LA-UR-77-2748

CONF-7706106--1

TITLE:

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SUBMITTED TO:

To be published in the Proceedings of the Biological Stain Commission Annual Symposium on Uses and Problems in the Application of Dyes to Tissue Culture, held in New Orleans, Louisiana (June 10, 1977). To be published in Stain Technology

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CONTRACT W-7405-ENG. 36

DETAILED STUDIES ON THE APPLICATION OF THREE FLUORESCENT ANTIBIOTICS FOR DNA STAINING IN FLOW CYTOMETRY

Running title: Antibiotic Staining of DNA

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ABSTRACT. The effects of various fixative agents, pH, ionic strength, stain concentration, and magnesium concentration on DNA staining with the fluorescent 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 cytometry (FCM) provided DNA distribution patterns with a high degree of resolution. Glutaraldehyde-fixed cells exhibited about one-half the fluorescence intensity of 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. Cell staining over a pH range of 5-9 in solutions containing 0.15-1 M NaCl and 15-200 mM $MgCl_2$ provided optimal results based on the DNA profiles obtained by FCM. 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, spectrophotofluorometric analysis of mithramycin-magnesium-DNA complexes in solution revealed no significant changes in fluorescence intensity over a range of 0-1.75 M NaCl. These results and those obtained by FCM 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 larger number of binding sites for the dye-magnesium complex.

Three fluorescent antitumor antibiotics have been examined recently and found useful for staining and analyzing DNA in flow systems (Crissman et al. 1976). 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 here 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 the chromatin structure of intact cells by FCM analysis.

MATERIALS AND METHODS

Spectrophotofluorometric Assay. The excitation and emission spectra for mithramycin (Pfizer Co.), chromomycin A3 (Calbiochem), and olivomycin (DCT/NCI) in (a) aqueous solution (3.3 µg/ml), (b) complexed with magnesium (15 mM $MgCl_2$), and (c) complexed with magnesium plus calf thymus DNA (final concentration 1 mg/ml saline) were obtained using the automatic recording Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Springs, Md.). 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 (Crissman et al. 1975); (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 3-10 (pH 3-6 acetic acid-acetate buffer; pH 7-8 Tris-HCl buffer; and pH 9-10 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 0-1.75 M NaCl (saline). Ethanol-fixed cells were also stained with mithramycin solutions containing 10-250 µg/ml of mithramycin. In other experiments, ethanol-fixed cells were stained with mithramycin solutions containing 0-1 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) (Van Dilla et al 1969, Holm and Cram 1973) 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 (1974) 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 (Ward et al. 1965). 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 (Waring 1970).

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 5-10 nm toward the red region and the emission peak 5-10 nm toward the blue region of the spectrum. Spectral shifts of similar magnitude 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 mg/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. (1965) 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%, chromomcyin 4.7%, mithramycin 4.5%).

Effects of Fixative Agents. In our original protocol (Crissman and Tobey 1974), ethanol was added to the mithramycin staining solution so 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 FCM 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 FCM 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 3-10 and analyzed by FCM showed a 4-fold increase in fluorescence intensity between pH 3-5 (Fig. 4). From pH 5-9, there was little change in fluorescence; however, between pH 9 and 10, there was an additional 13% increase in fluorescence intensity. Above pH 10, 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 so that the variations in accessibility of dye-binding sites under conditions in which chromatin is undergoing mild and gradual alkaline hydrolysis can be examined.

Effects of Ionic Strength. The fluorescence intensity of cells stained in solutions of mithramycin (Fig. 5), chromomycin, or olivomycin (100 μg/ml in 15 m$\underline{M}$ $MgCl_2$) containing 0-1.75 $\underline{M}$ NaCl increased significantly from 0-1 $\underline{M}$ NaCl but then changed only slightly from 1-1.75 $\underline{M}$ 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 FCM between the range of 0.15-1.0 $\underline{M}$ 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 FCM 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 chromatin are only transient and produce no permanent observable changes in chromatin structure in the absence of the dye.

