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# Kinetics of Histone Endocytosis in Chinese Hamster Ovary Cells

## A FLOW CYTOFLUOROMETRIC ANALYSIS*

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**The endocytosis of histones by cultured cells was examined by flow cytofluorometry. Monolayer cultures of Chinese hamster ovary cells were incubated with fluorescein-labeled histone for various periods of time and then trypsinized to remove surface-bound protein. Internalization followed first order kinetics with a half-time of 45 min, and was linear in histone concentration up to 80 μg/ml. Since fluorescein fluorescence decreases with decreasing pH, the fluorescence of labeled histone contained in lysosomes was expected to be decreased relative to its fluorescence at neutral pH. This was demonstrated by using chloroquine to increase the lysosomal pH of intact cells. The fluorescence of labeled histones incorporated into cells increased when those cells were incubated with 50 μM chloroquine and remeasured. This provides a method for measuring the kinetics of entry of a fluorescent probe into lysosomes. Internalization into lysosomes began almost immediately upon addition of histone, but stable nonlysosomal fluorescence appeared only after a lag of 1 h. Using suspension cultures, the short term binding and internalization kinetics were also measured. In pulse-chase experiments, lysosomal fluorescence decreased with a half-time of 30 min, but nonlysosomal fluorescence decreased with a half-time of almost 12 h, probably as a result of cell division. These results demonstrate the usefulness of flow cytometry for the quantitation and characterization of endocytosis in cultured cells.**

The nucleosome core histones, H2A, H2B, H3, and H4, facilitate the uptake of exogenous protein when added to the growth medium of cultured mammalian cells (15). Histones share this carrier property with other basic macromolecules such as poly(L-ornithine), polylysine, protamine, and DEAE-dextran (14). Large size and abundance of positively charged residues increase the stimulating effect of these molecules. At concentrations of 100 μg/ml, calf thymus histone stimulates a 15-fold increase in the uptake of albumin into sarcoma 180 cells (15). The sensitivity of the albumin internalization process to temperature is altered by the addition of histones, suggesting that the histone initiates an uptake process that differs qualitatively from that of albumin alone (15). The precise mechanism of this uptake has yet to be elucidated.

Flow cytometric systems allow the rapid measurement of cellular properties on a cell by cell basis (for reviews, see Ref. 7). We have used this method to measure the incorporation of fluorescently labeled histone into cultured cells. In this paper, we demonstrate the advantages of flow cytometry for the study of endocytic processes.

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### EXPERIMENTAL PROCEDURES

*Cells and Reagents*—The Chinese hamster ovary cell line K1 (4) was maintained in monolayer culture in plastic Petri dishes at an average cell density of $7 \times 10^4$ cells/cm$^2$. Ham's F12 medium (3), supplemented with 5% fetal calf serum (Gibco), 100 units/ml of penicillin and 100 μg/ml. streptomycin sulfate, was used for all experiments.

Sodium iodoacetate and chloroquine (diphosphate salt) were obtained from Sigma.

*Isolation and Labeling of Chicken Erythrocyte Histones*—Total chicken erythrocyte histones were prepared from purified nuclei (9) by salt extraction, ultracentrifugation, and BioRex 70 chromatography (17). The fluorescent labels IAAF[1] (Molecular Probes, Inc., Plano, TX) and IATR (Research Organics, Inc., Cleveland, OH) were used to label specifically cysteine 110 of H3, since chicken histones contain no other cysteine residues. A 0.5 to 1.5 mg/ml solution of histone in 0.2 mM EDTA, 10 mM Tris, pH 8.0 (Tris/EDTA), was reduced with a 100-fold molar excess of β-mercaptoethanol for 30 min at 37 °C. The reduced histones were dialyzed at 4 °C for 90 min against 1000 volumes of Tris/EDTA containing 2 M NaCl and enough β-mercaptoethanol to result in a 10-fold molar excess.

A 100-fold molar excess of IAAF was dissolved in a volume of 2 M NaCl, Tris/EDTA equal to that of the histone solution, and filtered through a Millex-HA 0.45 μm filter (Millipore Corp., Bedford, MA). The histone and dye solutions were mixed and kept at room temperature for 2 h in the dark. The solution was dialyzed against 1000 volumes of 2 M NaCl, Tris/EDTA four times for 90 min or until the dialysate contained no fluorescence. A 100-fold molar excess of β-mercaptoethanol was added to the first dialysis to remove any unreacted dye. Labeling was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (6). The extent of labeling was measured spectrophotometrically; more than 90% of the H3 molecules were labeled. Fluorescence measurements were made using a Schoeffel RRS 1000 spectrofluorimeter interfaced to a Tektronix 31 programmable calculator.

IATR labelings were performed similarly, except that a 10-fold molar excess of dye was used, and β-mercaptoethanol concentrations were reduced by a factor of 10.

