FLOW CYTOFLUOROMETRIC ANALYSIS OF CELL CYCLE DISTRIBUTIONS USING PROPIDIUM IODIDE

Properties of the Method and Mathematical Analysis of the Data

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ABSTRACT

In order to better characterize the new rapid staining method for flow cytofluorometry proposed by Krishan, we have tested its stability and several other properties, and have carried out a quantitative comparison of the fluorescence histograms obtained using propidium iodide or the acriflavine-Feulgen staining procedure.

Using a human hematopoietic cell line in the logarithmic phase of growth, and analyzing the data by means of a mathematical method we have devised, we found that the fluorescence intensity of cells stained with propidium iodide remains stable for at least 48 h; it is insensitive to dye concentration between 0.025 and 0.10 mg/ml (37–150 μM); it is not affected by incubation with ribonuclease before staining; propidium iodide in 0.1% sodium citrate remains stable for at least 20 days; and quantitative estimates of the fractions of cells in the different phases of the cell cycle are in good agreement with those obtained from acriflavine-Feulgen staining and from autoradiography after pulse labeling with tritiated thymidine. We conclude that this method is useful for the measurement of relative DNA content by flow cytofluorometry, although modifications in the technique are necessary for some cell types which grow in monolayers.

Because it can be used to measure selected properties of large numbers of individual cells in a population rapidly and conveniently, flow cytofluorometry has become increasingly popular as a research tool, and its potential for clinical application is being intensively explored. In principle, the intercellular distribution of any cellular component can be measured provided an appropriate fluorescent dye can be used which binds stoichiometrically to the component in sufficient quantity to be detectable by the available instrumentation. Most applications of the method have until now been concerned with the determination of the relative distribution of DNA content among the cells of a population. A method that permits one to carry out rapid determinations of relative DNA content has important implications not only as a more convenient and potentially more accurate means of cell cycle analysis of populations in culture, but also for monitoring the status and kinetic response to therapy of patients with leukemia or other neoplasms (8, 9, 12, 13).

Until very recently, the fluorescent staining methods used by most investigators were the acri-

flavine-Feulgen (17, 20) and the ethidium bromide (6) procedures. In 1973, Crissman and Steinkamp (3) showed that propidium iodide, an analog of ethidium bromide, could be employed together with fluorescein isothiocyanate, a protein-binding fluorescent dye, for the simultaneous determination of DNA and protein content (the two dyes have separable fluorescence emission spectra, although both absorb strongly at 488 nm).

All of these methods, however, involve considerable manipulation entailing significant loss of cells, and require several hours for processing. To overcome the limitations of these staining methods, Crissman and Tobey (4) recently introduced a staining method using mithramycin, which requires only a few minutes for preparation and minimizes cell loss. However, because it has a peak excitation wavelength of 395 nm and negligible excitation at 488 nm, mithramycin cannot be used by laboratories possessing equipment operating at the latter wavelength.

Krishan (15) has very recently proposed a new rapid staining technique using propidium iodide, which utilizes a hypotonic solution of sodium citrate to rupture the cell membrane, enabling the dye to reach the nucleus. Most of the cytoplasm is removed in this process, and we have observed, with the aid of fluorescence microscopy, that the residual cytoplasm is unstained. The complete procedure requires only a few minutes, aside from preliminary procedures which may be needed for clinical specimens (8, 15).

Because of both its simplicity and its capability of being used with any flow cytofluorometer for determination of relative DNA content, Krishan's method will likely be used by many investigators in preference to the currently standard techniques. For this reason, we believe it is important to investigate certain fundamental characteristics of this method relative to its stability and reliability for quantitative work.

In this study, we report on the following properties of the propidium iodide method: (*a*) stability of the DNA histograms with respect to staining time up to 48 h; (*b*) stability of the hypotonic dye solution for periods up to 3 wk; (*c*) sensitivity of the DNA histograms and cell phase distributions to dye concentration; (*d*) quantitative comparison of the Krishan method with the acriflavine-Feulgen technique; (*e*) comparison of the predicted S phase fraction with autoradiographical pulse-labeling indices using tritiated thymidine; and (*f*) the effect of ribonuclease incubation on the histograms. In all cases, quantitative comparisons of the data were obtained using a mathematical method we have devised for estimating the fractions of cells in the G_1, S, and G_2+M phases of the mitotic cycle.