Spectral analysis of the mithramycin-magnesium-DNA complex in solutions over a range of 0-1.75 M NaCl revealed no significant change in fluorescence intensity of the complex as a function of increasing saline concentration. Addition of calf thymus DNA to the mithramycin-magnesium solutions did produce the fluorescence enhancement noted in Fig. 1; however, there was no significant difference in fluorescence intensity of the solution lacking NaCl, compared to the 1.75 M NaCl solution. These results indicate that, although variations in ionic strength do not affect the intensity of mithramycin bound to DNA in solution, such variations 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 FCM, 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 0-70 mM $MgCl_2$ showed a sharp increase in fluorescence intensity as a function of increasing $MgCl_2$ concentration (data not shown). 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 even at $MgCl_2$ concentrations up to 200 mM. 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 and analyzed 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, regardless 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 (FCM) 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, regardless of the resuspending solutions used for analysis; however, the G_1 peaks of cell populations analyzed in solutions lacking mithramycin were about 2-3 times larger than for control cells.

DISCUSSION

The three antibiotics, olivomycin, chromomycin A3, and mithramycin, exhibit similar spectral patterns when analyzed as (a) the free dye; (b) complexed with magnesium ions; or (c) complexed with both magnesium and DNA in solution. Likewise, cells stained and analyzed by FCM 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 complete 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.

Optimal staining with mithramycin can be achieved in the pH range 5-9. At present, conditions above pH 10 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., 3-4); however, the significant increase in fluorescence intensity in stained cells between pH 9 and 10, as noted by FCM, could not be explained on the basis of an increase in the intensity of free dye at elevated pH. Mild alkaline hydrolysis of the DNA most likely is providing more dye-binding sites at pH 10.

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., 1-1.75 M) increased the fluorescence intensity of cells and produced irreversible effects on dye-chromatin interaction based on FCM 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 (Crissman et al. 1976), we demonstrated that cells stained for 24 hr with concentrations of mithramycin as low as 10 µg/ml could provide DNA distributions 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 for 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.

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-DNA complex in solution. These results lend promise to the possibility of studying changes in chromatin structure by FCM. Interestingly enough, Swartzendruber (1977) has recently shown that the incorporation of bromodeoxyuridine into cycling cells during S phase can significantly increase the fluorescence of mithramycin-stained cells, enabling the distinction between traversing and nontraversing cells by FCM 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.

ACKNOWLEDGMENTS

The authors thank Nathan Belcher of the Pfizer Company for the generous supply of mithramycin; J. H. Jett for assistance in computer analysis of the FCM data; M. S. Oka for help in spectral analysis of the dyes; P. F. Mullaney and J. A. Steinkamp for helpful criticism of the manuscript; and E. M. Sullivan for manuscript preparation. This work was supported by the U. S. Energy Research and Development Administration and the National Cancer Institute.

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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.

