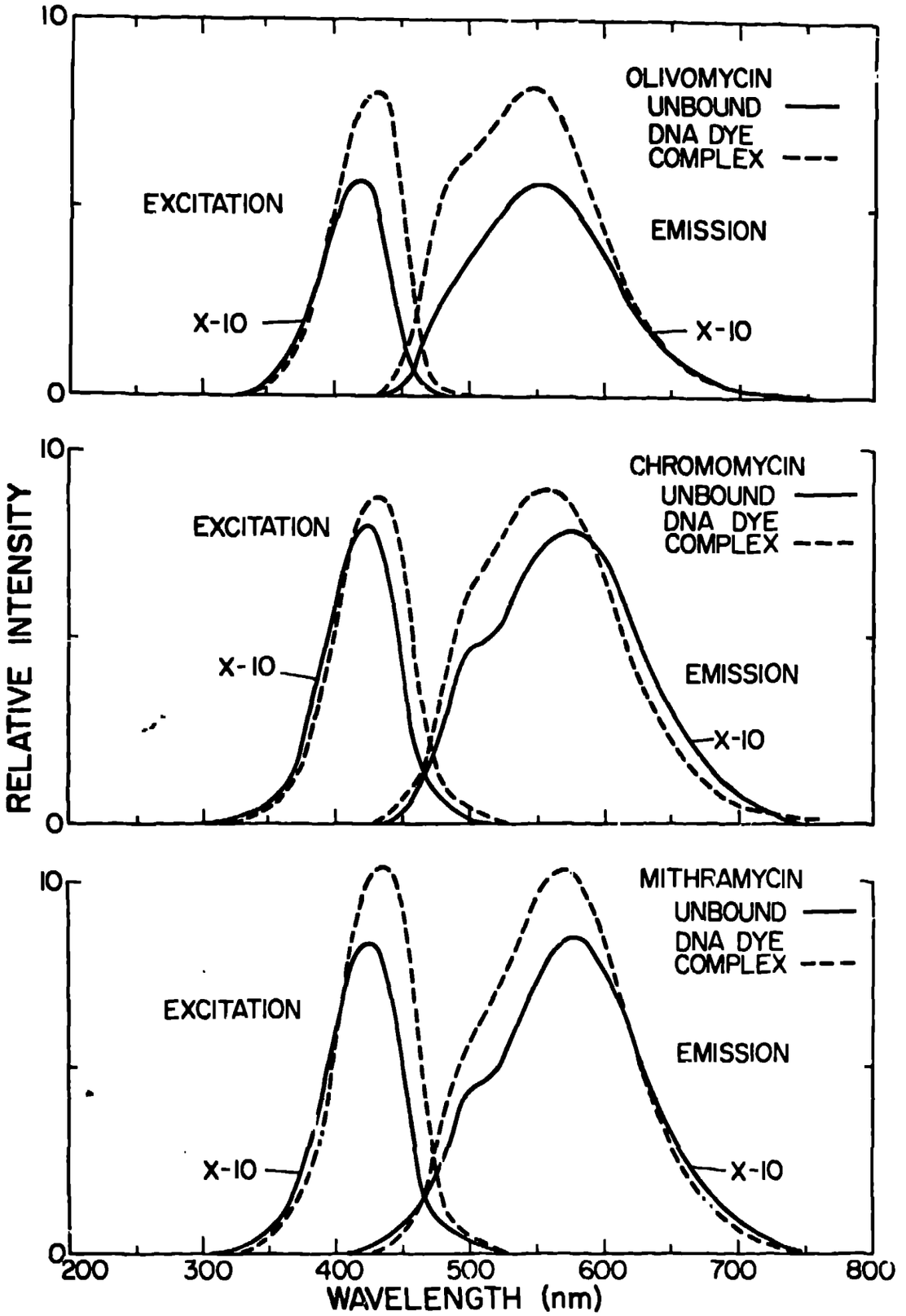

10
0
OLIVOMYCIN
UNBOUND
DNA DYE
COMPLEX
EXCITATION
EMISSION
X-10
X-10
10
0
CHROMOMYCIN
UNBOUND
DNA DYE
COMPLEX
EXCITATION
X-10
EMISSION
X-10
RELATIVE INTENSITY
10
0
MITHRAMYCIN
UNBOUND
DNA DYE
COMPLEX
EXCITATION
EMISSION
X-10
X-10
200
300
400
500
600
700
800
WAVELENGTH (nm)

Fig. 2. DNA distribution patterns obtained for Chinese hamster cells (line CHO) stained with olivomycin, chromomycin A3, or mithramycin (100 µg/ml in 0.15 M NaCl containing 15 mM $MgCl_2$). The CVs of the G_1 peaks were 4.3%, 4.7%, and 4.5%, respectively, for olivomycin-stained, chromomycin-stained, and mithramycin-stained cell populations.