MATERIALS AND METHODS

Cell Line and Growth Conditions

Detailed descriptions of the origin and properties of the cell line (SK-L7) used in these studies were published previously (1, 18). The cell line was derived in 1966 from the peripheral blood of a child with acute myelomonocytic leukemia but may actually have arisen from the patient's residual normal lymphoid cells (2). Cells are maintained at 37°C in McCoy's 5A medium, modified (Grand Island Biological Company, Grand Island, N. Y.) containing 30% fetal calf serum (Grand Island Biological Co.) without antibiotics. Experiments described herein were carried out with cells in log phase, at concentrations below 3.2×10^5 cells/ml.

Labeling Procedure and Autoradiography

SK-L7 cells were labeled for 30 min or 1 h in medium containing 2 μCi/ml [^{3}H]TdR (sp act 6 Ci/mmol, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) at 37°C. At the end of the labeling period, unlabeled thymidine was added to a final concentration of 0.4 mM, which corresponds to more than a 1,000-fold dilution of the label. The cells were then centrifuged, the supernate was aspirated to 0.25 ml, the cells were resuspended and smears were made. Autoradiographs were then prepared as previously described (9). 1,000 cells were usually examined from each slide for labeling index determination, and at least five slides were counted in a single experiment. The background grain count was usually about one grain per nuclear area, and a counting threshold of five grains was employed.

Stability of Propidium Iodide Solutions

In order to determine whether the prepared PI solution (0.050 mg/ml in 0.1% sodium citrate) is stable over a 20-day period, we prepared solutions 1, 7, 14, and 20 days before the time of the experiment. Propidium iodide was weighed with a Mettler Model B6 balance (Mettler Instrument Corp., Highstown, N.J.). Solutions were kept refrigerated in the dark during storage. On the day of the study, dye concentrations were determined by absorbance measurements at λ = 494 nm (extinction coefficient ϵ = 5900), using a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Effect of Ribonuclease Treatment

In order to determine whether significant binding of propidium iodide to RNA occurred under the conditions employed, we incubated cells with ribonuclease before

staining. 500,000 SK-L7 cells in log phase were centrifuged at 150 *g* at room temperature for 5 min, and the supernate was decanted. The cells were resuspended in 5 ml of either distilled water or 0.1% sodium citrate solution, with or without ribonuclease (3210 U/ml, Worthington Biochemical Corp., Freehold, N. J.), and placed in a 37°C water bath for either 45 or 105 min. After incubating, the tubes were centrifuged and the supernate was decanted and tested for ribonuclease activity as discussed below. The cells were then stained with propidium iodide, and fluorescence was determined by flow cytofluorometry as described later.

To insure that the ribonuclease was active and that it was not denatured during incubation with the cells either in distilled water or in the 0.1% sodium citrate solution, we proceeded as follows. SK-L7 cells in log phase were prelabeled with tritiated uridine by the addition of 0.1 ml of a stock solution of the tracer ([5-^{3}H]uridine, 1 mCi/ml, sp act 2 Ci/mmol, Schwarz/Mann) to a tissue culture flask containing about 3×10^7 cells in 100 ml of medium. The cells were then incubated for 30 min at 37°C, placed in an ice bath for 5 min, and centrifuged at 240 *g* for 10 min. The supernate was then discarded; the cells were rinsed twice with 20 ml of medium and were resuspended in 40 ml of unlabeled medium. Portions of 5×10^5 cells were centrifuged and fixed overnight at 4°C in 10% formalin. The cells were then rinsed twice with distilled water, and the ribonuclease solutions obtained following treatment of the unlabeled cells (see above) were added. The tubes were then incubated in a 37°C water bath for 60 min and were subsequently placed in an ice bath. The cells were then filtered on 45 μm pore size Millipore filters (Millipore Corp., Bedford, Mass.) and rinsed with 25 ml of cold 5% trichloroacetic acid. The radioactivity of the RNA was then determined with a liquid scintillation counter (Beckman Model LS-200 B, Beckman Instruments).

