0022-1554/79/2701-0044$02.00/0
THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY

Vol. 27, No. 1, pp. 44-48, 1979
Printed in U.S.A.

Cellular Autofluorescence — Is It Due To Flavins?[1, 2]

R. C. BENSON, R. A. MEYER, M. E. ZARUBA, AND G. M. McKHANN

Applied Physics Laboratory, Johns Hopkins University, Laurel, Maryland 20810 (R.C.B., R.A.M.) and Department of Neurology, Johns Hopkins Medical School, Baltimore, Maryland 21205 (M.E.Z., G.M.M.)

Received for publication June 13, 1978

For many cell types, cellular autofluorescence in the 500-600 nm spectral region can result in a significant background signal for measurements of weakly fluorescent probes. Measurements of fluorescence intensity and spectra of a variety of cell types and assays of neuronal homogenates are presented to demonstrate that this autofluorescence is most likely due to endogenous flavoproteins.

Fluorescence-activated cell sorters are commonly used to study cells having weakly fluorescent tags (e.g., fluorescein-coupled antibodies). Although the detection limit of cell sorters is about 3000 fluorescein molecules per cell (11), in practice the detection limit is often significantly higher than this owing to the native fluorescence of the cells which acts as a background noise level. A more complete understanding of this cellular autofluorescence is necessary to determine if modifications to either cell preparation (e.g., culture medium constituents) or sorter instrumentation (e.g., spectral properties of the fluorescence detection filter) can improve the sensitivity of fluorescence-activated sorters.

In this paper, we report measurements on the cellular autofluorescence in the yellow-green spectral region (500–600 nm). We present evidence that this cellular autofluorescence is due to endogenous flavins found within all cells. The fluorescence spectra (λ_{ex} = 488 nm) of various cell types were similar and consistent with the spectra of flavins. Calculations of flavin concentration within the cells, based on sorter autofluorescence measurements, were consistent with published flavin concentrations in various tissues. In addition, removal of the predominant fluorescent species from the culture medium did not significantly alter either the spectrum or intensity of neuronal autofluorescence, indicating that the autofluorescence was not due to uptake of medium. Finally, fluorescence and absorption spectra, hydrolysis properties, reduction-oxidation (redox) properties, and thin-layer chromatograms of neuronal homogenates were consistent with the presence of flavins.

MATERIALS AND METHODS

Materials: Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), phenol red, trypsin, and chymotrypsin were obtained from Sigma Chemical Co., St. Louis, MO. Riboflavin was obtained from Aldrich Chemical Co., Metuchen, NJ. Sodium hydrosulfite ($Na_2S_2O_4$) was obtained from Fisher Scientific Co., Fair Lawn, NJ. Trichloroacetic acid (TCA) was obtained from J. T. Baker Chemical Co., Phillipsburg, NJ. Phosphate buffered saline (PBS) and Dulbecco's modified Eagle's medium (with, and without, riboflavin and phenol red) were obtained from Grand Island Biological Co., Grand Island, NY. All reagents were used without further purification, although the solutions were filtered (0.45 μm, Millipore) in the low-level fluorescence measurements. The various cell types that were studied are listed in Table 1.

Instrumentation: A Becton-Dickinson FACS-I cell sorter was used in this study (Becton-Dickinson, Mountain View, CA). The cellular autofluorescence excited by an argon laser (λ_{ex} = 488 nm) was detected at 90 degrees from the incident beam by using an EMI 9524A photomultiplier tube (Emitronics, Inc., Plainview, NY). Two high-pass filters (Ditric 520 and D530, Ditric Optics, Marlboro, MA) were used to absorb the excitation light. Fluorescence distributions were triggered by low-angle scatter. The fluorescence signals were calibrated with glutaraldehyde-fixed chicken red blood cells (3).

