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Autofluorescence of Viable Cultured Mammalian Cells

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The autofluorescence other than intrinsic protein emission of viable cultured mammalian cells has been investigated. The fluorescence was found to originate in discrete cytoplasmic vesicle-like regions and to be absent from the nucleus. Excitation and emission spectra of viable cells revealed at least two distinct fluorescent species. Comparison of cell spectra with spectra of known cellular metabolites suggested that most, if not all, of the fluorescence arises from intracellular nicotinamide adenine dinucleotide (NADH) and riboflavin and flavin coenzymes. Various changes in culture conditions did not affect the observed autofluorescence intensity. A multiparameter flow system (MACCS) was used to compare the fluorescence intensities of numerous cultured mammalian cells.

The application of fluorescent flow cytometry and sorting techniques requires the knowledge of the location of various probes. Since numerous fluorescent probes may not be entirely specific for their intended targets, it is especially important to determine their locations when whole, viable cells are being studied. A very rapid and useful first determination of a probe's cellular milieu may be obtained with the use of a fluorescence-light microscope. In this way, various incubation conditions can be screened and the localization of the probes ascertained in at least a general sense: *i.e.*, plasma membrane, cytoplasm or nucleus.

In attempting to screen fluorescent probes which might label specifically the plasma membrane portion of cultured mammalian cells, I have been struck by the relatively high autofluorescence of unlabeled cells immediately after their removal from culture. This autofluorescence, outside the range of intrinsic emission from the aromatic side chains of amino acids, covered a wide range of excitation and emission wavelengths, and obscured the specific visualization of probes with similar excitation and emission wavelengths. The perturbation was particularly evident when cells were labeled only lightly or the probe fluorescence was relatively weak.

Cellular autofluorescence has been noted previously in a variety of cell types (2, 7, 11, 13). It is especially noticeable in plant tissues where, besides the fluorescence from metabolites shared with animal cells, fluorescence arises also from plant materials such as the chlorophyls, alkaloids and flavonoids (11, 13). Collagen and elastin in animal cells connective tissue and lipofuscin have strong autofluorescence, in the blue-green and yellow spectral regions respectively (2, 7, 11, 13). Intracellular porphyrin fluorescence (red) has been used diagnostically in primary human biopsy material (11). It has been suggested that most intracellular fluorescence in the blue spectral region may be due to the reduced form of nicotinamide adenine dinucleotide (NADH) (3, 4, 13). However, although extensive work has been done on the fluorescent components of isolated, purified subcellular fractions such as the mitochondria (2, 3, 13), and some on whole tissues (13), the autofluorescence of various cultured viable cells has not been well-characterized.

The observation and partial characterization of the autofluorescence in cultured mammalian cell lines is reported here. Results from different cell lines and various culture conditions are included. It is evident that this autofluorescence is a normal characteristic of all viable mammalian cells and apparently cannot be removed by any nontoxic procedure.

MATERIALS AND METHODS

Three different clones of Chinese hamster ovary (CHO) cells with different growth properties have been used. AuxB1 CHO cells (a gift from V. Ling), CHO SC1 cells (provided by J. Gray), and CHO Cl4 cells were grown routinely at 37° in suspension cultures in α-MEM (minimal essential medium) (Flow Labs, Inc., Rockville, Md.) supplemented with 10% fetal calf serum (Flow Labs) as previously described (9). The mouse fibroblast cell line, 3T3B, was propogated in monolayer cultures in Dulbecco's MEM (Flow Labs) supplemented with 10% fetal calf serum. B8, a line of Friend erythroleukemia cells (provided by W. Ostertag), were grown in monolayer cultures in Myeloma medium as previously described (10). Various other sera and media tested were as follows: Gibco fetal calf serum and dialyzed fetal calf serum, Flow Labs newborn calf serum, Microbiological Associates (Bethesda, Md.) fetal calf serum; McCoy's 5A medium (Flow Labs) and Ham's F-12 medium (Flow Labs).

Fluorescent components were removed from serum by dextran-charcoal treatment (15). Whole serum, 25 ml, was added to 50 ml packed, wet dextran-charcoal (Darco activated G-60 from Atlas Chemicals Industries, Wilmington, Del.) for 20 min at room temperature. Charcoal was removed by centrifugation and serum was further purified for cell culturing by filtration through a 0.45 μm followed by a 0.20 μm Millipore filter (Millipore Corp., Bedford, Mass.). This treatment removed >95% of the fluorescent component(s) detected in the serum.