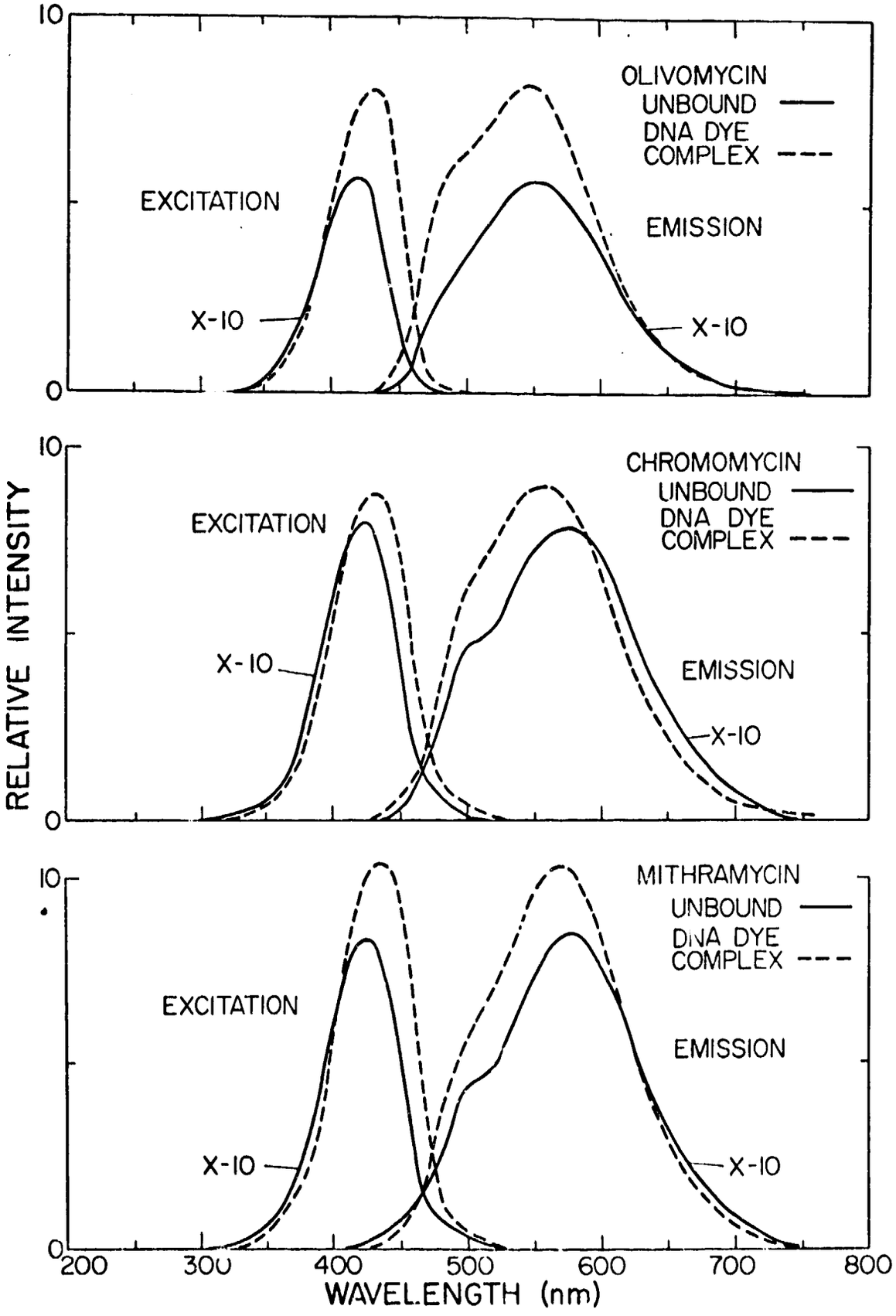

10
0
OLIVOMYCIN
UNBOUND
DNA DYE
COMPLEX
EXCITATION
EMISSION
X-10
X-10
10
0
CHROMOMYCIN
UNBOUND
DNA DYE
COMPLEX
EXCITATION
X-10
EMISSION
X-10
RELATIVE INTENSITY
10
0
MITHRAMYCIN
UNBOUND
DNA DYE
COMPLEX
EXCITATION
EMISSION
X-10
X-10
200
300
400
500
600
700
800
WAVELENGTH (nm)

FIG. 2. DNA distribution patterns obtained for Chinese hamster cells (line CHO) stained with olivomycin, chromomcyin 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.