The apparatus shown in Figure 1 was used to measure the fluorescence spectra of bulk samples. A Spectra-Physics model 164 argon laser (Spectra-Physics, Inc., Mountain View, CA) was used as the excitation source. The laser output was filtered to eliminate light at wavelengths greater than 500 nm. The laser beam was mechanically chopped and used to excite samples that were contained in a 100 μm-thick quartz cuvette. Thus, sample volumes could be small, and self-absorption was minimized because front-surface detection was used. The sample fluorescence was initially filtered by a No. 12 Wratten filter (Eastman Kodak Co., Rochester, NY) to attenuate the scattered 488 nm excitation light (typically 100 mW incident on the sample). The fluorescence was focused with a 50 mm Nikon lens onto the entrance slit (typically 5 mm, 20 nm bandpass) of a SPEX Industries, Inc. (Metuchen, NJ) model 1670 monochromator (grating: 1200 lines/mm, 500 nm blaze). The monochromator was scanned at a rate of 50 nm/min. The detector was an RCA 4832 photomultiplier tube which has a rather constant spectral responsivity from 400–800 nm. The detector output was preamplified and phase-sensitive detected at the modulation frequency using a lock-in amplifier. The output of the lock-in was then recorded on a strip chart. The spectra were measured after an irradiation time of at least 5 min to minimize any distortion in the spectra due to fluorescence fading during the scan of the spectrum. The shape of the fluorescence spectra was not affected by the fluorescence fading; this was verified by using an optical multichannel analyzer (model 1205, Princeton Applied Research Corp., Princeton, NJ) to display the entire fluorescence spectrum continuously (i.e., updated every 33 msec.) from the initiation of the irradiation. All measurements were made at room temperature. Cell

[1] This work was supported in part by the National Institute of Neurological Disease and Stroke, National Institutes of Health, USA, under grant NS12491.

[2] Presented at the VIth Engineering Foundation Conference on Automated Cytology, Schloss Elmau, Bavaria, West Germany, April 23-29, 1978.

TABLE I
Cell Types Studies

Rat neurons[a]
Bovine oligodendroglia[a]
Chinese hamster ovary[b]
Mink fibroblast (lung)[c]
Murine fibroblast (adult kidney)[c]
Murine lymphoma (EL-4)[c]
Murine macropahge (P388D1)[c]
MCA murine sarcoma (methylcholanthrene-induced)[c]

[a] Prepared by the gradient centrifugation technique (12).
[b] Provided by Dr. Robert Durand, Johns Hopkins Oncology Center.
[c] Provided by Susan Sharrow, National Cancer Institute.

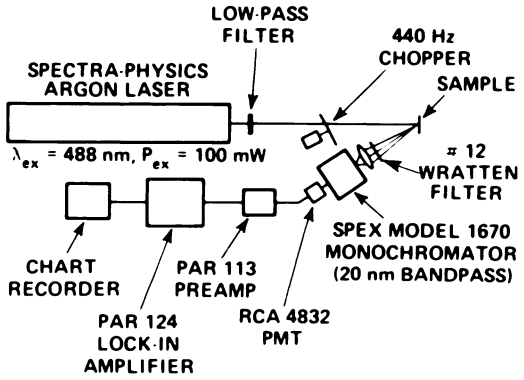

FIG. 1. Apparatus used in measurement of fluorescence spectra.

suspensions were washed twice with PBS (free of Mg^{++} and Ca^{++} in some cases) to remove any fluorescent species in the culture medium, and the resultant pellet was loaded into the cuvette.

In the flavin assay of neurons, it was necessary to measure accurately fluorescence intensities which were rather weak. The low-level fluorescence measurements were made on a model 111 Turner fluorometer (G. K. Turner Associates, Palo Alto, CA) equipped with a 20 nm bandpass excitation filter centered at 436 nm and a high-pass ($\lambda > 475$ nm) detection filter. The fluorometer was calibrated by using standard FAD and FMN solutions in the same solvent as the assay samples.

Neuron assay: The fluorescent compounds ($\lambda_n > 480$ nm) in neurons were assayed by the procedure of Bessey *et al.* (1) for acid-extractable compounds and of Cerletti *et al.* (4) and Wilson and King (15) for acid-nonextractable compounds. Additional tests were also used to determine whether flavins were present. The assay solutions were protected from undue exposure to light to minimize photolysis.