*Incubation of Cells with Labeled Histone*—All experiments (except the measurement of initial binding and internalization kinetics using cell suspensions) were performed on nonconfluent monolayers of CHO cells at an average cell density of $5 \times 10^4$ cells/cm$^2$. Labeled histone was added in phosphate-buffered saline to a maximum of 20% of the final volume. All incubations were at 37 °C and in an atmosphere of 5% $CO_2$ (except where noted). At the end of the incubation period, the plate was washed with 2 ml of 0.05% trypsin in phosphate-buffered saline, and 0.3 ml of the same solution was added. After 10 min at 24 °C, 2 ml of medium plus serum was added, and the cells were removed from the plate by repeated pipetting. Fluorescence

[1] The abbreviations used are: IAAF, 5-iodoacetamidofluorescein; IATR, iodoacetamido-tetramethyl-rhodamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHO, Chinese hamster ovary.

microscope observation revealed negligible surface fluorescence, with the vast majority of the fluorescence in small endocytic vacuoles.

To test further the effectiveness of these conditions at removing surface-bound histone, monolayer cultures were prechilled for 30 min at 4 °C and then incubated with 20 μg/ml IAAF-histone for 60 min at 4 °C. Cells were then harvested with trypsin at 24 °C as described above, but after incubation for from 3 to 20 min. The total amount of histone per cell under these conditions was determined by removing the cells from a duplicate plate using 0.5 mM EDTA in phosphate-buffered saline (10 min incubation at 24 °C) instead of trypsin. The samples were then analyzed by flow cytometry at 0 °C. The amount of the total bound histone (from EDTA measurement) which remained associated with the cell reached a minimum of 38% after 3 min of trypsinization. This amount probably represents histone which was slowly internalized at 4 °C, as well as some which was internalized during the trypsinization. This histone which was not removed by trypsin was only 15% of the total internalized during 60 min when incubation was at 37 °C instead of 4 °C.

Samples were analyzed by flow cytometry at room temperature (unless otherwise specified) within 60 min after trypsinization. No significant changes in total fluorescence were detected when samples were reanalyzed as much as 5 h later.

Short term binding and internalization was measured using suspensions of trypsinized cells. Cells were incubated with histone in fresh medium at 37 °C and then diluted 10-fold into 0 °C phosphate-buffered saline and analyzed immediately at 0 °C.

For some experiments, nuclei were purified from a fraction of the cells as previously described (9) with the exception that only 0.1% Nonidet P-40 was used. Nuclei were analyzed at 0 °C in 10 mM Tris, pH 8, 10 mM NaCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40.

The temperature dependence of histone uptake was determined using medium containing 14 mM HEPES, pH 7.0, instead of carbonate (to prevent changes in pH caused by changes in $CO_2$ concentration). Petri dishes containing growing monolayers were preincubated at either 4 °C or 37 °C for 30 min, and then the medium was replaced with medium (at the appropriate temperature) containing HEPES. Plates were incubated with labeled histone at the previous temperature and then harvested by trypsinization as described above.

*Flow Cytometry*—A FACS-IV cell sorter (Becton Dickinson Co., Mountain View, CA) interfaced with a VAX-11/780 computer (Digital Equipment Corp., Maynard, MA) was used for all analyses. Excitation was with a Spectra-Physics Model 164-05 argon ion laser. For cells labeled with IAAF-histone, excitation was with the 488 nm line, and emission was measured using a 530 nm long pass optical glass filter. For combined IAAF and IATR measurements, excitation was with the 497 nm line. The 530 nm long pass filter and a 540 nm short pass filter were used to detect IAAF emission, and a 580 nm long pass and a 590 nm long pass filter were used for IATR. Dual parameter data were corrected digitally for 37% leakage of IAAF fluorescence into the IATR detector. Optical filters were purchased from Ditric Optics (Marlboro, MA). Fluorescent microspheres (Polysciences, Warrington, PA; No. 9719, Lot 4-1591) were used to align and calibrate the instrument. For 1.333 μm diameter microspheres (coefficient of variation = 1.2%), typical coefficients of variation were 3.9% for scatter and 3.4% for fluorescence. At least 10,000 cells were analyzed for each sample. Interactive least squares fitting was used for analysis of kinetics (8).

Mean fluorescences are expressed as bead equivalents. To determine the amount of IAAF-histone corresponding to one bead equivalent, monolayers were incubated with and without 20 μg/ml of IAAF-histone and then trypsinized as above. The mean fluorescence per cell (in bead equivalents) was determined for both labeled ($F_1$) and unlabeled ($F_2$) samples by flow cytometry, and the concentration of cells was measured using a hemacytometer. The spectrofluorimeter was then used to measure the fluorescence of three samples: a known concentration ($c_1$) of labeled ($F_3$) and unlabeled ($F_4$) cells, and a known concentration ($c_2$) of IAAF-histone ($F_5$). The mass of IAAF-histone equal to one bead equivalent was then calculated:

$$\frac{(F_3 - F_4)c_2}{(F_1 - F_2)c_1F_5}$$

Using this method, one bead equivalent was determined to be equal to the fluorescence of $2.33 \times 10^{-12}$ g of IAAF-histone at pH 7.4.