Cells were prepared for spectra and photography as described in the figure legends. Uncorrected excitation and emission spectra of all materials were recorded on a Perkin-Elmer fluorescence spectrophotometer (model MPF 4) (Maywood, Ill.), with a Xenon XPO 150 lamp (Xenon Corp., Medford, Mass.). This instrument incorporates right angle optics. Although scatter signals therefore do arise from cell samples, these did not adversely affect the measured spectra. Various

dilutions of cell numbers, resulting in a gradation of optical densities for the cell suspensions, indicated that the observed measured fluorescence was quantitative with cell number. In order to test further for possible distortions due to self-quenching, self-absorption or scattering, standard solutions of riboflavin and NADH (Sigma Chemicals Co., St. Louis, Mo.), were analyzed in the presence of known numbers of cells and the measured fluorescence remained relatively constant. This ability to make valid fluorescence measurements in highly scattering environments even with right angle optics has been noted previously for NADH (4) and other metabolic products (13, 14). Spectra shown have the scatter peaks removed for clarity of comparison of cell *versus* standard solution spectra.

Another potential source of artifact in the spectra may result from the Raman peaks due to water which become increasingly evident at the very high instrument sensitivities required for cells. These have been identified by producing spectra at various excitation wavelengths (4, 13) and spectra shown have been corrected for their presence.

Scan speeds were such that any possible bleaching appeared to be minimal during the course of the recording of spectra.