Acid-extractable compounds: The neurons were washed twice with an isotonic PBS-sugar solution (5% fructose, 0.6% glucose) to remove any fluorescent species in the culture medium. Before the final centrifugation, an aliquot was taken for a cell count. The neuron pellet was brought up to 1.0 ml volume with ice water and then homogenized at 0°C. The cold suspension was immediately mixed with 1.0 ml of ice-cold 20% TCA and kept on ice. After 15 min, the suspension was centrifuged to remove the precipitated protein, and the supernatant was divided in half. One half was immediately neutralized with 0.25 ml 4M K_2HPO_4. The other half was maintained at 40°C for at least 1 hr to allow hydrolysis of FAD to FMN and then neutralized and assayed in the same manner as the unhydrolyzed aliquot. The fluorescence intensity, absorption and fluorescence spectra, and fluorescence fading were measured. (Absorption spectra were measured on a Beckman DK-2 spectrometer.) An aliquot was used to determine the effect of oxidation and reduction on the fluorescence and absorption spectra. Reduction was accomplished by adding $Na_2S_2O_4$ and oxidation by bubbling air. The remainder of the sample was analyzed by thin-layer chromatography (TLC). The silica gel plates were heated to 100°C for 1 hr before each run. The solution was lyophilized and then solubilized with H_2O to a minimal volume before spotting on paper (Whatman No. 1) and silica gel plates (Eastman, 100 μm coating on plastic). Two solvent systems (10,2) were used: organic phase of 1-butanol:acetic acid:water (40:10:50); and pyridine:acetic acid:water (19:2:79).

Acid-nonextractable compounds: The TCA precipitated protein from above (containing covalently bound flavoproteins) was treated with a combination of trypsin and chymotrypsin (4,15) in the following manner: 3 ml of 50 mM PBS were added to the precipitate, and the pH was adjusted to 7.5–8.0 with 2.5N NaOH. Portions of 40 mg each of crystalline trypsin and chymotrypsin were added, and the volume was brought up to 6.0 ml with water. The mixture was placed in a metabolic shaker at 37°C for 3.5 hr in the dark, with the pH monitored and adjusted if necessary. The digest was centrifuged, and the supernatant was analyzed in the same manner as the supernatant of the acid-extractable compounds. A check of the precipitate of the trypsin/chymotrypsin digest revealed that it was still strongly fluorescent, indicating that the enzymatic digestion had not completely liberated the fluorescent species. This precipitate was digested again with 20 mg each of the trypsin and chymotrypsin; the sample was placed in a metabolic shaker at 37°C overnight in the dark. Approximately the same amount of flavins were solubilized in the second digestion as in the first, and the precipitate was still fluorescent. This incomplete liberation of the covalently bound flavins by a trypsin/chymotrypsin digest has also been observed by us and others (4) in assays of entire rat brain. Thus, the reported concentrations of covalently bound flavin in rat brain based on a trypsin/chymotrypsin digestion (4) will be low.

RESULTS

The fluorescence spectra of the various cell types are shown in Figure 2. The shape of the curves and the location of the peaks are similar, indicating the presence of a common chromophore. The fluorescence spectra are slightly distorted because a No. 12 Wratten filter was used to absorb the scattered 488 nm light. This distortion is demonstrated in Figure 3 where the neuronal fluorescence was excited at 488 nm and at 458 nm with a No. 12 Wratten filter in front of the detector and at 458 nm without this filter. Clearly, there is only one major fluorescence peak in this spectral region. These spectra are similar to that of flavoproteins which have a single fluorescence peak that is located in the 540–560 nm region, with the peak location and the fluorescence intensity dependent on the type of flavoprotein and its environment (6). The fluorescence peak of a flavoprotein is typically red-shifted 10–30 nm relative to the unbound flavin (8).

The medium contains several fluorescent compounds in this spectral region (500 nm $< \lambda_n <$ 600 nm) whose uptake might account for the cellular fluorescence. The spectra of the predominant fluorescent constituents (i.e., riboflavin, fetal calf serum, and phenol red) are shown in Figure 4. When neurons were maintained in medium without these fluorescent species, the intensity and spectral properties of the neuronal fluorescence were not significantly changed. Thus, this autofluorescence is due to an endogenous species rather than uptake of medium.