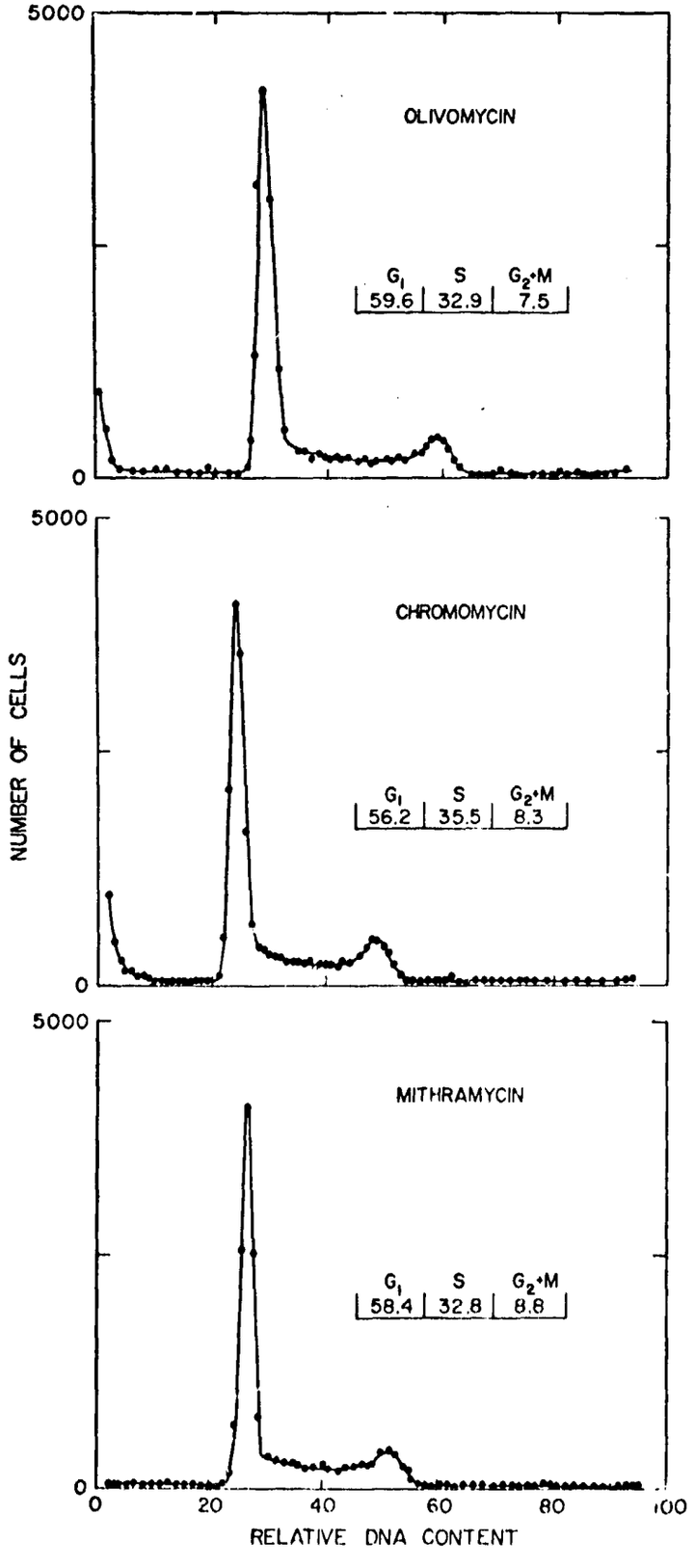

5000
OLIVOMYCIN
G1 S G2•M
59.6 32.9 7.5
0
5000
CHROMOMYCIN
G1 S G2•M
56.2 35.5 8.3
0
NUMBER OF CELLS
5000
MITHRAMYCIN
G1 S G2•M
58.4 32.8 8.8
0
0 20 40 60 80 100
RELATIVE DNA CONTENT

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.