Fluorescent Staining Procedure

PROPIDIUM IODIDE: 500,000 cells were transferred to 15-ml plastic centrifuge tubes and centrifuged at room temperature for 5 min at 150 *g*. The supernate was then aspirated to 0.25 ml and the cells were resuspended in the residual medium. 5 ml of propidium iodide (PI) solution (0.05 mg/ml of PI (Calbiochem, San Diego, Calif.) in 0.1% sodium citrate [15]) were added to each tube and mixed gently. The tubes were then placed in an ice bath, and cells were counted in the flow cytofluorometer after a minimum of 5 min.

ACRIFLAVINE-FEULGEN: Three million cells per tube were employed for the acriflavine staining procedure. After the cells were collected in 15-ml plastic centrifuge tubes, they were centrifuged at 400 *g* for 10 min, aspirated to 0.25 ml, and the cells were resuspended in the residual medium. Cold phosphate-buffered saline (PBS) was then added (to 2.5 ml), followed by 2.5 ml of cold 20% formalin in PBS. The cells were mixed gently and fixed for at least 18 h at 4°C. They were then rinsed twice with 5 ml of distilled water and stained as described by Deaven and Petersen (5).

Instrumentation

Cell fluorescence was measured on a Cytofluorograf, Model 4802 (Bio/Physics Systems, Inc., Mahopac, N. Y.), using an argon ion laser at 488 nm. Data were stored on a Northern Model NS-602 multichannel pulse height analyzer (Tracor-Northern Scientific, Inc., Middleton, Wisc.), recorded on a teletype printer and paper tape punch, Model ASR 33, and analyzed on an IBM 1800 digital computer.

Mathematical Analysis of Fluorescence Histograms

The method used to estimate the distribution of cells among the phases of the mitotic cycle is adapted from one we have previously described (7, 10, 11). The method can be applied to data from synchronous as well as asynchronous cell populations, and is based on a mathematical model of the cell population having the following properties and assumptions: (*a*) The population is separated into compartments, each consisting of cells having approximately the same DNA content. Cells in the G_1 phase comprise one compartment, those in G_2 or M are grouped in another, and S phase cells are classified into several compartments, according to their positions within S. (*b*) The fluorescence intensity of cells in each compartment is normally distributed with the mean intensity of the G_2+M compartment equal to twice that of G_1. Means of the S phase compartments are proportional to their average DNA contents and are spaced at equal intervals between those of G_1 and G_2+M. (*c*) Coefficients of variation of fluorescence intensity of the G_2+M and S compartments are adjusted relative to G_1 to yield a minimum sum of squares value. In the majority of cases, we have found the optimal ratio of CV of G_2+M to G_1 to be less than 1.

In carrying out the analysis, the CV and the channel locations of the G_1 and G_2+M compartments are estimated from the data, and the sizes of the various compartments are computed by a least squares procedure. A theoretical histogram is then generated corresponding to the solution and is compared with the experimental data points. Depending on the agreement between the two, either the result is accepted or specified parameters are altered and the program is rerun. The process is repeated until the fit of the model to the data is deemed satisfactory.

RESULTS

Effect of Dye Concentration and Staining Time

To determine whether the DNA histograms and/or the computed fractions of cells in the dif-

ferent phases of the cycle are sensitive to staining time or to concentration of propidium iodide in the suspending medium, a single experiment was performed in which both these parameters were varied. The dye concentrations used were 0.025, 0.050, and 0.100 mg/ml; the concentration of sodium citrate was 0.1% in all cases. Although the effects of changes in pH were not investigated, pH was approximately 7.2 at all dye concentrations. For each dye concentration, cells were analyzed in the flow cytofluorometer at 5 min and at 1, 5.5, 25, and 48 h after addition of propidium iodide. Cells were kept in an ice bath until analyzed.