## RESULTS

*Internalization of Exogenous Histone by CHO Monolayers*—Treatment of chicken erythrocyte histones with IAAF results in the specific labeling of cysteine 110 of H3. When CHO cells are incubated in the presence of this fluorescent-labeled histone mixture and then trypsinized and analyzed by flow cytometry, light scattering and fluorescence distributions such as those in Fig. 1 are obtained. Dead cells and debris have been eliminated from the analysis by using a light scattering threshold. (However, the number of dead cells observed using a lower light scattering threshold was negligible.) The cellular fluorescence distribution has a fairly sharp peak at a value much higher than the autofluorescence of untreated control cells. The mean fluorescence per cell is

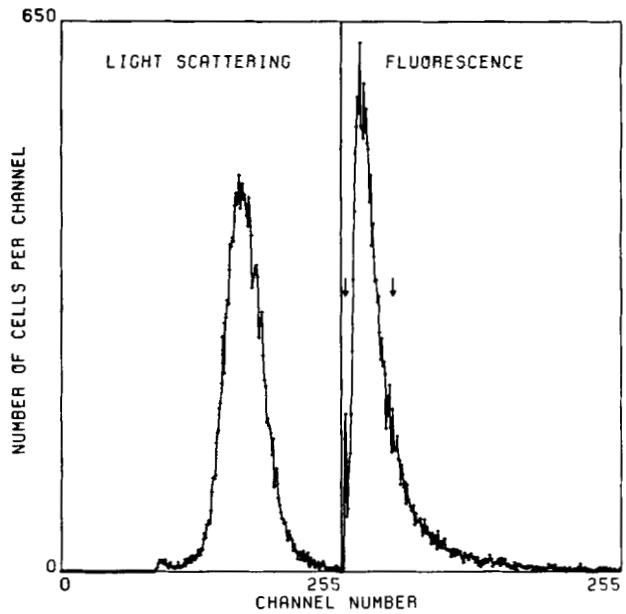


FIG. 1. **Typical light scattering and fluorescence histograms.** Cells were labeled with 20 μg/ml of IAAF-histone for 24 h and then trypsinized and analyzed as described under "Experimental Procedures." The coefficient of variation of the light scatter peak is 13%. Only cells with a light scatter greater than channel 85 are displayed in *both* histograms. This eliminates dead cells and debris. *Arrows* at fluorescence channels 4 and 42 show the peak position for control cells (autofluorescence) and fluorescent microspheres, respectively.

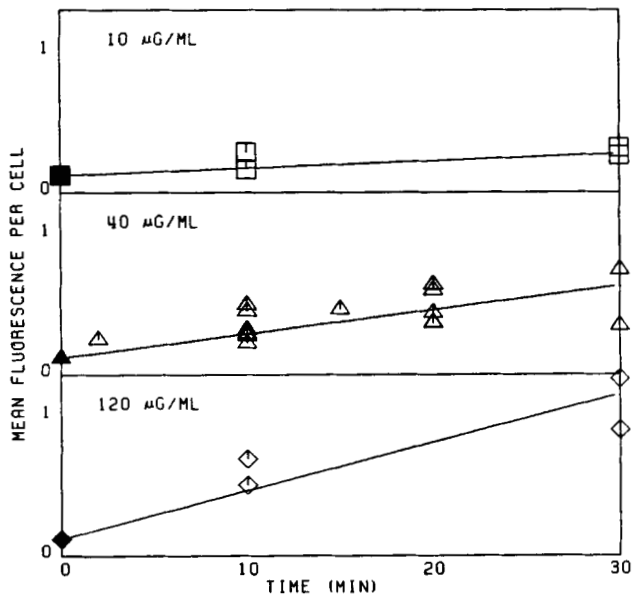


FIG. 2. **Initial kinetics of internalization of IAAF-histone at 37 °C.** The *closed symbols* represent control (untreated) cells.

calculated from this distribution, and is found to be close to the peak (modal) fluorescence value. (The mean values are calibrated using the values for a sample of fluorescent microspheres analyzed at the same time; in this way, data from different experiments can be combined and compared.) For the experiments described below, the calibrated mean fluorescence per cell has been used as a measure of the amount of histone bound to and/or incorporated by cultured cells under various conditions. The fluorescence of IAAF-labeled H3 is assumed to be proportional to amount of total histone.

To examine histone internalization (while disregarding surface binding), monolayer cultures were incubated for 10 or 30 min with from 10 to 120 μg/ml of IAAF-histone. The cells were then harvested by trypsinization (which removes the majority of histone bound to the cell surface; see "Experimental Procedures") and analyzed by flow cytometry. The initial time course of incorporation is linear, and the initial rate is roughly proportional to histone concentration (Fig. 2 and Table I).

To determine whether saturation was occurring at high histone concentration, monolayers were incubated with from 10 to 120 μg/ml of IAAF-histone for 10 min, 30 min, or 2 h, and then trypsinized and analyzed as before. The amount of histone internalized in a fixed time levels off between 80 and 120 μg/ml (Fig. 3).

We found that 100 μM iodoacetate had no effect on the amount of histone internalized at up to 80 μg/ml for up to 60 min (data not shown). Since phagocytosis is inhibited by iodoacetate (12), and since soluble protein was used, it would appear unlikely that phagocytosis is involved in histone internalization under our conditions.