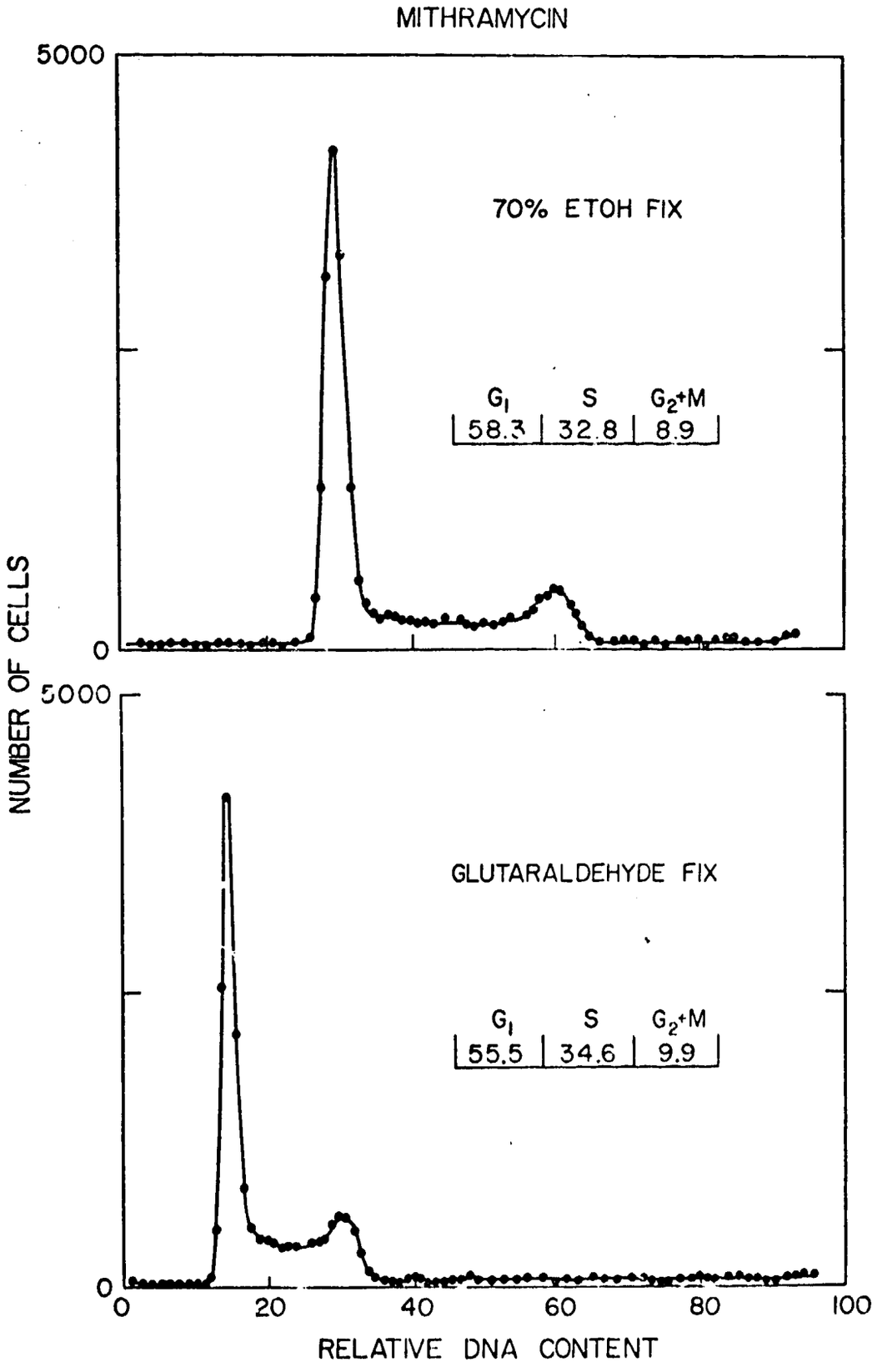

MITHRAMYCIN
5000
70% ETOH FIX
G1 S G2+M
58.3 32.8 8.9
0
5000
GLUTARALDEHYDE FIX
G1 S G2+M
55.5 34.6 9.9
0
NUMBER OF CELLS
0 20 40 60 80 100
RELATIVE DNA CONTENT

FIG. 4. Effects of pH on the relative intensity (G_1 modal channel) of mithramycin-stained CHO cells. Ethanol-fixed cells were stained with mithramycin solutions in the pH range 3-10. The G_1 modal channel values were derived from the DNA distributions obtained by FCM.