DNA histograms are shown in fig. 1, and the distributions of cells among the phases of the cell cycle are listed in Table I, together with the coefficients of variation of fluorescence intensity of the G_1 and G_2+M compartments. It is evident from the appearance of the histograms and from the Table that there is some variability from one run to another, but there is no consistent difference related to either dye concentration or to standing time within the ranges surveyed. The CV's of the G_1 cells were almost always larger than those of G_2+M; their respective means were 7.4% and 5.8%.

Comparison of Propidium Iodide with Acriflavine-Feulgen Staining and with [^{3}H]TdR Labeling Indices

In order to evaluate the quantitative differences between results obtained with the propidium iodide method and those obtained with the widely used but tedious acriflavine-Feulgen staining procedure, an experiment (A) was performed in which log phase populations were stained by both techniques. At the same time that cells were collected for staining, samples were also exposed to tritiated thymidine for 30 min at 2 μCi/ml and processed for autoradiography as described previously. In another experiment (B), cells were

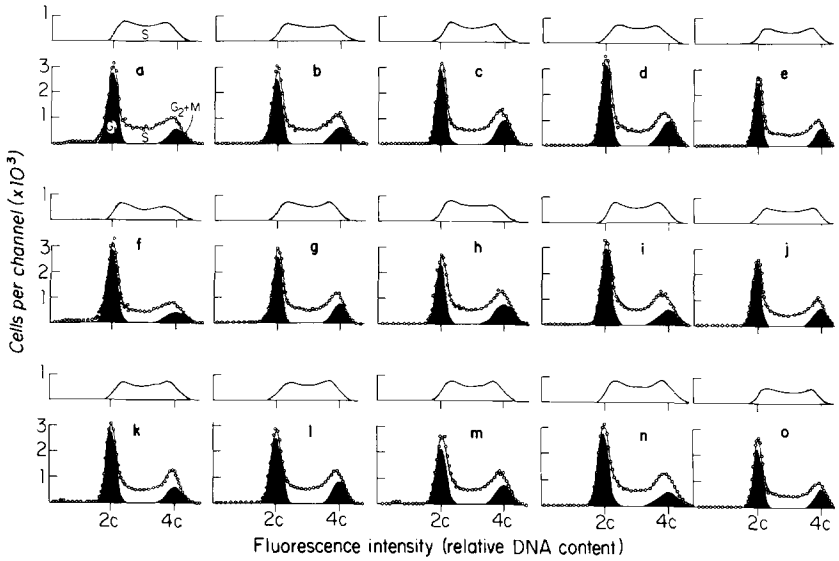

FIGURE 1 Histograms representing distributions of fluorescence intensity among cells in the population after staining with propidium iodide. The ordinate gives the number of cells per channel, and the abscissa gives the relative fluorescence intensity, which is proportional to DNA content. Data were normalized to 50,000 cells before computer analysis to facilitate comparison between samples. Normalized data points are shown as open circles, outer solid lines are the theoretical histograms resulting from mathematical analysis of the data, shaded areas show the predicted distributions of fluorescence intensity of G_1 and G_2+M cells, and the unshaded areas between them represent S phase cells. The last are redrawn in the upper portions of the diagrams for clarity. Mean fluorescence intensity of G_1 cells (diploid DNA content) is indicated as 2c, that of G_2+M (tetraploid DNA content) as 4c. The first row (histograms *a* through *e*) corresponds to a propidium iodide concentration of 0.025 mg/ml, the second row (*f* through *j*) were stained at 0.05 mg/ml, and the third row (*k* through *o*) at 0.10 mg/ml. The first through fifth columns represent samples analyzed after staining times of 5 min, 60 min, 5.5 h, 25 h, and 48 h, respectively.