The internalization of IAAF-histone is reduced at 4 °C relative to 37 °C (Fig. 4). This is in agreement with previous results for other endocytic processes (1, 2, 16, 18). We conclude that the internalization of IAAF-histone results from saturable binding to the cell surface, followed by temperature-dependent internalization.

*Detection of Histone in Lysosomes using Chloroquine*—The fluorescence of fluorescein is sensitive to pH (10). Table II shows that the relative fluorescence yield of IAAF-histone increases approximately 8-fold from pH 4 to pH 7.4. Since the pH of lysosomes is approximately 4.5 (10), we expect that the fluorescence yield of histone incorporated into lysosomes would be decreased relative to that at neutral pH. We have used the drug chloroquine, which increases the pH of lysosomes of intact cells from 4.5 to 6.5 (10), to determine the fraction of total internalized histone that is present in lysosomes.

Monolayer cultures were incubated with 20 μg/ml of IAAF-

TABLE I

*Effect of histone concentration on internalization*

| Histone | Initial slope[a] | $k^b$ | Plateau value[c] | $N^d$ |
|---|---|---|---|---|
| *μg/ml* | *pg/min/cell* | *min*$^{-1}$ | *pg/cell* | |
| 10 | 0.0119 | 0.0220 | 1.11 | 6 |
| 20 | 0.0154 | 0.0090 | 2.12 | 51 |
| 40 | 0.0401 | 0.0104 | 2.49 | 24 |
| 80 | 0.0576 | 0.0173 | 4.65 | 12 |
| 120 | 0.0806 | 0.0110 | 7.52 | 6 |

[a] From linear fit from 0 to 30 min.
[b] From nonlinear least squares first order fit to all data. The root mean square errors of the fits were between 0.4% and 2.3% of the maximum fluorescence. Similar rate constants result from dividing the initial slope by the maximum fluorescence.
[c] From nonlinear fit.
[d] Number of samples used in determination.

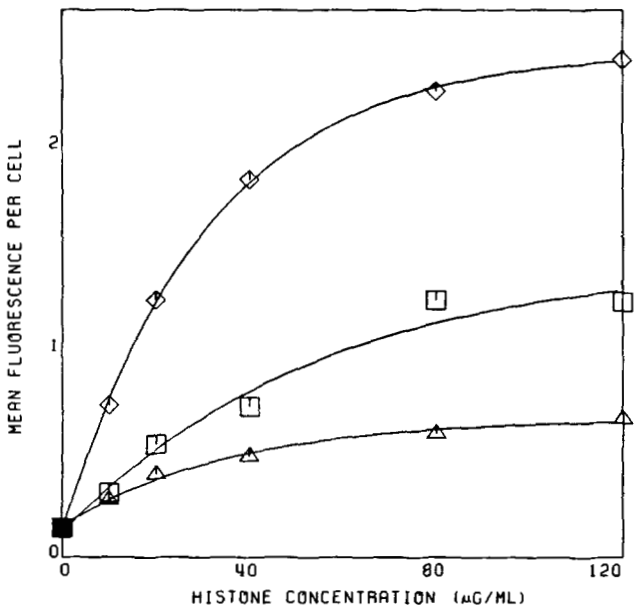


FIG. 3. **Concentration dependence of histone endocytosis at 37 °C.** Cells were labeled with varying concentrations of IAAF-histone for 10 min (△), 30 min (□), or 2 h (◇). The *closed symbol* represents untreated cells. The lines are first order fits to the data (8).

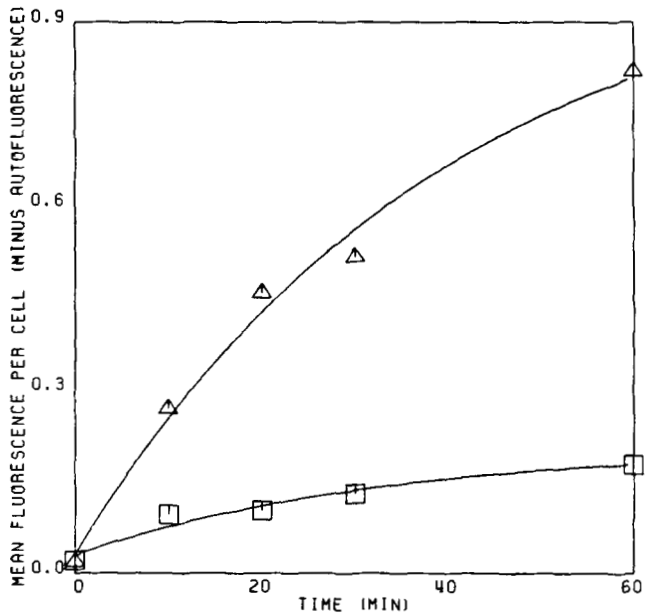


FIG. 4. **Temperature dependence of histone endocytosis.** Cells were labeled with 20 μg/ml of IAAF-histone for varying times at either 4 °C (□) or 37 °C (△) and then trypsinized and analyzed. The lines are first order fits to the data (8). The autofluorescence of untreated cells has been subtracted from each point.