The endogenous fluorescent species in this wavelength region are the previously mentioned flavins, which are involved in many of the redox reactions within cells. Lipofuscin has also been reported to have fluorescence peaks at 450 and 545

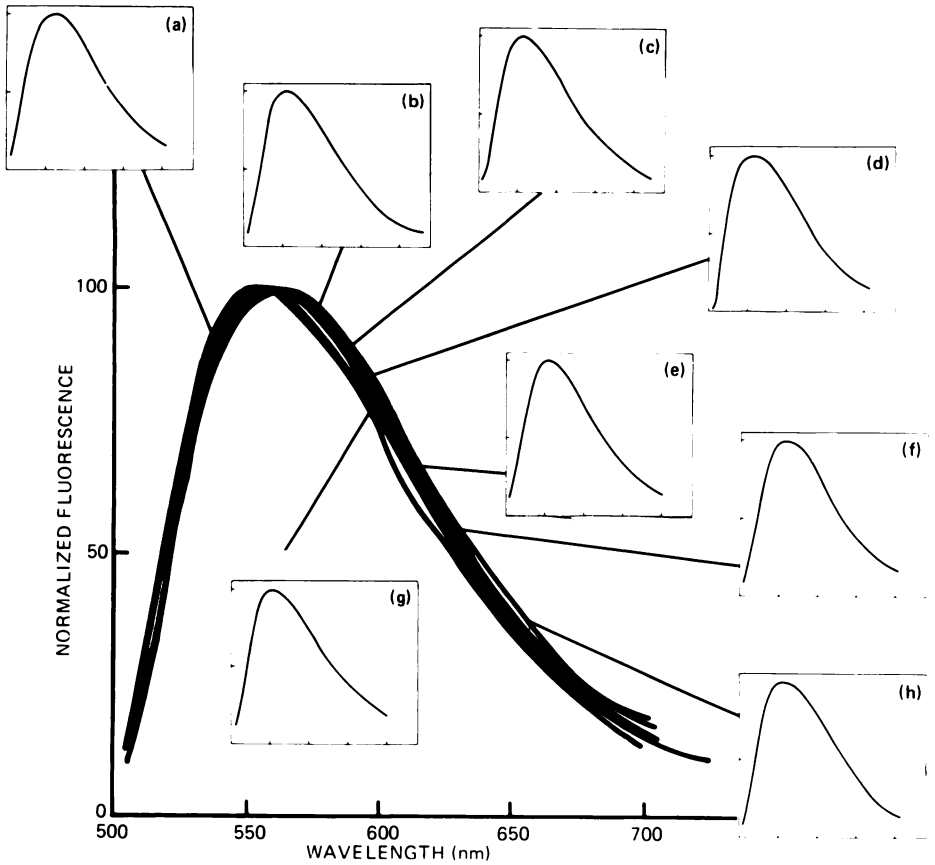

FIG. 2. Fluorescence spectra of various cell types. A No. 12 Wratten filter was in front of the detector. a, murine fibroblast; b, rat neurons; c, murine macrophage; d, bovine oligodendroglia; e, mink fibroblast; f, murine lymphoma; g, Chinese hamster ovary; and h, MCA murine sarcoma.

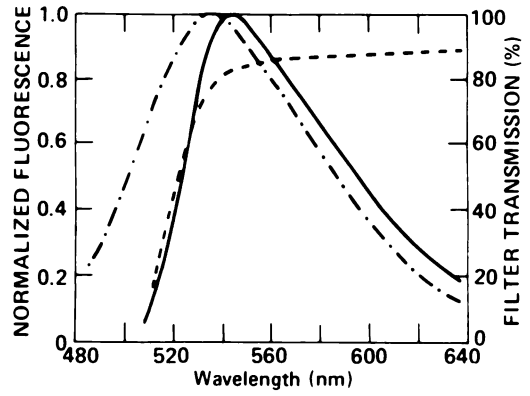

FIG. 3. Neuronal autofluorescence for λ_{ex} = 488 nm and 458 nm with a No. 12 Wratten filter in front of the detector (——), and for λ_{ex} = 458 nm without this filter (·–·–). The transmission of the No. 12 Wratten filter is also shown (-----).

nm (9). However, in these studies the samples probably contained both flavins and lipofuscin so that the contribution of lipofuscin is not known. More recent work on the isolation of lipofuscin indicates that only the 450 nm peak is observed (16). Thus, flavins appear to be the most likely sources of the autofluorescence.