TABLE I

Effect of Staining Time and Dye Concentration on Computed Percentages of Cells in G_1, S, and G_2+M and on CV Values

PI concentration	Phase	Staining time					Mean (SD)
		5 min	60 min	5.5 h	25 h	48 h	
mg/ml		%	%	%	%	%	%
0.025	G_1	37.3	35.8	35.6	39.7	36.0	36.9 (1.7)
"	S	50.2	47.5	45.9	41.3	49.8	46.9 (3.6)
"	G_2+M	12.5	16.7	18.5	19.0	14.2	16.2 (2.8)
"	CV(G_1)	7.5	7.4	7.0	7.5	6.0	7.1 (0 6)
"	CV(G_2+M)	6.0	6.6	5.6	6.0	4.2	5.7 (0.9)
0.050	G_1	43.2	36.0	32.4	38.5	37.5	37.5 (3.9)
"	S	45.1	49.3	48.2	48.7	47.5	47.8 (1.7)
"	G_2+M	11.7	14.7	19.4	12.8	15.0	14.7 (3.0)
"	CV(G_1)	7.5	7.0	7.5	7.5	7.0	7.3 (0.3)
"	CV(G_2+M)	7.5	4.9	6.8	6.8	4.9	6.2 (1.2)
0.010	G_1	39.6	38.0	33.4	37.2	35.7	36.7 (2.5)
"	S	48.3	47.2	50.8	51.0	49.3	49.3 (1.7)
"	G_2+M	12.1	14.8	15.8	11.8	15.0	14.0 (1.8)
"	CV(G_1)	7.5	8.0	8.0	8.5	6.5	7.7 (0.8)
"	CV(G_2+M)	5.3	4.8	5.6	7.7	4.6	5.6 (1.2)

stained by the propidium iodide technique only, and labeling indices were determined after 30-min labeling in [^{3}H]TdR. Finally, in a third experiment (C), cells were stained and analyzed by the acriflavine-Feulgen method, and labeling indices were determined after 60-min incubation in [^{3}H]TdR.

Representative histograms of propidium iodide and acriflavine-Feulgen stained cells from the experiment in which both were performed are shown in Fig. 2. Table II gives the distributions of cells among the phases of the cell cycle, together with CV values, from all three experiments. Labeling indices and their standard deviations following [^{3}H]TdR exposure were 53.4(±2.2)% (A), 56.7(±2.8)% (B), and 62.6(±2.7)% (C).

It is noted that in experiment A the results obtained with the propidium iodide staining method are in excellent agreement with those obtained with the acriflavine-Feulgen procedure. While the labeling indices were in all cases higher than the mean computed percentages of cells in S phase, only in experiment C were the two values farther apart than two standard deviations (which is a rough estimate of the 95% confidence interval). Possible reasons for this discrepancy will be discussed later.

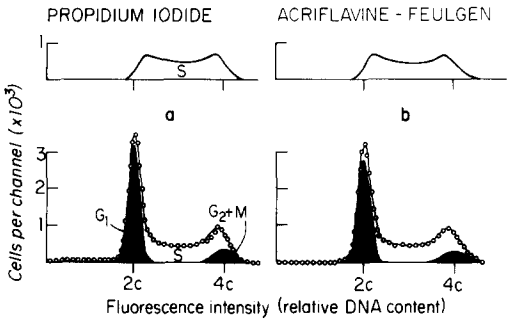

FIGURE 2 Fluorescence histograms following staining with 0.05 mg/ml propidium iodide (*a*) or with the acriflavine-Feulgen method (*b*). Abscissa scale factors were adjusted to obtain G_1 peaks at the same positions in the two cases. Refer to the legend for Fig. 1 for further description.

Stability of Propidium Iodide Solution

For practical reasons, it is useful to know the stability of the propidium iodide solution during storage. It would be convenient if the prepared stain could be used for relatively long periods without deteriorating. Even if the dye had a short storage life, it would be important to know how long a storage period would be considered safe.