TABLE II

*pH dependence of IAAF-histone fluorescence*

| pH | $F_{530}$[a] | $F/F_{pH\ 4}$ |
|---|---|---|
| | *10*$^{-6}$ *be* | |
| 4.0 | 0.57 | 1.0 |
| 5.0 | 1.21 | 2.1 |
| 6.0 | 2.80 | 4.9 |
| 7.4 | 4.29 | 7.5 |

[a] Fluorescence emission at 530 nm for 10 μg/ml of IAAF-histone in phosphate-buffered saline (converted to bead equivalents). Excitation at 490 nm.

histone for from 10 min to 24 h and prepared for analysis as described above. The mean fluorescence per cell for each sample was first determined in medium and then again at least 10 min after the addition of 50 μM chloroquine. Fig. 5*a* shows that the addition of the chloroquine produces an increase in fluorescence. The ratio of intensities with and without chloroquine is related to the fraction of histone contained in lysosomes. As can be seen in Fig. 5*b*, this ratio reaches a peak between 1 and 2 h after addition of histone, and then subsides before slowly increasing again from 2 to 24 h.

Using previously determined values for the pH of lysosomes with and without chloroquine treatment (10), the amount of lysosomal and nonlysosomal IAAF-histone can be calculated. The total fluorescence observed before ($F_n$) and after ($F_c$) chloroquine treatment can be expressed in terms of the relative fluorescence yields ($Q$) at the lysosomal pH (4.5), the lysosomal pH after the addition of chloroquine (6.5), and the nonlysosomal pH (assumed to be 7.4):

$$F_n = M_L{}^*Q_{4.5} + M_V{}^*Q_{7.4} \quad (1)$$

$$F_c = M_L{}^*Q_{6.5} + M_V{}^*Q_{7.4} \quad (2)$$

where $M_L$ and $M_V$ are the total amounts of lysosomal and nonlysosomal fluorescent histone (per cell), respectively. Solving for these amounts, we obtain

$$M_L = \frac{F_c - F_n}{Q_{6.5} - Q_{4.5}} \quad (3)$$

and

$$M_V = \frac{Q_{6.5}{}^*F_n - Q_{4.5}{}^*F_c}{(Q_{6.5} - Q_{4.5})^*Q_{7.4}} \quad (4)$$

The results of these calculations are presented in Fig. 5, *c* and *d*. Lysosomal incorporation of histone increases almost linearly from the earliest time point. On the other hand, nonlysosomal incorporation occurs only after a lag of more than 1 h.

*Measurement of Surface Binding and Subsequent Internalization using Cell Suspensions*—In the previous experiments, histone bound to the cell surface was removed during harvesting of cells by trypsinization. While surface binding could be measured using methods of detachment that do not use trypsin (for example, incubation in EDTA), the rapidity with which histone is internalized makes the use of these methods difficult. Instead, we have analyzed the early steps in the internalization process using cell suspensions.

Trypsinized cells were incubated in medium at 37 °C in the presence of 20 μg/ml of IAAF-histone for from 0 to 30 min, and then analyzed as described under "Experimental Procedures" (Fig. 6). The binding is extremely rapid, so that the fluorescence of the earliest sample (approximately 5 s) is many times the autofluorescence of the untreated control sample. The fluorescence increases up to 5 min, after which it begins to decrease. That this drop is due to entry of the histone into lysosomes was demonstrated by adding chloroquine to the same samples and remeasuring the fluorescence (Fig. 6*a*, *upper line*). The ratio of fluorescence in the presence and absence of chloroquine (corrected for autofluorescence) increases after a lag of approximately 5 min (Fig. 6*b*).

*Processing of Histone after Internalization*—To determine the fate of internalized histone, monolayer cultures were incubated for 1 h with IAAF-histone and then for various periods of time with unlabeled histone. The amount of labeled histone per cell decreases rapidly during this chase (Fig. 7*a*), with a half-time of approximately 30 min. The amount of histone degradation caused by this process was determined by incubating monolayers with fluorescent histone for various times and then measuring the total and acid-soluble fluorescence of the culture medium. The amount of histone degraded increased linearly with incubation time, and by 24 h, only 35% of the fluorescent label was still attached to intact histone (data not shown).