If we assume that the fluorescent species are flavins, we can determine whether the known concentration of flavins in tissue can account for the observed fluorescence intensity on the cell sorter. The autofluorescence of the various cell types (except rat neurons) was typically $^1/_{20}$ the fluorescence of the glutaraldehyde-fixed chicken red blood cell standard. Using a fluorescein calibration of our sorter (13) and assuming that the autofluorescence is entirely due to free FAD, the autofluorescence of the various cell types corresponds to ~ 1×10^7 FAD molecules/cell; for rat neurons (maintained in culture medium for one day) the value is ~ 4×10^7 FAD molecules/cell. The value for neurons was independently verified by measuring the neuronal autofluorescence in the Turner fluorometer. The neuron concentration was sufficiently low that scattered excitation light was negligible. The neuronal autofluorescence corresponded to ~ 2×10^7 FAD molecules/cell, which is the same order of magnitude as that obtained from the sorter. The concentration of acid-extractable flavins ranges from 1 to 40 μg/g wet weight depending on the type of tissue (7). Assuming that the cell diameters range from 7.5 μm to 15 μm, this concentration range corresponds to a range of ~ 10^5 to 10^8 FAD molecules/cell. Since most of the FAD and FMN in tissue are associated with flavoproteins (not all of which are acid-extractable) and the relative fluorescence efficiencies of these bound flavins are quite variable,

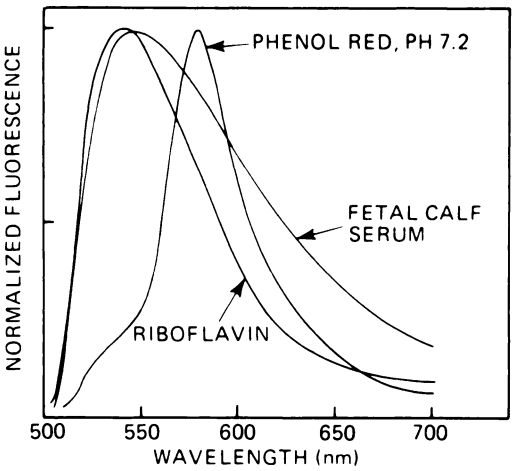

FIG. 4. Predominant fluorescent species in culture media (λ_{ex} = 488 nm, and a No. 12 Wratten filter was in front of the detector).

calculations of the cellular autofluorescence from tissue assay can only be approximate. The concentration of flavins in tissue is sufficient to account for the observed cellular fluorescence intensity.

To further verify that flavins are present, several assays were made on neuronal homogenates. The acid-extractable species (free molecules and those which are weakly bound to protein) exhibited absorption and fluorescence spectra, and also fluorescence fading rates characteristic of FAD and FMN. The fluorescence spectrum of the extracted species is essentially the same as that of the free flavins as shown in Figure 5. (It should be noted that the fluorescence efficiency of FAD is less than that of FMN or riboflavin, but the normalized fluorescence spectra are the same.) Upon hydrolysis of the extracted species, the fluorescence intensity and the fluorescence fading rate increased which would be expected when FAD is hydrolyzed to FMN (1). When $Na_2S_2O_4$ was added, the fluorescence intensity decreased markedly and the 450 nm absorption peak disappeared, and when air was bubbled through the reduced solution the fluorescence intensity rapidly returned to the previous level in accord with the redox properties of flavins. The only yellow-fluorescent compounds observed when using TLC were at R_f values corresponding to flavins (10,2). The following average concentrations from three neuronal assays were determined for the acid-extractable flavins: ~3 × 10^6 FAD molecules/cell and ~ 6 × 10^5 FMN molecules/cell. These values are significantly lower than predicted by the sorter measurement which suggests that the majority of flavin molecules in neurons are tightly bound. The trypsin/chymotrypsin digest was only partially effective in liberating the covalently bound flavins (see Methods), and thus an accurate concentration for the acid-nonextractable compounds could not be determined. However, the species that were solubilized exhibited the properties of flavins, and the concentration was comparable to that of the acid-extractable species. In addition, the residual precipitate had a fluorescence spectrum similar to the whole cell (Fig. 5).