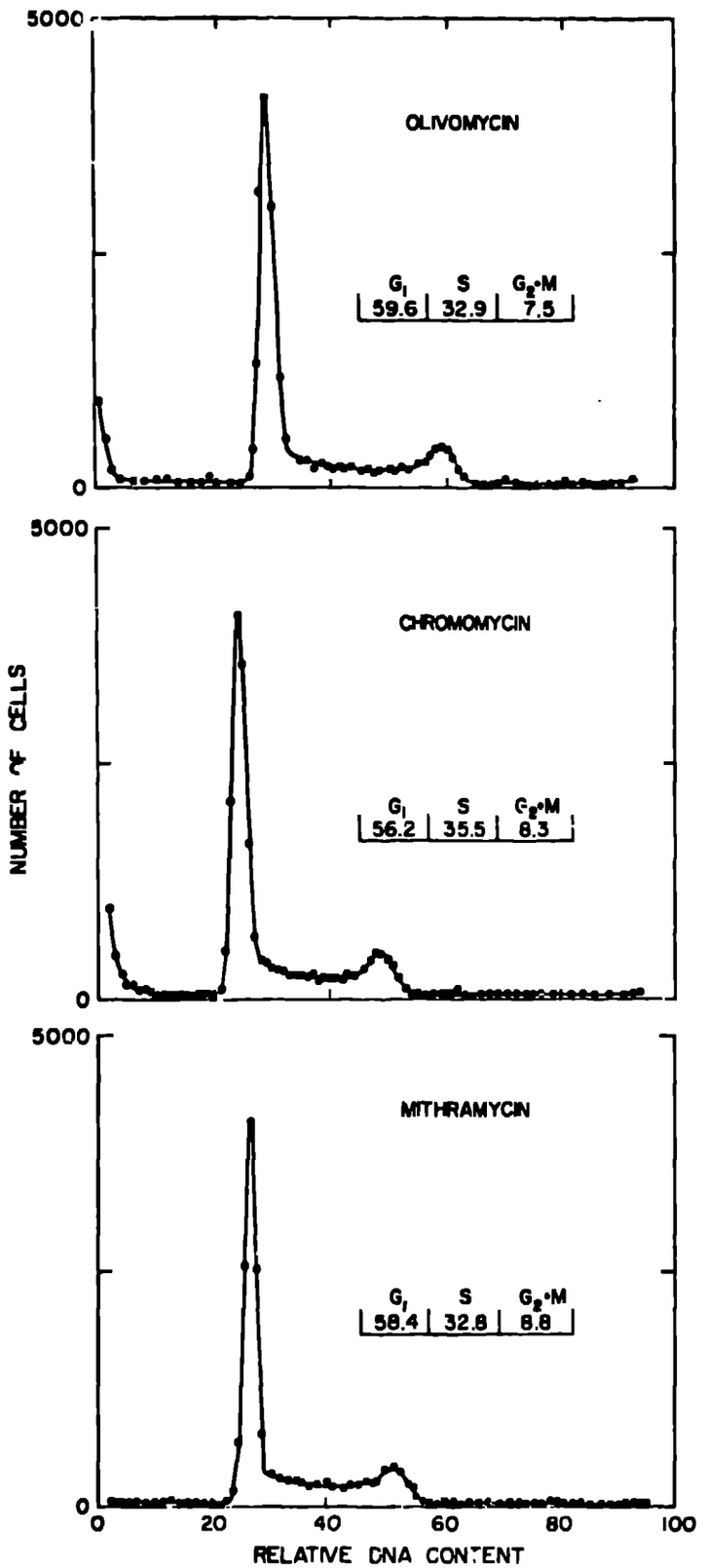

5000
OLIVOMYCIN
G1 S G2•M
59.6 32.9 7.5
0
5000
CHROMOMYCIN
G1 S G2•M
56.2 35.5 8.3
0
NUMBER OF CELLS
5000
MITHRAMYCIN
G1 S G2•M
58.4 32.8 8.8
0
0 20 40 60 80 100
RELATIVE DNA CONTENT

Fig. 3. DNA distribution patterns obtained for Chinese hamster cells (line CHO) fixed in either 70% ethanol or 1% glutaraldehyde for 24 hr prior to staining with mithramycin (100 μg/ml in 0.15 M NaCl containing 15 mM $MgCl_2$). The CVs of the G_1 peaks were 4.5% and 6.4%, respectively, for ethanol-fixed and glutaraldehyde-fixed cell populations.