Levels of autofluorescence were monitored for cell populations in

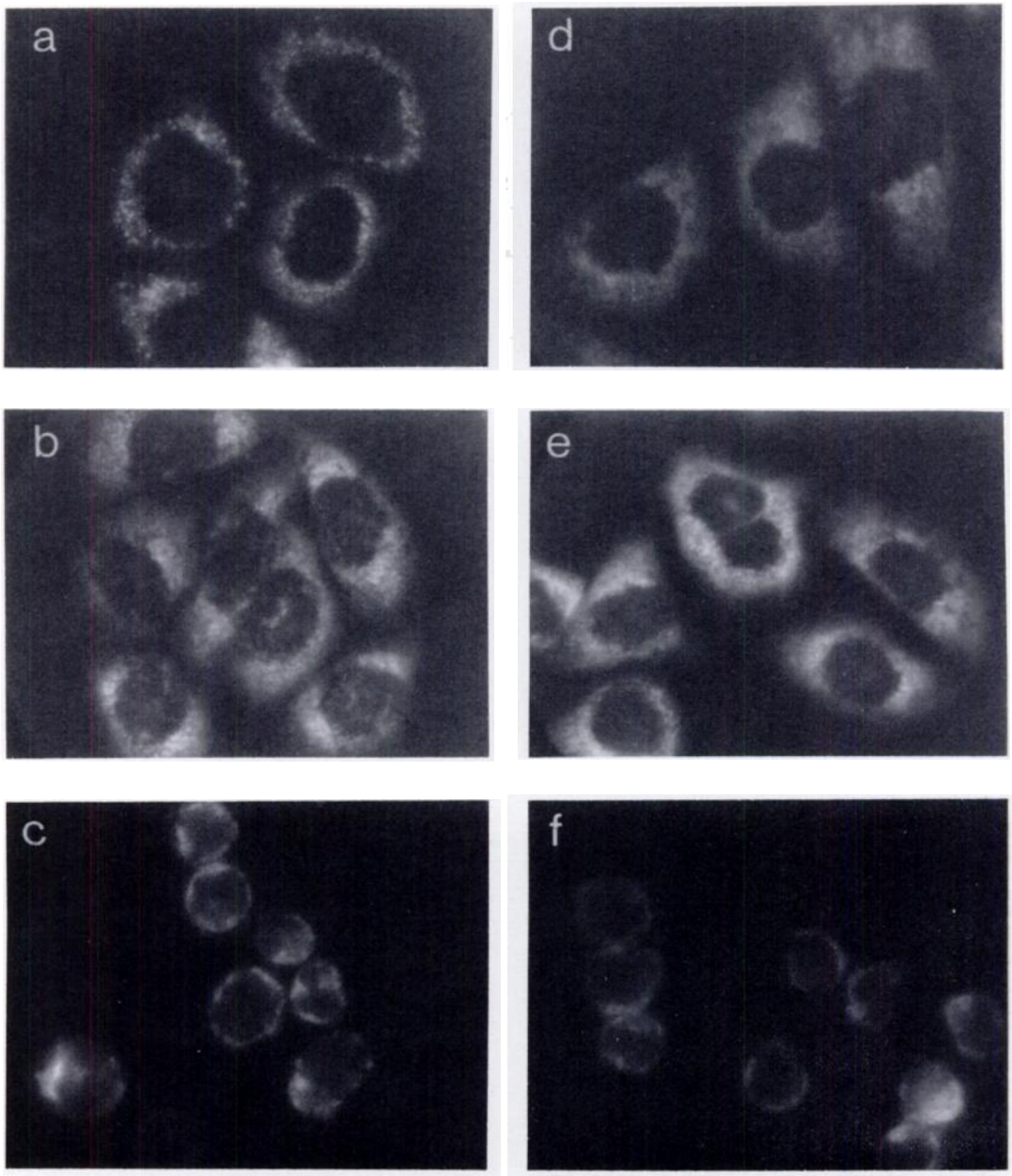

FIG. 1. Cellular autofluorescence visualized in the fluorescence microscope. CHO (Chinese hamster ovary) Cl4 cells and 3T3B cells, grown on cover slips (10 mm diameter), and B8 Friend erythroleukemia cells harvested from monolayer, were washed three times in phosphate-buffered saline (PBS) and immediately viewed without fixation in a Zeiss fluorescence microscope with epi-illumination. a, CHO Cl4 cells viewed with excitation filter UG 1 (360 nm), a 420 nm dichroic filter, and emission filter LP 418 nm; b, c, 3T3B cells and B8 cells respectively, as seen with the same filter combinations as in a; d, CHO Cl4 cells viewed with excitation filter BG 12 (400 nm), dichroic filter 510 nm, and emission filter IF 500 nm; e, f, 3T3B cells and B8 cells respectively, as seen with the same filters as in d. Film was Kodak Tri-X (Eastman Kodak Co., Rochester, N.Y.), exposure time 30–45 sec.

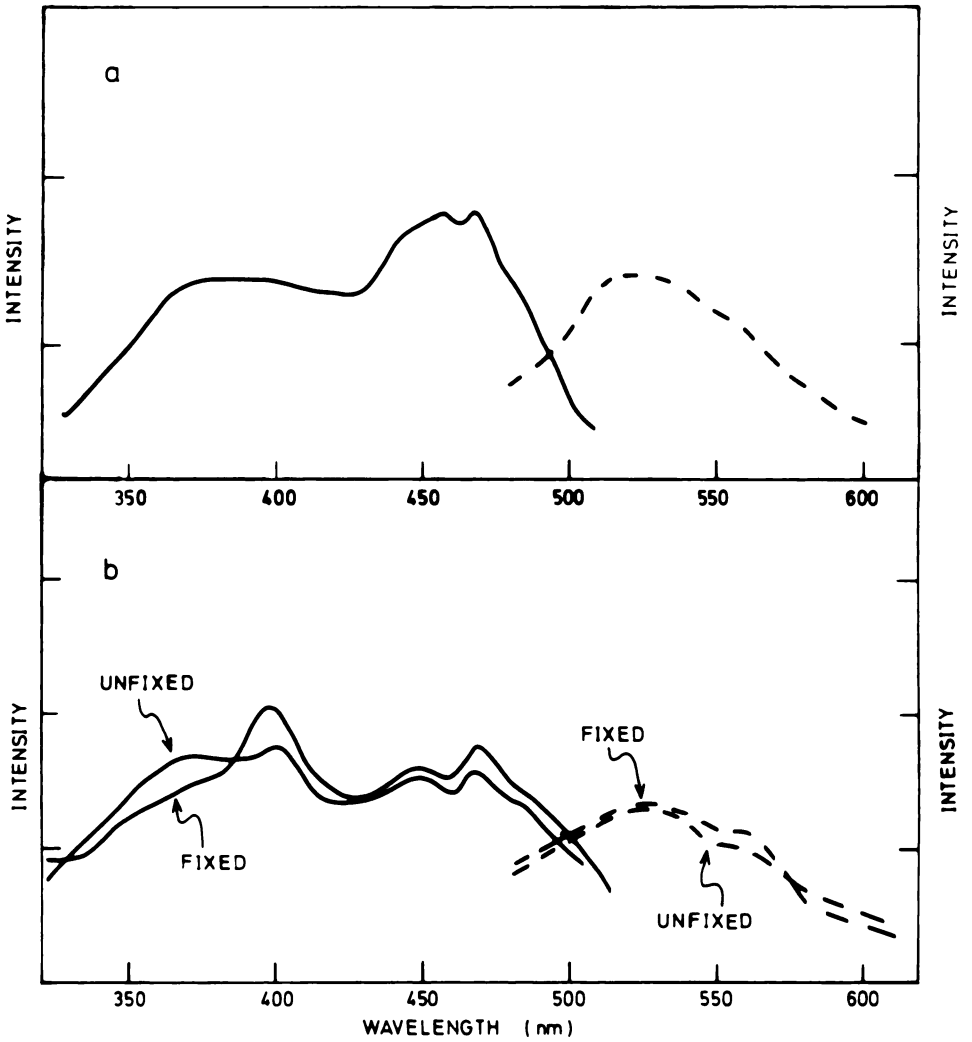

FIG. 2. Excitation and emission spectra of CHO (Chinese hamster ovary) SC1 cells from suspension culture and B8 cells. Exponentially growing cells of both types were centrifuged to a tight pellet, washed three times in phosphate-buffered saline (PBS), and then resuspended to a final cell concentration of 10^6 cells/ml in PBS. a, CHO SC1 cells. The uncorrected excitation spectrum was recorded at 20° with emission set at 520 nm (slit 10 nm) and excitation slit 10 nm. The emission spectrum was recorded with excitation at 440 nm, slits 10 nm. Both spectra were recorded with sensitivity 100. b, B8 cells, unfixed and fixed in 70% ethanol at 4° and prepared as above. Conditions for recording the spectra were as in a. (solid line, excitation spectrum; dashed line, emission spectrum).

either the fluorimeter or with the multiparameter flow system (MACCS; 1). Solutions of riboflavin and NADH, as well as Polysciences fluorescent polystyrene beads (POS, 1.75 μm) (Polysciences, Inc., Warrington, Pa.) were used as standards against which the cells were measured, as described in the figure legends.