The experiment discussed below consisted of two parts. First, propidium iodide solutions were prepared as described previously at 1, 7, 14, and 20 days before the study and were kept refrigerated in the dark until the time of the experiment. Dye concentrations were determined with a Beckman DU spectrophotometer as described earlier

TABLE II

Mathematical Analysis of Data after Propidium Iodide or Acriflavine-Feulgen Staining

(A) Propidium Iodide vs. Acriflavine-Feulgen

Phase	Propidium Iodide			Acriflavine-Feulgen			
	1	2	Mean (SD)	3	4	5	Mean (SD)
	%	%	%	%	%	%	%
G_1	40.7	43.4	42.1 (1.9)	41.8	43.0	39.7	41.5 (1.7)
S	50.5	49.4	50.0 (0.8)	49.8	46.0	52.2	49.3 (3.1)
G_2+M	8.8	7.2	8.0 (1.1)	8.4	10.9	8.2	9.2 (1.5)
CV(G_1)	6.7	6.3	6.5 (0.3)	7.0	7.0	6.5	6.8 (0.3)
CV(G_2+M)	4.7	5.0	4.9 (0.2)	5.6	5.6	6.5	5.9 (0.5)

(B) Propidium Iodide Only

Phase	Sample no.			Mean (SD)
	1	2	3	
	%	%	%	%
G_1	35.7	36.5	33.8	35.3 (1.4)
S	54.9	48.8	51.6	51.8 (3.1)
G_2+M	9.4	14.7	14.6	12.9 (3.0)
CV(G_1)	8.0	8.0	8.0	8.0 (–)
CV(G_2+M)	7.2	6.4	5.6	6.4 (0.8)

(C) Acriflavine-Feulgen Only

Phase	Sample no.			Mean (SD)
	1	2	3	
	%	%	%	%
G_1	34.7	34.4	33.3	34.1 (0.7)
S	53.2	53.6	56.0	54.3 (1.5)
G_2+M	12.1	12.0	10.7	11.6 (0.8)
CV(G_1)	7.8	8.0	9.0	8.3 (0.6)
CV(G_2+M)	7.8	7.2	9.0	8.0 (0.9)

TABLE III

Effect of Age of Propidium Iodide Solution

Phase	Age of PI (days)				Mean (SD)
	1	7	14	20	
	%	%	%	%	%
G_1	36.3	36.2	32.8	35.8	35.3 (1.7)
S	52.4	49.6	55.7	48.1	51.5 (3.8)
G_2+M	11.3	14.2	11.4	16.1	13.3 (2.3)
CV(G_1)	6.0	6.0	7.0	6.7	6.4 (0.5)
CV(G_2+M)	4.5	3.9	5.0	3.4	4.2 (0.7)

and were found to be 73.9, 73.9, 74.4 and 75.4 μM, respectively. If these are converted to weights (mol wt of 668.4), the corresponding concentrations in grams per liter were 0.0494, 0.0494, 0.497, and 0.0504. Differences in these values are within the range of experimental error in weighing and mixing. Thus, it is clear that the stain is stable for at least 20 days and probably considerably longer.

To confirm this observation, we stained cells and analyzed them by flow cytofluorometry, using each of the prepared staining solutions. The resulting fluorescence histograms (not shown) were very similar; the numerical data are listed in Table III. It is evident that there is no significant difference in the results from the different stain preparations, and there is no suggestion of a trend in progressing from the freshest to the oldest preparations.

Effect of Ribonuclease Incubation

To determine whether there was significant binding of propidium iodide to RNA in our cell line, which might have the effect of altering the fluorescence histogram and the computed percentages of cells in the different phases of the cell cycle, we incubated cells for 45 or 105 min with or without ribonuclease, as described previously. The results obtained by mathematical analysis of the data are listed in Table IV. There appears to be no significant difference in the results between sam-

TABLE IV

Effect of Ribonuclease Incubation

Phase	45-min incubation			105-min incubation			Mean (SD)*
	Control	RNAse (Na Cit)	RNAse (H_2O)	Control	RNAse (Na Cit)	RNAse (H_2O)	
	%	%	%	%	%	%	%
G_1	32.8	31.9	31.6	34.2	30.2	32.5	32.2 (1.3)
S	53.8	52.0	56.1	54.1	51.6	48.6	52.7 (2.6)
G_2+M	13.4	16.1	12.3	11.7	18.9	18.8	15.2 (3.2)
CV(G_1)	7.0	7.5	7.0	7.0	7.5	7.0	7.2 (0.3)
CV(G_2+M)	5.0	6.5	6.0	5.0	7.5	7.0	6.2 (1.0)

* These were averaged over all six samples.

ples incubated with and without ribonuclease, or between samples incubated for different times.