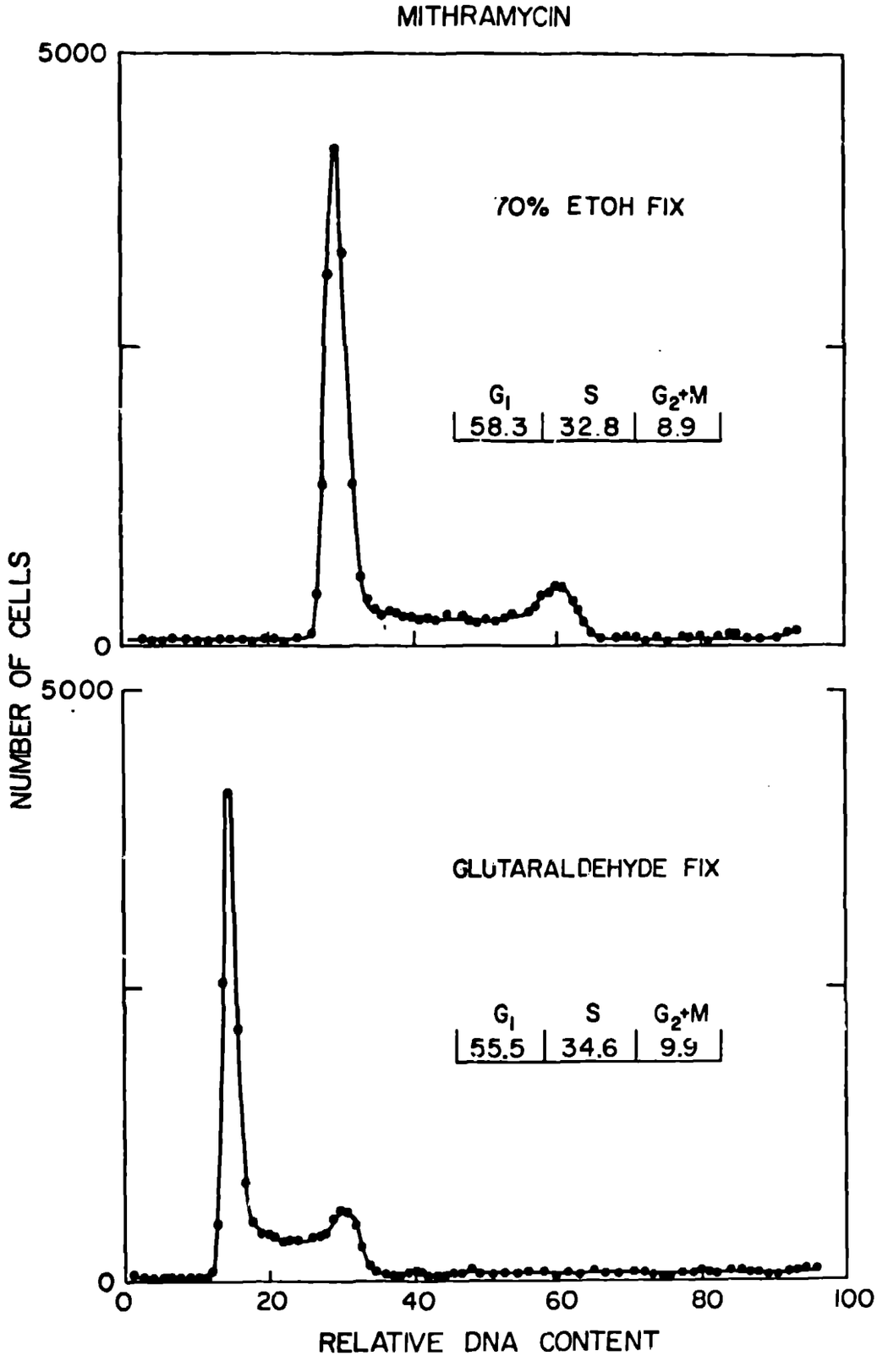

MITHRAMYCIN
5000
70% ETOH FIX
G1 S G2+M
58.3 32.8 8.9
0
5000
GLUTARALDEHYDE FIX
G1 S G2+M
55.5 34.6 9.9
0
NUMBER OF CELLS
0 20 40 60 80 100
RELATIVE DNA CONTENT

Fig. 4. Effects of pH on the relative intensit, (G_1 modal channel) of mithramycin-stained CHO cells. Ethanol-fixed cells were stained with mithramycin solutions in the pH range 3.0 to 10.0. The G_1 modal channel values were derived from the DNA distributions obtained by FMF.