RESULTS

The autofluorescence of exponentially growing viable cells was visualized easily in a fluorescence Zeiss microscope with epi-illumination (Fig. 1). Various combinations of excitation and emission filters were employed and indicated the presence of at least two fluorescent species. One was maximally excited with a UG 1 (360 nm) filter and emission filter LP 420 nm (blue); the other was seen clearly with excitation with a BP 400 nm and emission filter IF 500 nm (green).

The fluorescence was detected in both wavelength ranges in discrete cytoplasmic regions, while the nucleus remained dark (Fig. 1). Due to this cytoplasmic accumulation, it was particularly visible in cell lines with large cytoplasmic to nuclear ratios (*e.g.*, CHO and mouse fibroblasts), while it was less evident, and then only as a narrow ring of fluorescence, in cells with much smaller cytoplasmic to nuclear ratios (*e.g.*, Friend erythroleukemia cells).

The cellular components of the autofluorescence for the two distinct spectral regions were identified as follows.

Autofluorescence—emission at 520 nm: Excitation and emission spectra of exponentially growing, washed, viable CHO cells revealed an excitation spectrum with broad maxima centered at 380 nm and 460 nm (the xenon lamp line structure in the uncorrected spectrum has not been considered). The emission maximum in the fluorescence spectrum occurred at 520 nm (Fig. 2a). Very similar spectra were obtained for other mammalian cells, for example, mouse 3T3B cells as shown (Fig. 2b). It will be noted also that fixation with 70% ethanol

at 4° did not noticeably alter the excitation and emission profiles (Fig. 2b).

Numerous culture media and sera used as tissue culture supplements were tested for the presence of fluorescent components. All sera tested, including fetal calf serum and newborn calf serum, horse serum and dialyzed fetal calf serum, had excitation and emission spectra with maxima similar to those of cells (Fig. 3b), but with a large contribution from a