That the ribonuclease was active was demonstrated by incubating the supernate from the above samples with cells prelabeled with tritiated uridine, as described in Materials and Methods. Liquid scintillation counting showed that the radioactivity of ribonuclease-treated cells was less than 10% of that of cells not exposed to ribonuclease. There was no significant difference in the activity of samples incubated for 45 min vs 105 min, or in that of samples incubated in 0.1% sodium citrate vs. distilled water.

DISCUSSION

Examination of the figures and tables reveals that although no systematic changes with the various conditions of staining were noted, there appeared to be random fluctuation in the fluorescence histograms and in the computed fractions of cells in the G_1, S, and G_2+M phases of the cell cycle. Within a single experiment in which all cells were obtained from the same culture flask, the three factors that are probably responsible for these fluctuations were: (*a*) statistical fluctuations due to the limited number of cells recorded per channel; (*b*) instability in the electronic circuitry, the flow system, or the laser light intensity; and (*c*) sensitivity of the mathematical method used for the data analysis to small differences in the G_1 and G_2+M regions of the fluorescence histograms. The results obtained in different experiments were also influenced by slight differences in growth conditions or proliferative state.

Our experience with the rather complex equipment used in flow cytofluorometry indicates that instrumental instability is a major limiting factor in the accuracy with which a given histogram can be reproduced in a rerun of the same sample. Such instability can be reduced by appropriate adjustment and maintenance of the equipment. We found that a major source of instability in our instrument was fluctuation in the photomultiplier tube voltage supply; we were able to reduce this consideraby by providing an external regulated power supply for the PMT.

Statistical fluctuation can in principle be reduced by accumulating more cells in the histogram, but in practice this will be effective only if the machine instability is sufficiently low. The mathematical method used to evaluate the data is also sensitive, under certain conditions, to relatively small changes in the data in the regions of the G_1 and G_2+M compartments. While this sensitivity permits even small perturbation-induced changes in the phase distribution of the cells to be detected and quantitated, it can, on the other hand, lead to erroneous conclusions if the data are not sufficiently accurate.

Comparison of the propidium iodide technique with the acriflavine-Feulgen staining procedure shows that the two methods agree very closely with one another. Since the latter method is ordinarily accepted as being specific for DNA, this implies that if there is in fact any binding of propidium iodide to RNA or other macromolecules, such binding is either negligible or is proportional to the DNA content of the cells. Observations of propidium iodide-stained cells with a fluorescence microscope showed that, under the hypotonic conditions of staining, the nuclei were intact and only small fragments of cytoplasm adhered to them. While the nuclei exhibited strong fluorescence, none of the cytoplasmic material was fluorescent. When the stain was prepared in 1.12% sodium citrate rather than 0.1%, only the few cells in which the cell membrane was disrupted were fluorescent; the majority were intact and not fluores-

cent. These visual observations are in agreement with flow cytofluorometry with propidium iodide prepared in 1.12% sodium citrate; in this case, most cells appeared at very low channel locations, close to the fluorescence threshold.

The experiment in which cells were incubated with ribonuclease before staining demonstrated that such incubation did not significantly affect the results. While it has been found that a portion of the RNA in mammalian cell nuclei is resistant to degradation by ribonuclease, and that a part of this compartment may be double-stranded and hence could bind propidium iodide by intercalation, the relative amount of such RNA is probably very small in most cases (14, 16, 19).

Autoradiography after pulse labeling with tritiated thymidine indicated that the observed labeling indices agree reasonably well with the predicted percentages of cells in the S phase, although the former were somewhat higher. Only in experiment C was the computed percentage of cells in S more than two standard deviations from the mean labeling index. This discrepancy might be explained by noting that the period of incubation with tracer was 1 h. If we make the assumption that the rate of increase of labeling index during this incubation period (due to entrance of G_1 cells into S) is the inverse of the 16-h population doubling time, then a "corrected" estimate of labeling index would be 56.3%, which is very close to the cytofluorometrically derived value of 54.3%.