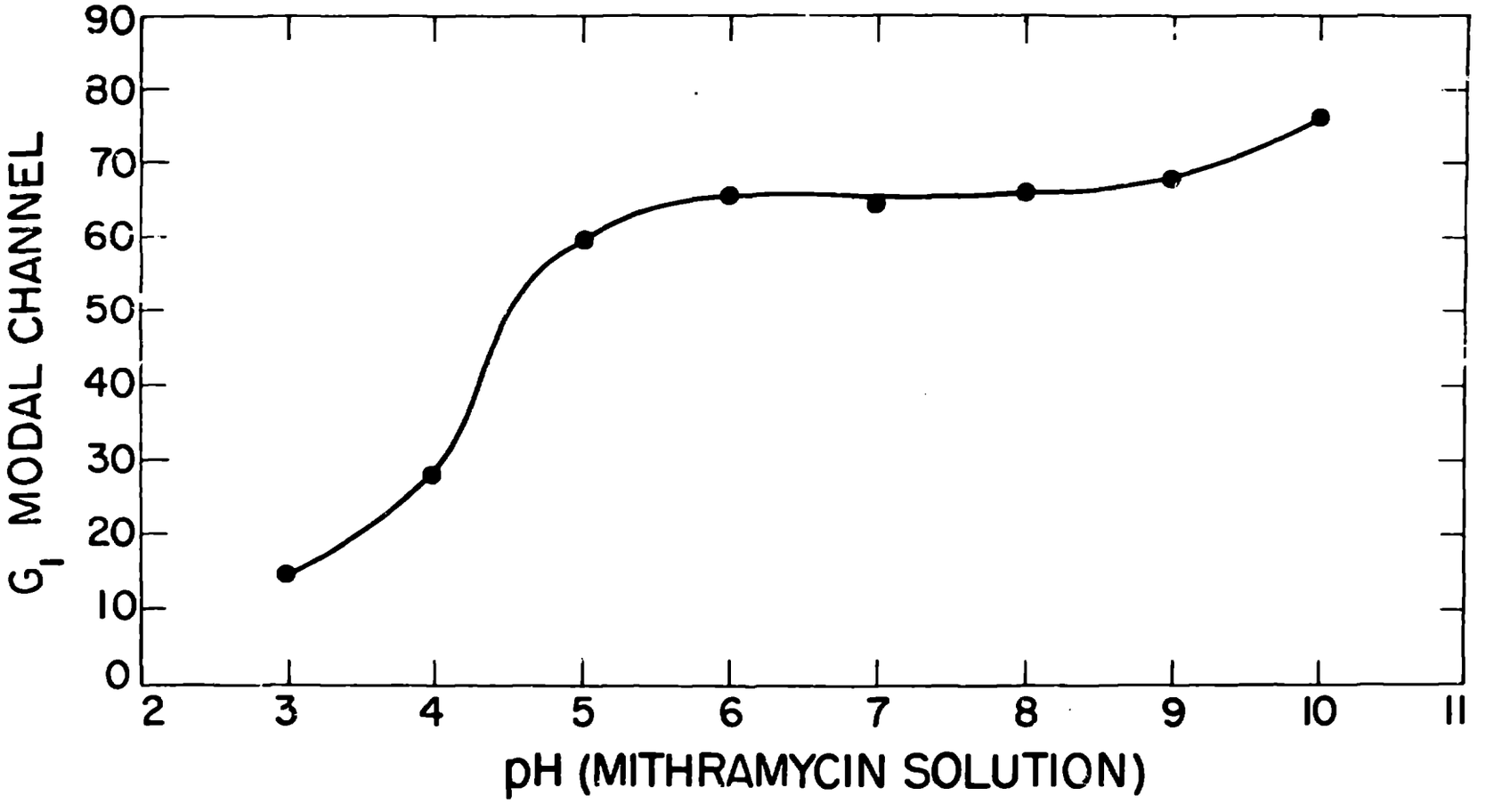

90
80
70
60
50
40
30
20
10
0
G_1 MODAL CHANNEL
2
3
4
5
6
7
8
9
10
11
pH (MITHRAMYCIN SOLUTION)

Fig. 5. Effects of ionic strength (NaCl molarity) on the relative intensity of mithramycin-stained CHO cells. Ethanol-fixed cells were stained with mithramycin solutions (100 μg/ml in 15 mM $MgCl_2$) over a NaCl concentration range of 0 to 1.75 M. The G_1 modal channel values were derived from the DNA distributions obtained by FMF.