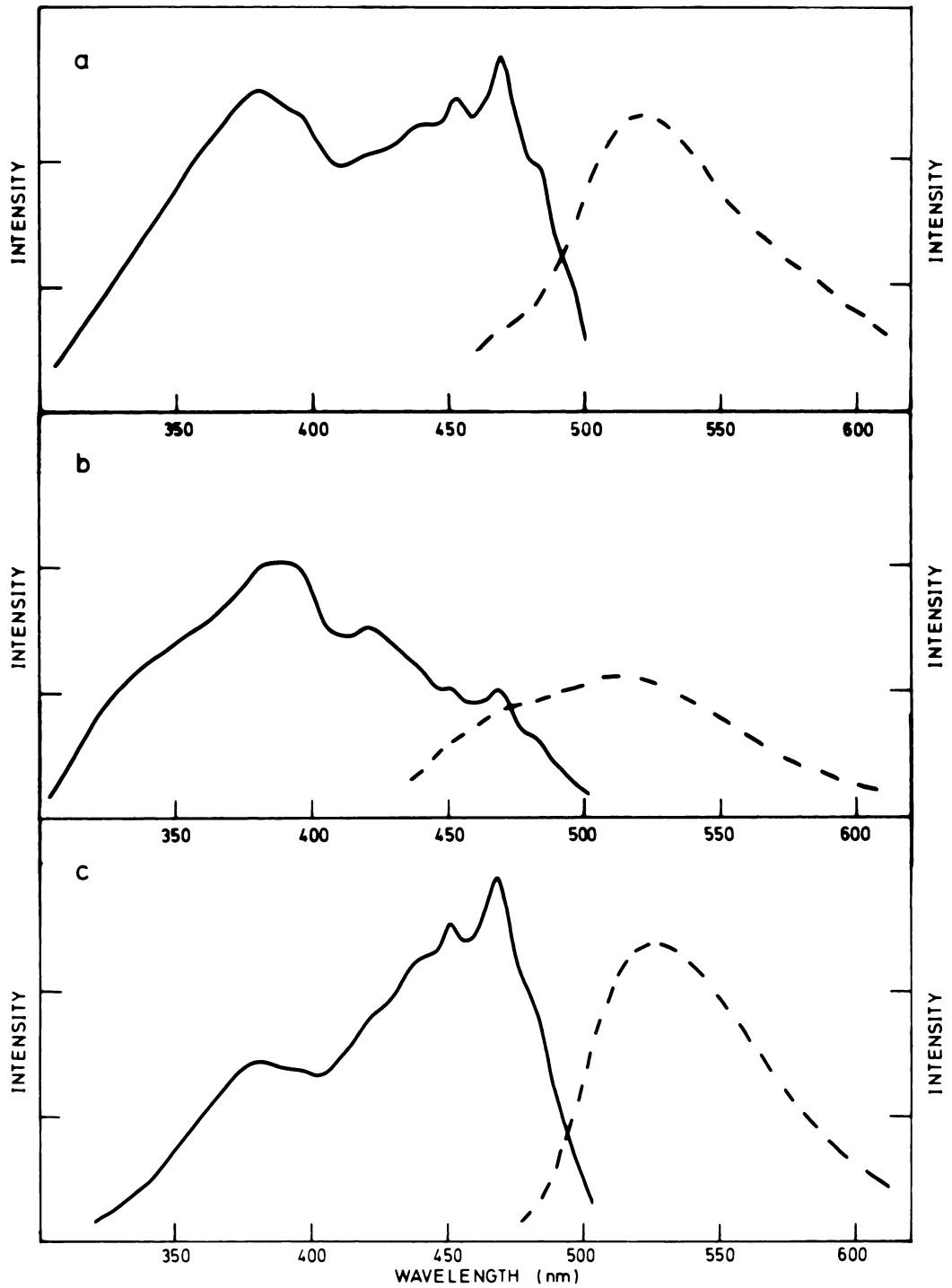

FIG. 3. Excitation and emission spectra of α-MEM (minimal essential medium), fetal calf serum, and riboflavin. a, Spectra of undiluted α-MEM (Flow Labs, Inc., Rockville, Md.) containing 0.1 mg/liter of riboflavin, with no serum present, were recorded at 20°. For the excitation spectrum, emission was set at 520 nm (slit 5 nm) and excitation slit 5 nm. The emission spectrum was determined with excitation at 440 nm and slits at 5 nm. Sensitivity in both cases was 30. b, The spectra of Flow Labs fetal calf serum were determined with conditions as in a, but with sensitivity 10. c, Riboflavin was prepared at 1 μg/ml in phosphate-buffered saline (PBS) and the spectra were recorded with conditions as in a, with sensitivity 10. (Solid line, excitation spectrum; dashed line, emission spectrum).

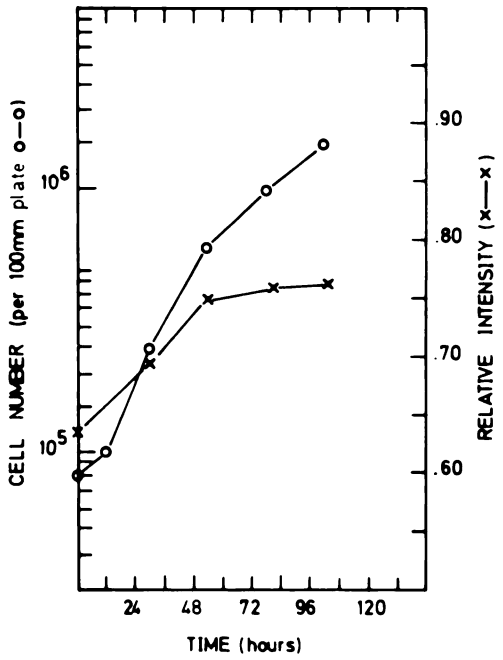

FIG. 4. Increase of autofluorescence (emission at 520 nm) with time of cells in culture. CHO (Chinese hamster ovary) SC1 cells were thawed from frozen stocks and replated at 37° in α-MEM (minimal essential medium) supplemented with 10% fetal calf serum. Their autofluorescence was determined at various times thereafter as cell numbers increased. Autofluorescence intensities were determined from emission spectra recorded on the spectrofluorimeter and from fluorescence distributions measured on multiparameter flow system (MACCS) using Polysciences fluorescent polystyrene beads (POS; Polysciences, Inc., Warrington, Pa.) microspheres as a standard as described in Table I. Data from MACCS measurements are shown here.

species with a lower wavelength in the excitation spectrum (see below). The actual amount of the fluorescent component(s) varied over a 2-fold range from serum to serum (data not shown). These fluorescent components could be removed from sera by treatment with dextran-charcoal. When either CHO SC1 cells or 3T3B cells were grown on medium supplemented with these treated sera, cell growth and population doubling times were essentially unchanged from that of control populations grown with untreated sera, but the level of autofluorescence also remained the same (data not shown).

As mammalian cells require riboflavin (Fig. 3c) as a culture medium supplement for growth, all culture media (Fig. 3a) have this vitamin as a constituent in concentrations varying from 0.10–0.50 mg/L. When CHO SC1 cells were analyzed for fluorescence after growth in media containing these various concentrations of riboflavin, their observed autofluorescence did not change (data not shown).

One further feature of the autofluorescence is its increase with time of cells in culture. When cells were removed from frozen stocks and reintroduced to culture, the observed cellular fluorescence increased with time until a plateau was reached as indicated in Figure 4.