While our results imply that Krishan's new propidium iodide staining procedure is accurate and reliable, preliminary work with two cell lines growing in monolayers has shown that the standard staining procedure must be modified in some cases, as described in the appendix. Comparison of the histograms after staining of cells either after trypsinization or while still attached to the growth vessel indicates that, while trypsinization can alter the fluorescence distribution of the cell population, it does not invariably do so.

APPENDIX

Application to Other Cell Lines

Although most of our work with propidium iodide has utilized the SK-L7 cell line, we have also applied the method to two cell lines growing in monolayers, i.e., Chinese hamster ovary (CHO) cells and the human embryonic diploid fibroblast line WI-38. These studies indicate that the method described above for cells in suspension culture (and which is also satisfactory for bone marrow and blood cells from patients with leukemia [7, 8]) must be altered for cells growing in monolayers. While more work must be performed to establish whether these altered methods yield accurate quantitative cell cycle distributions in these cell lines, it is relevant to summarize the revised staining procedures here.

CHO or 30th passage WI-38 cells were grown in Ham's F12 medium without antibiotics, supplemented with 10% fetal calf serum (GIBCO). When cells growing in log phase were harvested by trypsinization and stained by the method outlined above, very few cells were fluorescent. Examination of the cells by fluorescence microscopy revealed that almost all the cells were intact and nonfluorescent. In an attempt to render the cell membrane permeable to the dye, we carried out the following procedure. After trypsinization and centrifugation, the centrifuge tubes containing the cells were decanted rather than aspirated, and excess medium was removed from the lips of the tubes. Cold propidium iodide solution was then added, and the cells were resuspended and allowed to stand for 10 min at room temperature before being placed on ice. The cells were maintained in an ice bath for a minimum of 10 min before analysis by cytofluorometry. We noted that the incubation at room temperature was not always necessary, but the results appeared to be more consistent if this was done.

With these modifications, the CHO cells were adequately stained by propidium iodide, and the fluorescence histograms appeared to be satisfactory. However, this treatment did not yield acceptable results with WI-38 cells, as seen in Fig. 3 (bottom). For these cells, the staining procedure was further modified to avoid the use of trypsin. In this method, petri dishes containing the growing cells were removed from the incubator and rinsed three times with 5 ml of cold propidium iodide solution. 5 ml of the dye were then added and the dishes were refrigerated for 10 min. Cells were then dislodged by repeated pipetting, placed in a test tube in an ice bath, and analyzed by flow cytofluorometry. Short-term incubation at room temperature or further standing in ice did not affect the histograms. The results are shown in the upper portion of Fig. 3. The pattern was stable for at least 24 h. This method for staining attached cells without the use of trypsin was effective with CHO cells as well, and yielded histograms very

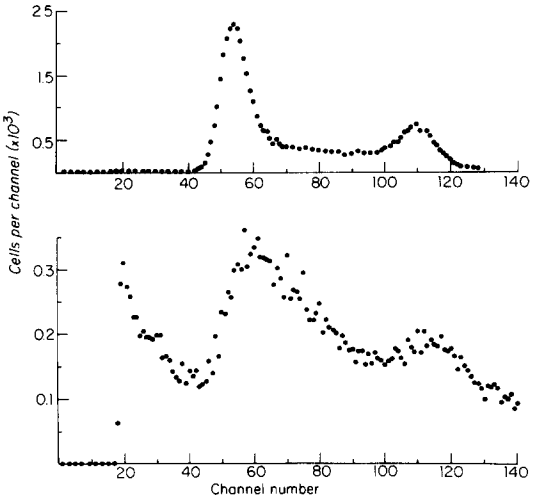

FIGURE 3 Propidium iodide staining of attached cells (top) or trypsinized cells (bottom). Histograms of WI-38 cells stained with propidium iodide according to the modified methods described in the Appendix. These represent the raw data, before analysis. The upper portion of the diagram corresponds to cells stained "in situ" i.e., while attached to the surface of the petri dish; the lower diagram represents cells detached by trypsinization prior to staining. Cell loss from the latter sample was about twice that of the former.

similar to those obtained when these cells were trypsinized.

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