The fluorescence which remains in the cell after longer

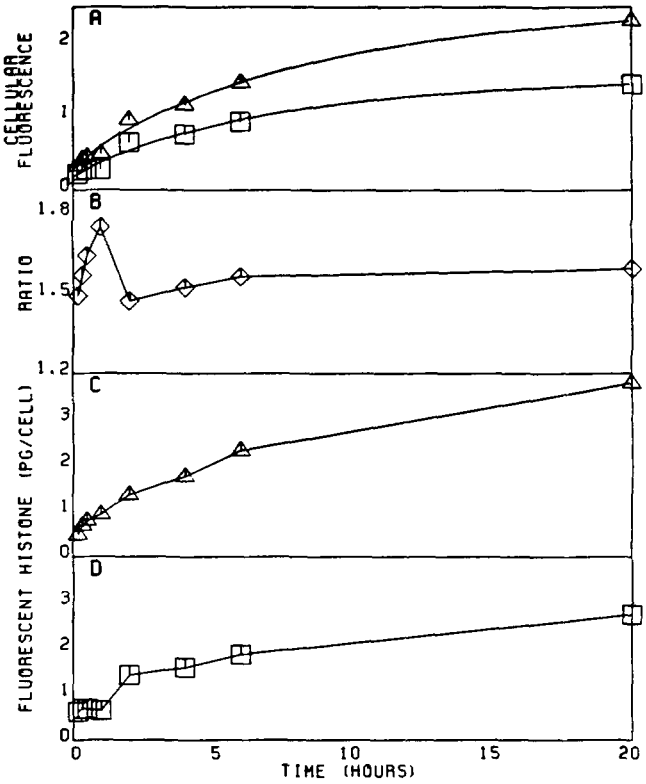


FIG. 5. **Detection of histone in lysosomes using chloroquine.** *A*, cells were incubated with 20 μg/ml IAAF-histone for various times and then trypsinized. Each sample was analyzed before (□) and after (△) the addition of 50 μM chloroquine. *B*, ratio of the measurements with and without chloroquine (corrected for autofluorescence). *C* and *D*, amount of lysosomal histone ($M_L$) and nonlysosomal histone ($M_V$) calculated using equations 3 and 4 with $Q_{4.5} = 0.946 \times 10^{11}$ bead equivalents/g, $Q_{6.5} = 3.23 \times 10^{11}$ bead equivalents/g and $Q_{7.4} = 4.29 \times 10^{11}$ bead equivalents/g (interpolated from Table II).

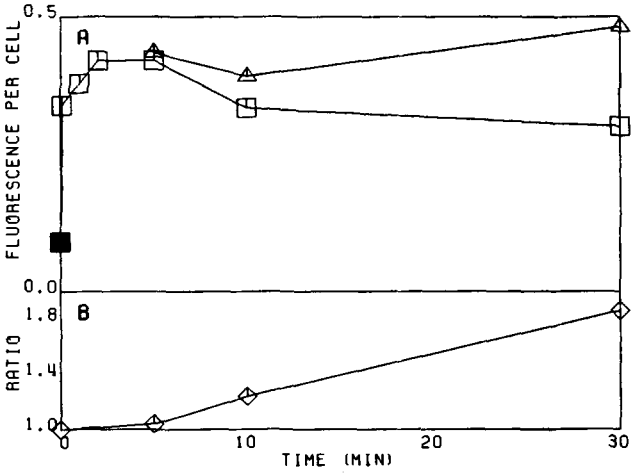


FIG. 6. **Surface binding and subsequent internalization by cell suspensions.** Trypsinized cells were incubated with 20 μg/ml of IAAF-histone for various times and diluted into cold phosphate buffered saline before analysis. *A*, The mean fluorescence per cell was measured before (□) and after (△) addition of 50 μM chloroquine. The fluorescence of control cells is also shown (■). *B*, ratio of the measurements with and without chloroquine (corrected for autofluorescence).

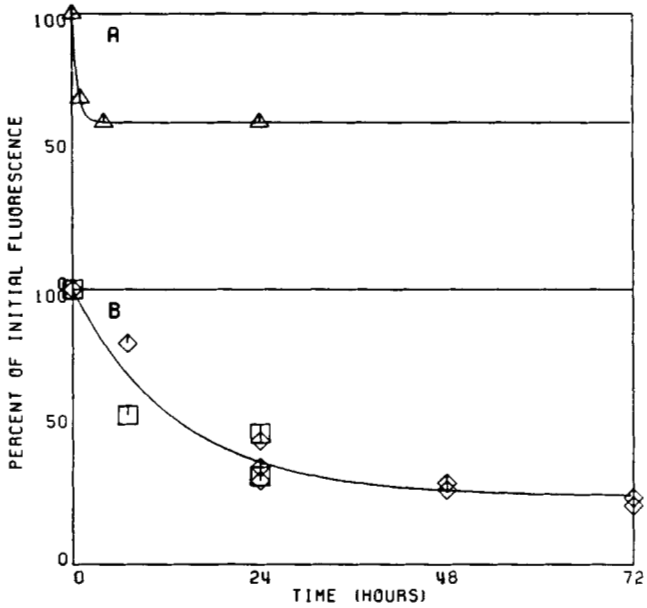


FIG. 7. **Subsequent processing of internalized histone.** *A*, Cells were incubated with 40 μg/ml of IAAF-histone for 1 h and then with 40 μg/ml of unlabeled histone for various times. *B*, cells were incubated with 20 μg/ml of IAAF-histone for 24 h (◇) or 48 h (□) and then without histone for various times.