A comparison of the relative intensities of fluorescence of various cell lines is shown in Table I. These data were obtained from measurements both on the spectrofluorimeter and MACCS. In this way, not only the relative intensities of the populations as a whole, but also the distributions of fluorescence from the individual cells were compared. Typical fluorescence profiles are shown in Figure 5. It can be seen that different cell lines had very different maximum intensities (*cf.*

TABLE I

Relative Autofluorescence of Mammalian Cell Lines[a]

Sample	MACCS[b] I	MACCS[b] II
POS	1.0 (1.0)[1, c]	1.0[d] (1.0)[2, c]
CHO SC1		
suspension	1.5 (2.0)	0.9
monolayer	1.1	0.8 (0.8)
CHO C14	1.3	ND[e]
CHO AB1	0.7	0.8
3T3B	ND	1.2
B8	ND (1.0)	0.5 (0.4)

[a] Relative autofluorescence of mammalian cell lines. Cells were prepared as for Figure 6 (all cells were grown in monolayer except for the one sample noted in the table) and their frequency distributions of fluorescence were determined. Two different sets of conditions were employed: (I) excitation was with the UV lines of the argon laser and all emission above 418 nm was collected; (II) excitation was with the 457 nm line of the argon laser and emission above 510 nm was collected. Distributions and peak positions of the cell populations were compared to those from POS beads to generate the table of relative intensities.

[b] The abbreviations used are: MACCS, multiparameter flow system; POS, polysciences fluorescent polystyrene beads; and CHO, Chinese hamster ovary.

[c] Numbers in brackets were determined from emission spectra of the same cell populations measured on the spectrofluorimeter. [1] Excitation was at 360 nm, emission at 440 nm; [2] excitation was at 457 nm, emission at 520 nm.

[d] A neutral density (0.3) filter was used to reduce the emission from the POS beads to the same scale as the cellular fluorescence at these wavelengths.

[e] ND, not done.

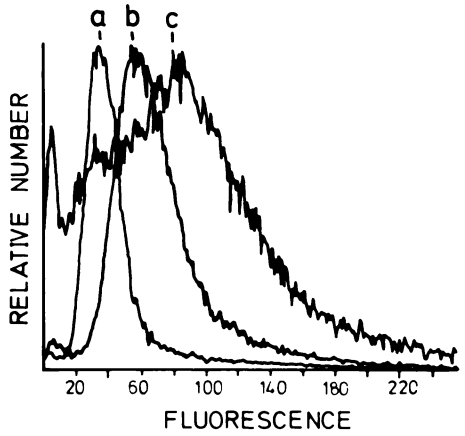

FIG. 5. Frequency distributions of the fluorescence of B8 cells, 3T3B cells, and CHO (Chinese hamster ovary) SC1 cells, as measured by a multiparameter flow system (MACCS). Cells were harvested, washed three times in phosphate-buffered saline (PBS) at RT and resuspended at 10^6 cells/ml in PBS at RT for the measurements. The ordinate is the number of cells per channel (35,000–40,000 total cells measured, normalized to peak values) and the abscissa is a linear scale of the fluorescence intensity of the individual cell types. Excitation was with the 457 nm line of an Argon Spectra physics model 171 laser, emission fluorescence above 510 nm was measured. a, B8 cells; b, CHO SC1 cells; c, 3T3B cells.

CHO SC1, 3T3B, B8), and also, interestingly, very different distributions in values of individual cells in the populations, with some lines showing a quite broad fluorescence distribution. It is also evident that one line (CHO SC1) growing in suspension displayed a higher autofluorescence than the same line growing in monolayer. That this may reflect a different metabolic rate for the cells under the two growth conditions is a possibility.

Autofluorescence—emission at 440 nm: Many fluorescent probes with useful spectral characteristics have chromophores which fall into the wavelength range with excitation maxima around 360 nm and emission maxima around 450 nm. All cultured mammalian cells tested in these experiments are highly autofluorescent in this spectral region. This fluorescence, like the higher wavelength fluorescence described above, is confined also to discrete cytoplasmic vesicle-like regions (Fig. 1). As is the case with the higher wavelength fluorescence, various cell lines express different amounts of the material as indicated in Table I.

Excitation and emission spectra of whole cells (Fig. 6a) suggested a close similarity between this cellular fluorescence and that resulting from NADH (Fig. 6b). This is in agreement with a number of earlier studies done in both isolated mitochondria and various whole tissues and cell types (3, 4, 13). As with the flavin fluorescence described above, there was a tendency for the fluorescence to be minimal in freshly thawed and reseeded cultures and increase with time as cells entered exponential phase of growth (data not shown).