The autofluorescence of the neurons was different than the autofluorescence of the other cell types studied. As shown in Figure 6, the fluorescence intensity was initially higher and also increased with time as the neurons were maintained in culture medium (R. A. Meyer *et al.*, in preparation). This increase in fluorescence was inhibited when the cells were maintained at 4°C (R. A. Meyer *et al.*, in preparation), suggesting that it is of metabolic origin. Fluorescence spectra and assays of homogenates obtained from neurons at maintenance duration times of 1, 3, and 5 days were similar and the results

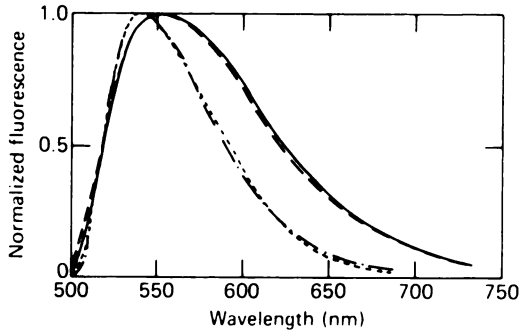

FIG. 5. Fluorescence spectra of species extracted from neuronal homogenates (-----), free flavins: FAD, FMN, and riboflavin (·—·—), residual precipitate of trypsin digest (— — —), and whole neurons (——). λ_{ex} = 488 nm and a No. 12 Wratten filter was in front of the detector.

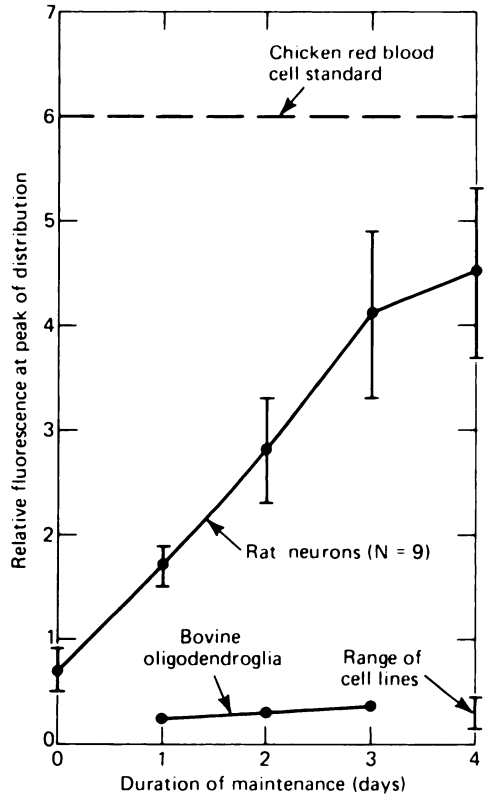

FIG. 6. Fluorescence intensity of various cell types *vs* duration of maintenance in culture media. The data was obtained with the cell sorter. Note that, with the exception of neurons, the autofluorescence of the various cell types (Table 1) was about 0.05 that of the glutaraldehyde-fixed chicken red blood cell standard.

were consistent with the flavins. Although the actual cause of this increase in neuronal fluorescence has not yet been determined, it does appear to be related to the flavoproteins.

DISCUSSION

We have presented several lines of evidence that flavoproteins are the predominant autofluorescent species (500 nm < λ_{fl} < 600 nm) for a variety of cell types. These flavoproteins are actively involved in a number of metabolic processes within the cell. Since the relative fluorescence efficiencies of flavoproteins are a function of their redox state (6,8), autofluorescence measurements on a sorter might provide information about the metabolic activity of single cells. Various tissues also exhibit autofluorescence at 450 nm (λ_{ex} = 366 nm) which is related to the redox state of nicotinamide adenine dinucleotide (NAD) within the tissue (5,6), and has been used as an indicator of cerebral ischemia (14). Thus, autofluorescence measurements on cell sorters over this spectral region (400 nm < λ_{fl} < 500 nm) could provide additional information about the metabolic state of single cells.