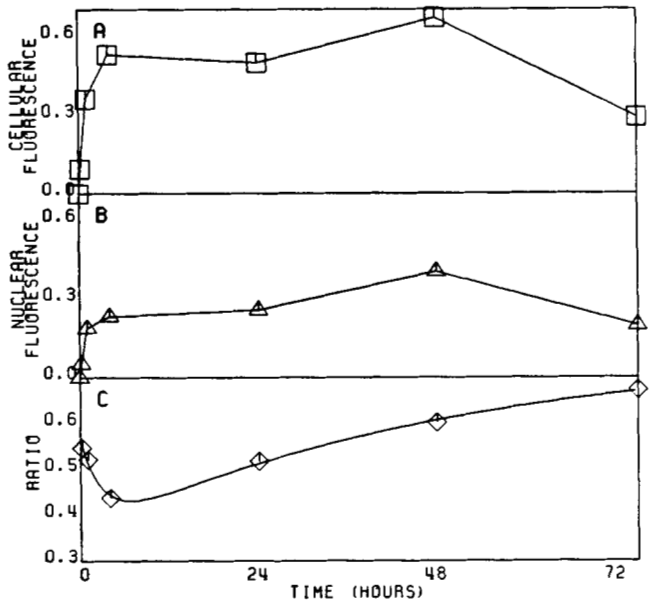


FIG. 8. **Association of internalized histone with nuclei.** *A*, mean fluorescence per cell after incubation with 20 μg/ml IAAF-histone for various times. *B*, mean fluorescence per nucleus for nuclei isolated from samples in *A*. *C*, Fraction of total cellular fluorescence associated with nucleus.

labelings also decreases when the culture medium is replaced with medium containing unlabeled histone (Fig. 7*b*). In contrast to the turnover of the histone in lysosomes, the decrease in nonlysosomal fluorescence is much slower ($t_{1/2}$ = 690 min), and probably results from a combination of exocytosis and cell division. The fluorescence stabilizes at approximately 25% of the amount present at the beginning of the chase.

The slime mold *Physarum polycephalum* internalizes fluorescent-labeled histone, and incorporates it into nucleosomes (13). To determine whether a similar process can occur in mammalian cells, the amount of cellular fluorescence associated with the nucleus was measured. Cells were labeled for various times and the fluorescence per cell was determined. Nuclei were then isolated from these samples and the fluorescence per nucleus was measured using the same fluorescence detection settings (Fig. 8; the light scattering detection settings were changed to measure only intact nuclei). Significant amounts of histone were found associated with isolated nuclei after short labelings, probably as a result of contamination due to lysosomal breakage during nuclear isolation. At longer times (when the amount of labeled histone in lysosomes has decreased), much of the cell fluorescence was nuclear. After 48 h, there was approximately 0.94 pg of IAAF-histone/nucleus (each nucleus contains approximately 5 pg of endogenous histone[2]). However, no fluorescence was found in isolated nucleosomes (data not shown), in contrast to the previous results with *Physarum* (13).

*Cellular Variation in Histone Internalization*—The experiments described above have utilized only the mean fluorescence per cell under various conditions. Additional information, however, is contained in the distribution of fluorescence per cell for any individual sample. Under most of the conditions we have used, the number of cells with a given amount of fluorescence decreases exponentially as the fluorescence increases above the peak value. We have used a dual-fluorescence technique to clarify the origin of this unusual distribution.

After short labeling periods, the amount of histone internalized would be expected to reflect statistical fluctuations, differences in cell state (such as position in the cell cycle) and genotypic or phenotypic variation in internalization capacity. After longer labelings, however, the first two factors should be less significant. If major differences in internalization capacity exist, we expect that the amount of histone internalized during one time period would be well correlated with the amount incorporated during a later period. To determine whether this was the case, we incubated monolayer cultures with IAAF-labeled histone for either 13 or 46 h, replaced the medium with medium containing IATR-labeled histone, and incubated for either 30 or 60 min. The cells were then harvested by trypsinization as above, and the amount of each chromophore per cell was determined. Fig. 9, *b* and *c*, presents the results for one 13-h sample in two different ways. The number of cells with specified amounts of each fluorescence are indicated either by apparent height in a perspective plot of a three-dimensional surface (Fig. 9*b*), or by a shade of gray tone in a density map (Fig. 9*c*). The histograms for the separate fluorescences are also shown (Fig. 9, *a* and *d*). The logarithm of the number of cells is plotted *versus* amount of fluorescence in these histograms, showing the exponential distribution described above.

If significant correlation existed between the amount of histone internalized during the two labeling periods, the number of cells per channel in Fig. 9, *b* and *c*, should decrease diagonally from the peak. On the other hand, if there was no correlation, then the number of cells per channel should decrease in both the horizontal and vertical directions from the peak. This is clearly the case for this sample. Similar results were obtained for the 46-h labeling period, and also when cells were incubated with IATR-labeled histone during the first period. Thus, the variation in amount of histone internalized under these conditions would appear to be due to statistical fluctuation.

## DISCUSSION

Previous studies of endocytosis have relied mainly on microscopic observation or radiolabeling. While the first method

[2] L. Chasin, personal communication.