It should be noted that the fluorescence of the 440 nm fluorescence was normally 50–100 times more intense than the 520 nm fluorescence. Although the flavins have an excitation band at 360 nm, excitation at 360 nm resulted almost

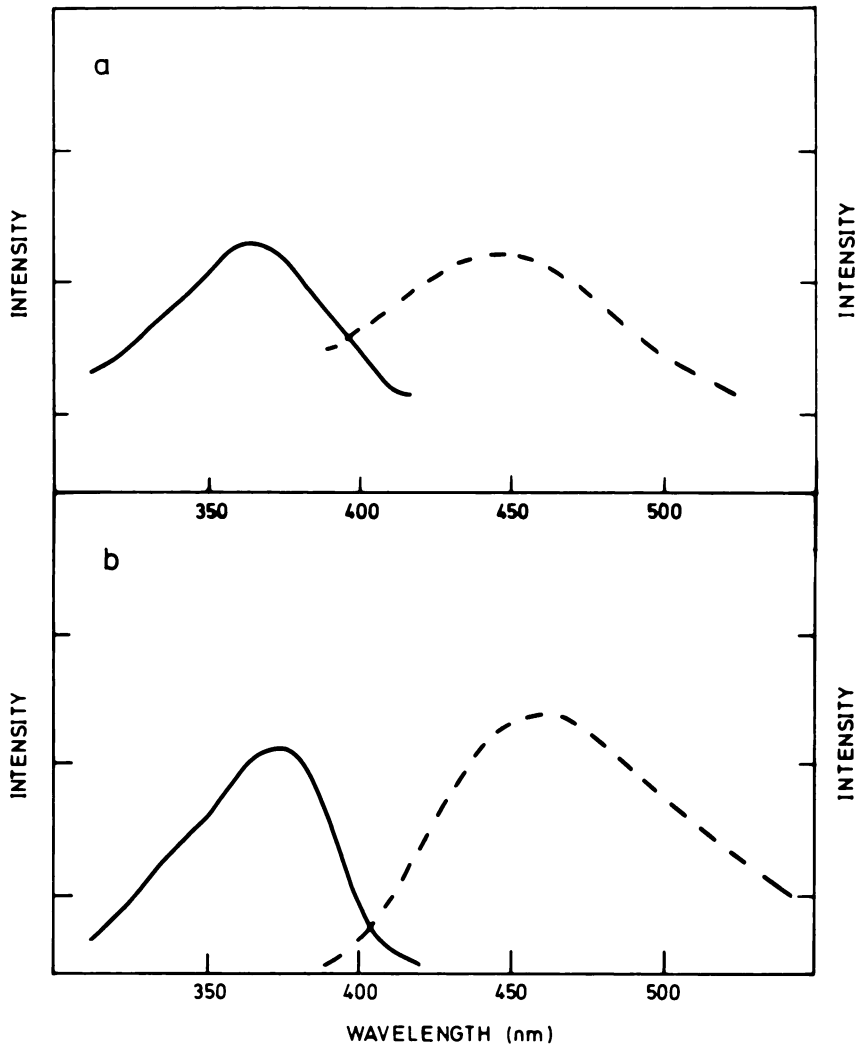

FIG. 6. Excitation and emission spectra of CHO (Chinese hamster ovary) SC1 cells and NADH. a, CHO SC1 cells were grown in suspension and prepared for spectra as described in Figure 2. Spectra were recorded at 20° with emission at 460 nm (slits 5 nm) for the excitation spectrum and excitation at 360 nm (slits 5 nm) for the emission spectrum. Sensitivity in both cases was 30. b, The spectra of reduced form of nicotinamide adenine dinucleotide (NADH) at 100 μg/ml in phosphate-buffered saline (PBS) were determined as those above with sensitivity three. (Solid line, excitation spectrum; dashed line, emission spectrum).

exclusively in fluorescence at 440 nm with the 520 nm band obscured. The flavin fluorescence as above was best observed with excitation at 440 nm and high instrument sensitivity.

DISCUSSION

Cellular autofluorescence can obscure the fluorescence arising from specific added probes in applications of fluorescence techniques in intact, viable cells. Mammalian cell autofluorescence outside the range of intrinsic protein fluorescence has been investigated here. The essential features of the fluorescence are as follows. First, the fluorescence can be divided into at least two distinct spectral regions, one exciting at λ_{max} 365 nm and emitting at λ_{max} 445 nm (blue); the other exciting at λ_{max} 380 nm and λ_{max} 440 nm and emitting at λ_{max} 520 nm (green). Second, the fluorescence from both sources is most intense in discrete cytoplasmic vesicles. Third, the fluorescence is low in freshly thawed and reseeded cultures, increasing with time to a maximum value at increased cell numbers. Comparison of the spectra of whole cells with spectra of known cellular metabolites and available literature values indicates that the fluorescence probably arises from intracellular NADH and riboflavin, flavin coenzymes, and flavoproteins bound in the mitochondria. Qualitatively the spectra of the free components NADH and riboflavin compare favorably with the spectra of whole cells. A comment may be in order on the differences that are observed however. The presence of and correction for Rayleigh light scattering and Raman bands in the whole cell spectra was necessary. Secondly, it has been established for both NADH and riboflavin, that enzyme and protein bound forms of these species exhibit fluorescence emission maxima shifted to shorter wavelengths by 20 nm and 5 nm respectively (4, 13). Blue shifts in the excitation maxima have also been observed (4, 13). Furthermore, nonspecific binding of NADH to serum components such as bovine serum albumin have been shown to lead to enhancement and blue shift of the emission spectrum of the NADH (4, 13). Such nonspecific binding may contribute to the whole cell spectra if serum proteins remain bound to the cell surfaces after their removal from culture, as well as to the spectra of the serum itself, where large amounts of serum albumin and other serum proteins are present. These considerations of multiple species and bound forms of the fluorophores and nonspecific binding could lead to wavelength shifts and band broadening of the spectra as observed.