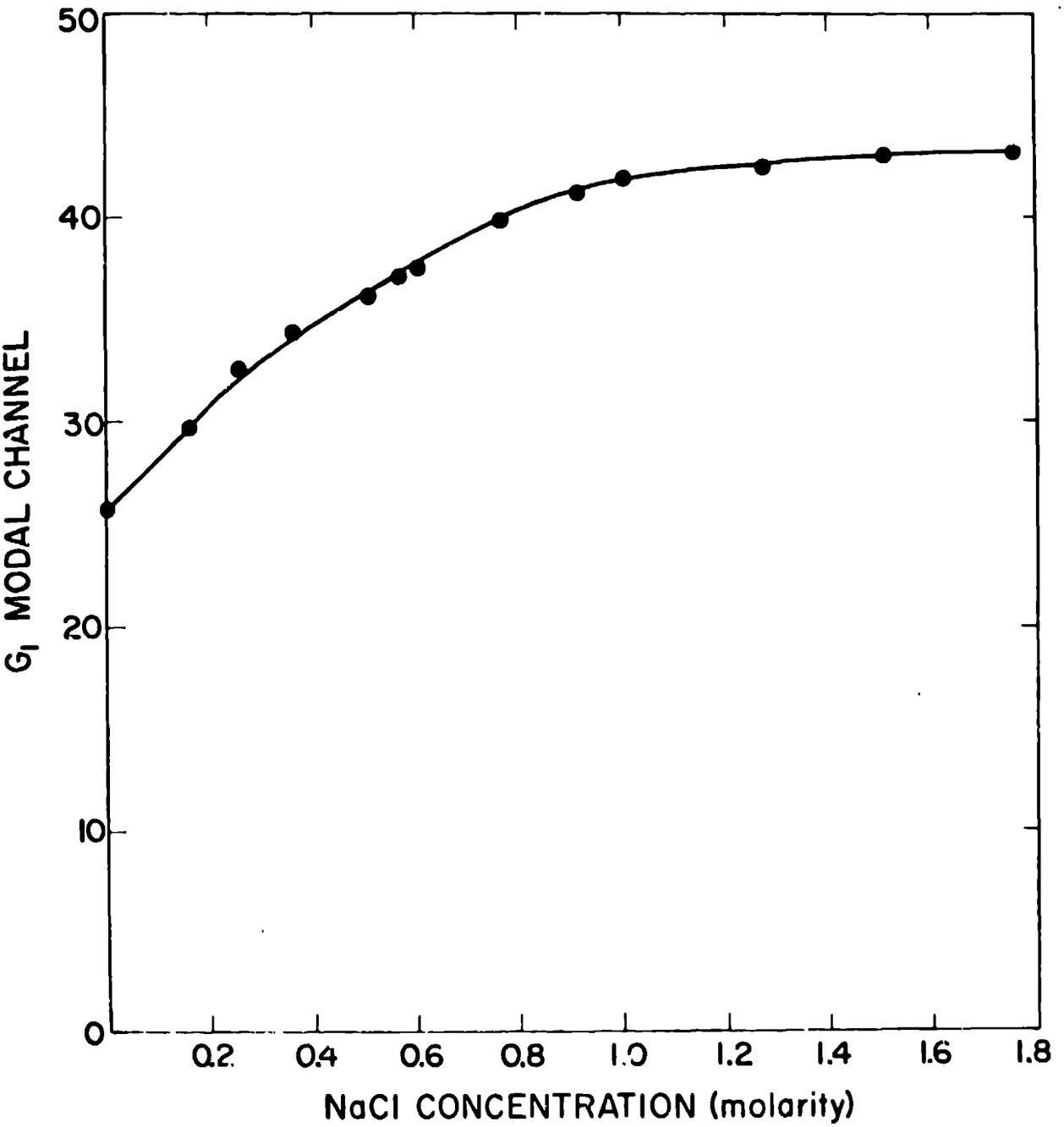
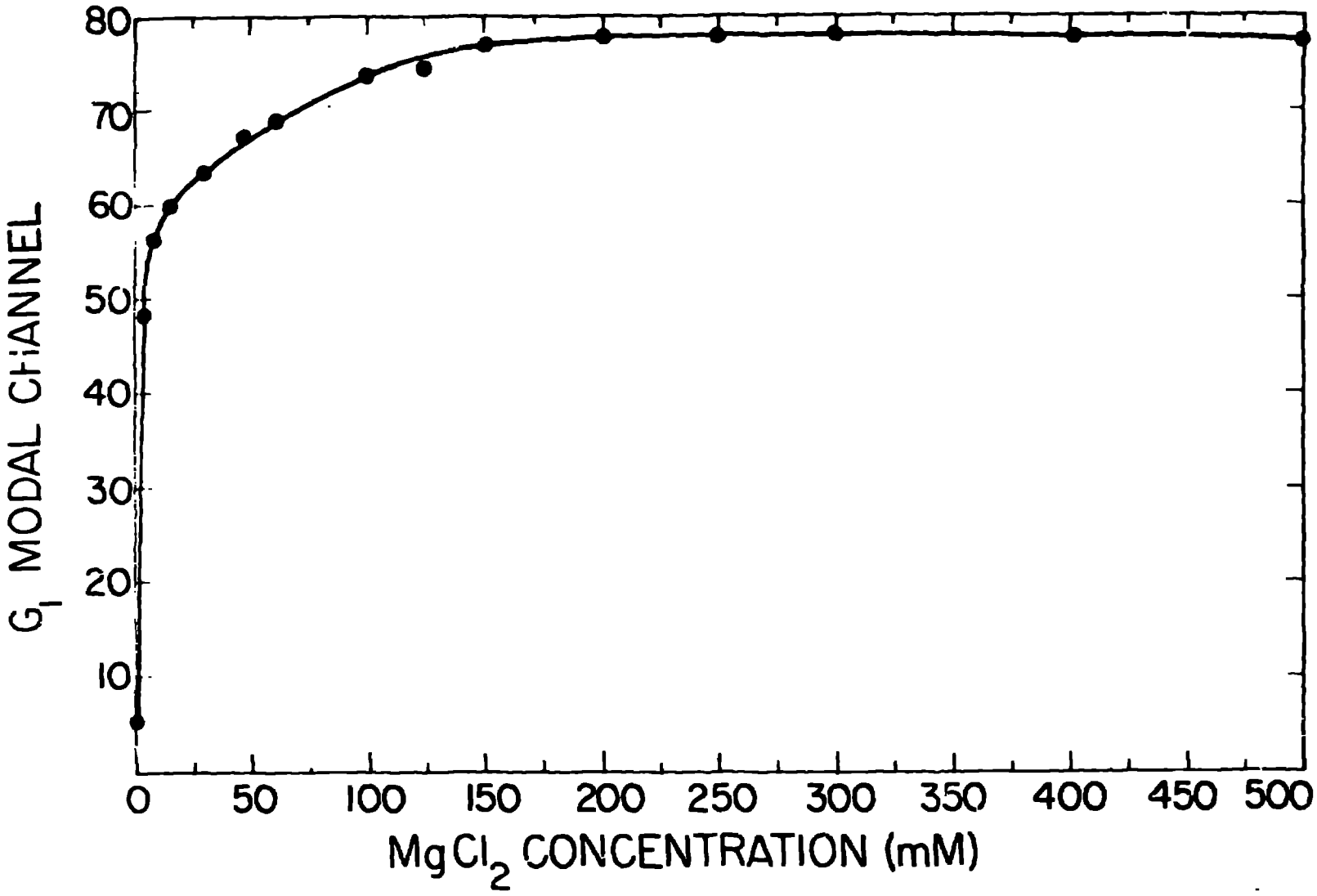

50
40
30
20
10
0
G_1 MODAL CHANNEL
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
NaCl CONCENTRATION (molarity)

Fig. 6. Effects of magnesium concentration ($MgCl_2$ molarity) on the relative intensity of mithramycin-stained CHO cells. Ethanol-fixed cells were stained with mithramycin-solutions (100 µg/ml in 0.15 M NaCl) over a $MgCl_2$ range of 0 to 500 mM. The G_1 modal channel values were derived from the DNA distributions obtained by FMF.


80
70
60
50
40
30
20
10
G_1 MODAL CHANNEL
0
50
100
150
200
250
300
350
400
450
500
$MgCl_2$ CONCENTRATION (mM)