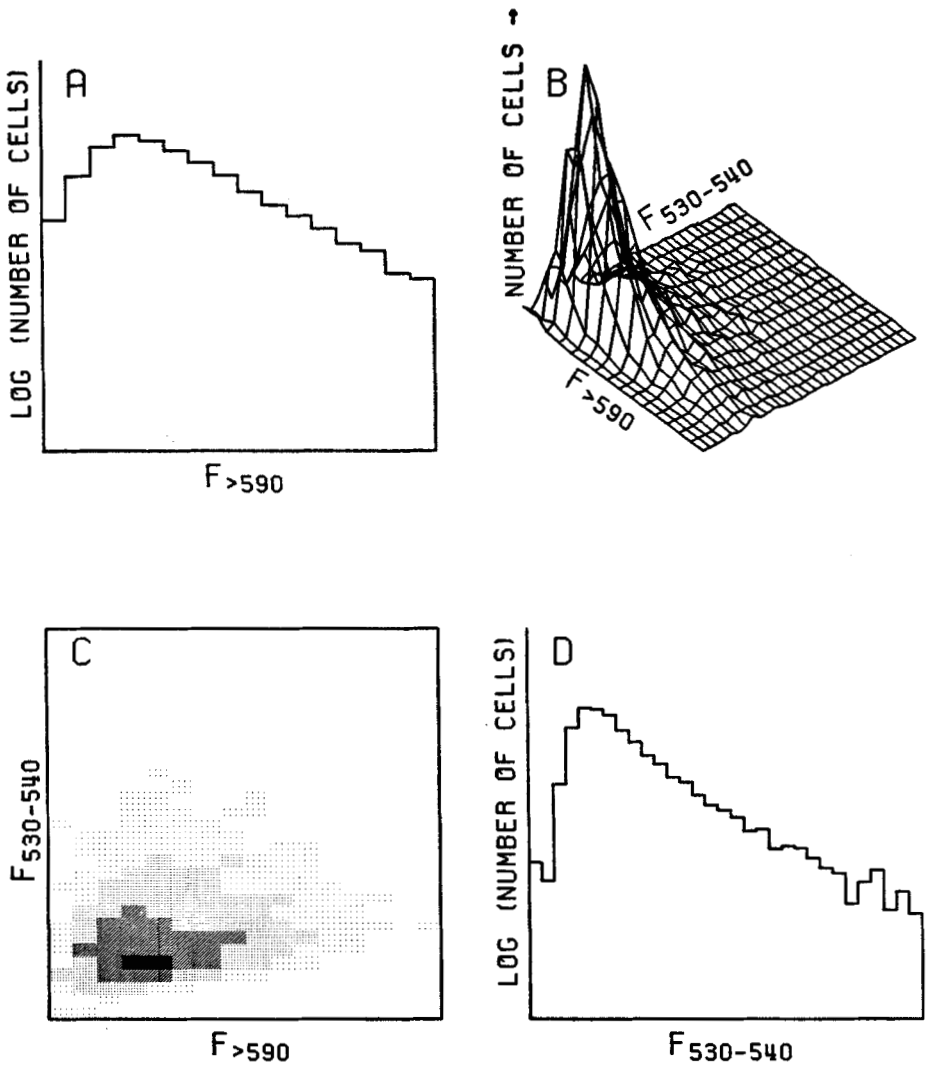


FIG. 9. **Lack of correlation between amount of endocytosis per cell at different times.** *B* and *C*, two-parameter histograms for cells labeled for 13 h with 20 μg/ml of IATR-histone and then for 30 min with 20 μg/ml of IAAF-histone. *B*, Perspective plot in which number of cells is represented by apparent height above a plane; *C*, density map in which the number of cells is represented by a shade of gray tone; *A* and *D*, single-parameter histograms produced from the data in *B* and *C*. *A*, IATR-histone fluorescence. *D*, IAAF-histone fluorescence.

has contributed significantly to the current understanding of endocytic mechanisms, it is difficult to quantitate and very time-consuming. The use of radiolabeled proteins avoids these problems to some extent, but cannot easily provide information on the distribution of protein in the cell population, and may produce incorrect estimates of degradation kinetics, due to the instability of the labeled proteins (11). The flow cytometric method we have described for measuring internalization of protein has significant advantages over these methods, and has been used to study the endocytosis of low density lipoprotein by fibroblasts (5). With this method, measured values do not have to be normalized by the total number of cells, and determinations can be made very rapidly using small numbers of cells. In addition, the variation in fluorescence per cell can be measured (eliminating for example, the possibility that most of the fluorescence is contained in a small fraction of the cells). Cells of interest can also be selected on the basis of measured parameters and physically separated for further analysis. The use of fluorescein-labeled probes allows the determination of the kinetics of entry of endocytosed material into lysosomes, using the chloroquine method we have described above.

The origin of the exponential fluorescence per cell distribution shown in Fig. 9 is at present unknown. The distribution may reflect variation in cell volume and/or surface area.

The results we have presented demonstrate the utility of flow cytometry for the study of endocytosis. Since endocytosis of a fluorescent probe may occur by a number of pathways with very different kinetics, including receptor-mediated endocytosis, non-receptor-mediated adsorptive endocytosis, and fluid phase endocytosis, the internalization rates observed for IAAF-histone may represent a combination of different rates. However, no specific receptor for histone has been demonstrated, and fluid phase endocytosis should be minimal at the concentrations of histone used. Therefore, IAAF-histone should provide a convenient marker for detection of adsorptive endocytosis, especially in combination with chloroquine treatment. Additional studies of the variation in endocytic capacity in cell populations are in progress.

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