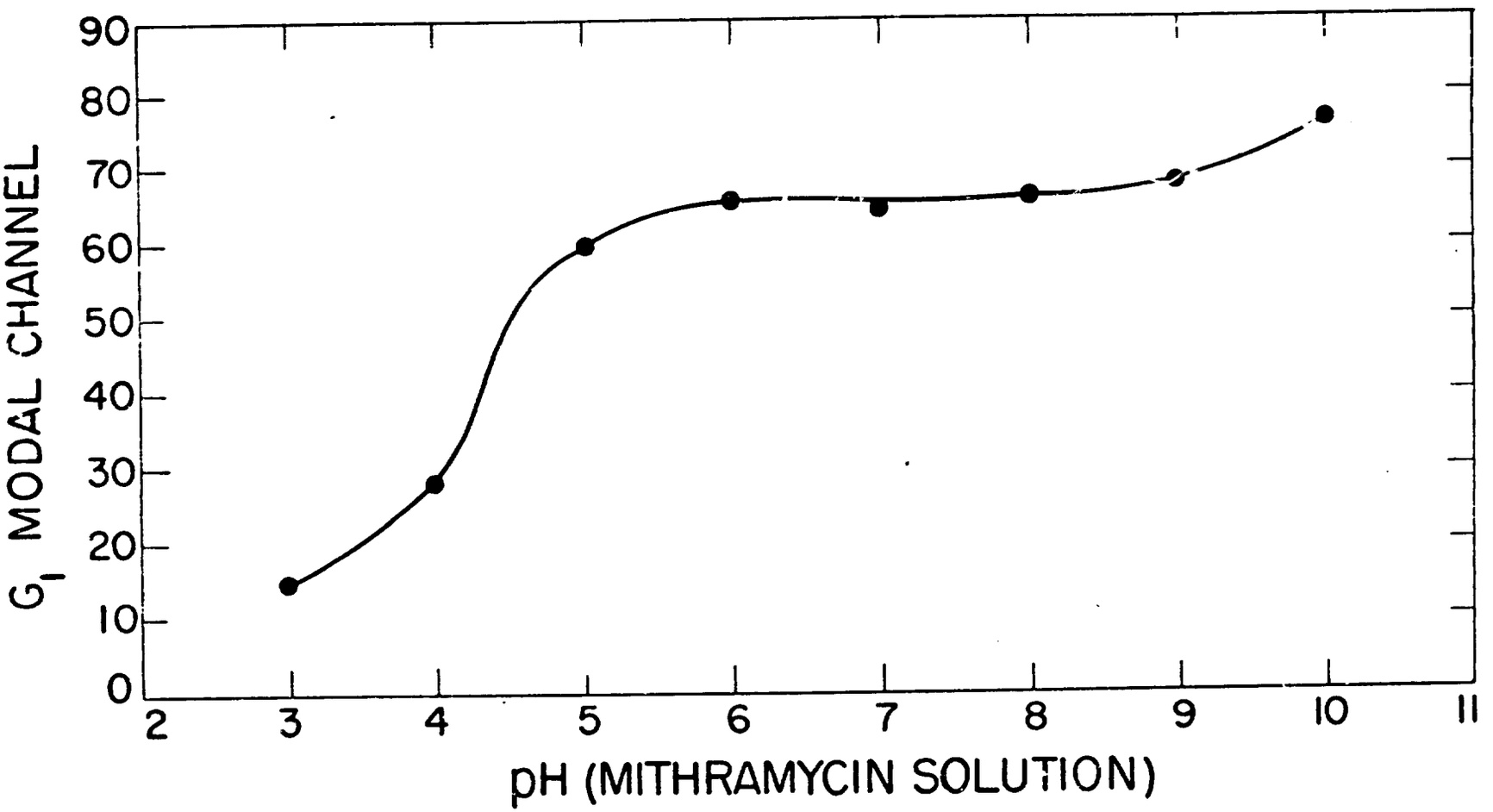
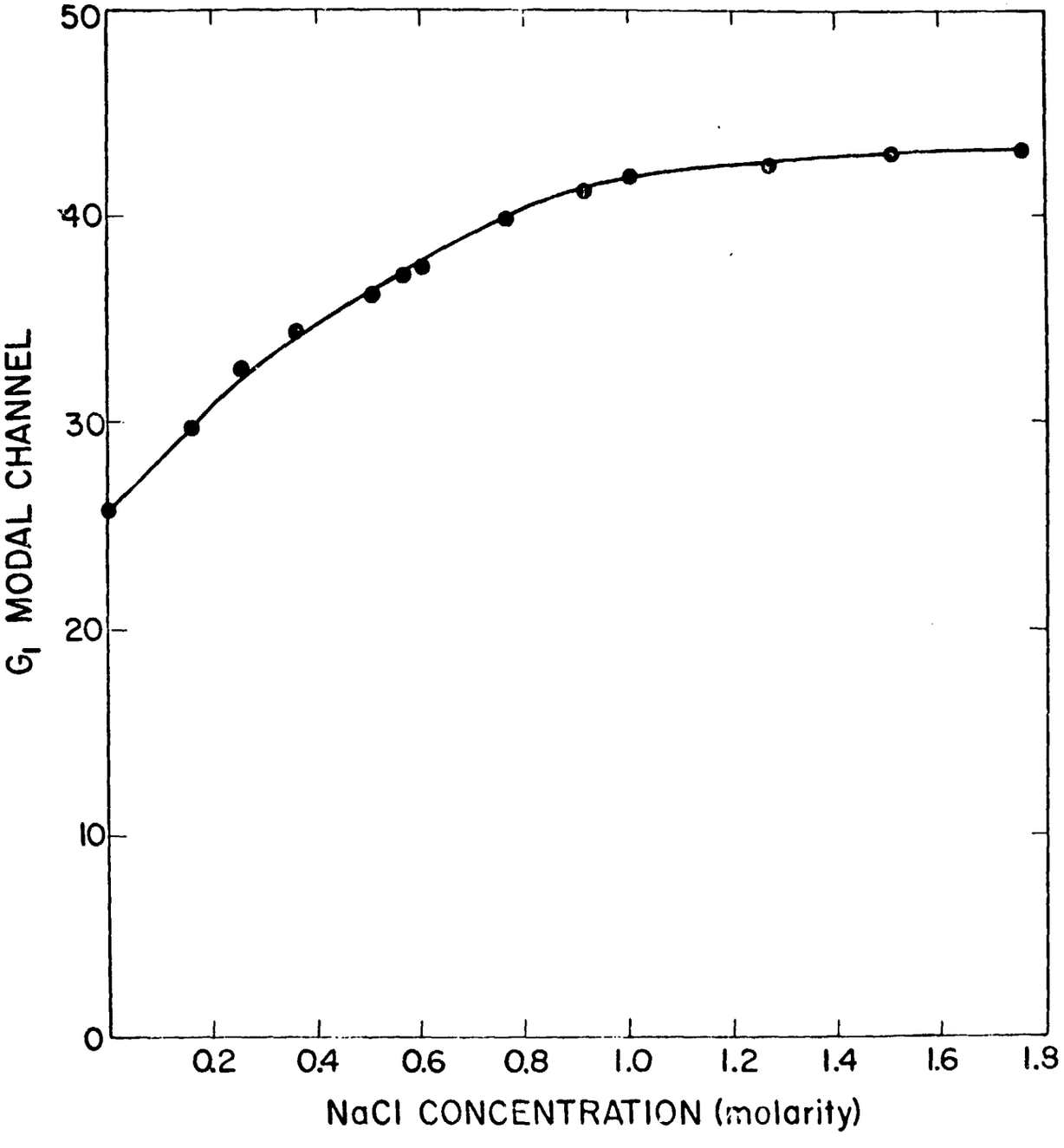

90
80
70
60
50
40
30
20
10
0
G_1 MODAL CHANNEL
2
3
4
5
6
7
8
9
10
11
pH (MITHRAMYCIN SOLUTION)

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 m$\underline{M}$ $MgCl_2$) over a NaCl concentration range of 0-1.75 $\underline{M}$. The G_1 modal channel values were derived from the DNA distributions obtained by FCM.


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)