That the fluorescence arises here mainly from bound species is supported by other observations as well. Their discrete cytoplasmic locations are consistent with the fact that the highest concentrations of these compounds would be expected in mitochondria, the organelles involved with their cellular utilization. A high degree of polarization is also consistent with tightly bound species (8, 13). The emission anisotropies (EA) of the autofluorescent species as measured on MACCS are quite high: EA 0.25 λ_{emis} 440 nm; EA 0.30 λ_{emis} 520 nm (J. Aubin, unpublished data). These data support the view that most of the NADH and flavin in these cells is in bound forms, as has been suggested for a variety of other tissues (2–4, 13, 14).

Some attempts were made to decrease the level of the autofluorescence without altering cell viability. However, due to the origin of the fluorescence, manipulations to decrease it and still maintain good growth, are probably not possible. NADH is a cellular compound required for growth and actively synthesized by growing cells from precursors which themselves are required for growth. Similarly, all culture media for mammalian cells must be supplemented with riboflavin to support the growth of cells. Varying this riboflavin concentration to minimal levels, and removal of the fluorescent components from serum, did not alter the observed autofluorescence, suggesting that the cells will take up and utilize the riboflavin to maximal levels.

It is not known why some cell populations are more highly autofluorescent than others, but it seems reasonable that if indeed the fluorescence arises from bound flavins and NADH, the cells' fluorescence intensities reflect the intracellular concentrations of these compounds. Different metabolic rates and different cytoplasmic to nuclear volumes could be reflected in larger concentrations of the compounds in one cell line than another. It can be seen readily that not all the difference in the fluorescence intensity can be accounted for by the volume differences themselves. The diameters of 3T3B cells, CHO SC1 cells, and B8, Friend cells, measured by Coulter Counter sizing, were 16, 12 and 8 μm respectively (D. Ardnt-Jovin, personal communication). A comparison of the relative fluorescence intensities per unit volume at 440 nm and 520 nm for these cell types, indicates that the fluorescence is not constant per unit volume. Other factors such as metabolic rate must play a role in the observed fluorescence. In this regard, the increase in autofluorescence with time after thawing and reseeding cultures, is also relevant. We have not pursued in detail the reasons for this increase. However, a substantial number of studies have been done to correlate the changes of fluorescence of reduced pyridine dinucleotides with metabolic activity in a variety of whole tissues, other cell types, and isolated mitochondria (3, 4, 13).

In attempting to screen by fluorescence microscopy the intracellular location of various fluorescent probes added to intact viable cells, we have been hampered by this cellular autofluorescence, which in some cases, completely obscured the specific probe fluorescence. Some of these difficulties in localization and detection of probes can be overcome in the case of covalently bound probes, where subcellular fractionation may still be useful to indicate the location of the majority of the probe. Moreover, appropriate and specific filter combinations for excitation and emission can in some cases decrease the contribution in the signal of the autofluorescence while enhancing the contribution from the probe in studies on both the fluorescence microscope and other fluorescence instrumentation. However, it is evident that in the case of very highly autofluorescent cell lines, the specific fluorescence from probes which may bind in very low quantities or which may have spectral properties similar to the background and/or low quantum efficiencies, may be completely or almost totally obscured. This we have found to be the case with a number of membrane probes utilized in the mammalian cells described here. For example, bis-ANS (12), the dimer form of 1-anilino-8-napthalene sulfonate (ANS), a membrane probe (5, 6), has excitation and emission maxima at 400 nm and 500 nm respectively, which brings it within the range of both the re-

duced pyridine nucleotide and flavin autofluorescence. Under many incubation conditions of bis-ANS with cultured cells, samples were obtained where probe fluorescence was at best 1.5–3.0 times greater than background as measured on MACCS, with excitation with the UV lines of the Argon laser and collection of the fluorescence emission above 418 nm.

Some efforts may have to be directed to the selection of probes with chromophores emitting at wavelengths outside the range of the autofluorescence. Where this is not possible, the results in this paper require that the contribution of autofluorescence be quantified and considered in all fluorescence and flow cytometry studies especially in particular kinds of measurements, for example, those for emission anisotropy of membrane bound dyes such as that mentioned above. In this regard, recently we have done calculations to subtract the contribution of background from viable cells in the measurements on MACCS of fluorescence intensities and emission anisotropies of several of the weakly labeling membrane probes where background contributed a significant fraction of the total signal. Such concepts of correcting for multiple species (or background) have been reviewed (8). In many cases, such corrections may allow studies in which the background fluorescence makes a significant contribution, but extensive limitations may be imposed by the autofluorescence in other experiments.

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