The cellular autofluorescence can be a significant background signal in measurements of weakly fluorescent probes. For immunofluorescence measurements, the relative contribution of the autofluorescence signal can be reduced by (1) using antibodies coupled to other fluorescent dyes (e.g., Rhodamine B, λ_{ex} = 550 nm, λ_{fl} = 580 nm) which have excitation and fluorescence spectra that are well separated from the flavins, or (2) using a bandpass fluorescence detection filter which is centered at the fluorescein emission peak (λ_{fl} = 520 nm).

ACKNOWLEDGMENTS

We would like to thank S. Sharrow for many helpful discussions as well as for donating cells; V. P. Pigiet and L. Brand for helpful discussions regarding the flavin assay and fluorescence measurements; and J. Weinstein for the use of his sorter calibration. We also appreciate the donation of oligodendroglia by S. Poduslo, K. Miller, and S. Deak and the donation of the CHO cells by R. Durand.

LITERATURE CITED

1. Bessey OA, Lowry OH, Love RH: The fluorometric measurement of the nucleotides of riboflavin and their concentration in tissues. J Biol Chem 180:755, 1949
2. Bolliger HR, König A: Vitamins, including carotenoids, chlorophylls and biologically active quinones, Thin-layer Chromatography. Edited by E. Stahl. Springer-Verlag, New York, 1969
3. Brown G, Capellaro D, Greaves M: Leukemia associated antigens in man. J Natl Cancer Inst 55:1281, 1975
4. Cerletti P, Strom R, Giordano MG: Flavin peptides in tissues: the prosthetic group of succinic dehydrogenase. Arch Biochem Biophys 101:423, 1963
5. Chance B, Cohen P, Jobsis F, Schoener B: Intracellular oxidation-reduction states *in vivo*. Science 137:499, 1962
6. Chance B, Lieberman M: Intrinsic fluorescence emission from the cornea at low temperatures: evidence of mitochondrial signals and their differing redox states in epithelial and endothelial sides. Exp Eye Res 26:111, and references therein, 1978
7. Christensen S: The biological fate of riboflavin in mammals. Acta Pharmacol Toxicol 32:27, 1973
8. Ghisla S, Massey V, Lhoste J-M, Mayhew SG: Fluorescence characteristics of reduced flavins and flavoproteins, Reactivity of Flavins. Edited by K Yagi. University of Tokyo Press, Tokyo, 1973
9. Hyden H, Linström B: Microspectrographic studies on the yellow pigment in nerve cells. Discussions Faraday Soc 9:436, 1950
10. Kilgour GL, Felton SP, Huennekens FM: Paper chromatography of flavins and flavin nucleotides. J Am Chem Soc 79:2254, 1957
11. Loken MR, Herzenberg LA: Analysis of cell populations with a fluorescence-activated cell sorter. Ann NY Acad Sci 254:263, 1975
12. Poduslo SE, Norton WT: The bulk separation of neurons and neuroglia, Research Methods in Neurochemistry. Edited by N Marko, R Rodnight. Vol. 1. Plenum Press, New York, 1972
13. Weinstein JN, Blumenthal R, Sharrow SO, Henkart PA: Antibody-mediated targeting of liposomes: binding to lymphocytes does not ensure incorporation of vesicle contents into the cells. Biochim Biophys Acta 509:272, 1978
14. Welsh FA, O'Connor MJ, Langfitt TW: Regions of cerebral ischemia located by pyridine nucleotide fluorescence. Science 198: 951, 1977
15. Wilson DF, King TE: The determination of the acid-nonextractable flavin in mitochondrial preparations from heart muscle. J Biol Chem 239:2683, 1964
16. Zeman W, Donahue S, Dyken P, Green J: Neuronal ceroid-lipofuscinoses, Handbook of Clinical Neurology. Edited by PJ Vinken, GW Bruyn. Vol. 10. North Holland, Amsterdam, 1970