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Characterization of the autofluorescence of rat liver plasma membranes

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The autofluorescence of isolated rat liver cell plasma membranes was characterized in vitro in relation to the autofluorescence used previously for fluorescence recovery after photobleaching (FRAP) studies. The fluorescence of membrane preparations displayed an emission pattern with a maximum at around 525 nm when excited with a 468 nm blue light. The excitation spectrum monitored at 525 nm closely resembled that of flavin compounds (riboflavin, FAD, FMN). The chloroform extract of the membrane fraction showed practically no fluorescence, whereas, both the water-soluble and water-insoluble protein fractions remaining after chloroform extraction were strongly fluorescent. The fluorescence disappeared almost completely under the effect of sodium hydrosulfite, and recovered after oxidation either by shaking in air or by adding buffered hydrogen peroxide solution. The fluorescence of the acid extract of the plasma membranes photolyzed in an alkaline medium was quite similar to that of lumiflavin obtained from the photolysis of riboflavin in an alkaline medium. The plasma membranes prepared from isolated hepatocytes (which were completely devoid of endothelial cell contamination) exhibited the same autofluorescence in the liver cell plasma membranes. The results suggest that the autofluorescence of the liver cell plasma membranes is most likely of a character similar to that of flavin, bound to hepatocyte plasma membrane proteins. This fluorescence is suitable for measuring the average lateral diffusion constant of proteins by means of FRAP methods.

Introduction

It was noted in our first report on the technique of fluorescence recovery after photobleaching (FRAP) that hepatocytes exposed to atmospheric oxygen in the form of a liver smear displayed a yellowish-green autofluorescence when excited with a 476.5 nm argon laser beam [1]. The intensity of this fluorescence was considerably increased and stabilized by treating the smear with a 1.0 mM hydrogen peroxide solution for 10 min at 37°C. We designated it, therefore, peroxide-induced autofluorescence (PIAF) [2,3].

Indirect evidence suggested that PIAF is localized on the proteins of the plasma membrane [2]. Nevertheless, further characterization was necessary.

In the present paper, we characterize the autofluorescence of isolated rat liver cell plasma membranes prepared in vitro. This autofluorescence appears to be due to an oxidized flavin compound bound to proteins of the hepatocyte plasma membrane.

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Abbreviations: FRAP, fluorescence recovery after photobleaching; PIAF, peroxide-induced autofluorescence.

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Materials and Methods

Liver cell plasma membrane preparations. Liver cell plasma membranes were isolated from livers of male Fischer 344 rats by the method of Boyer and Reno [4] with a minor modification [5]. Briefly, rats were killed by decapitation. The livers were excised, weighed and perfused with a cold saline solution to remove the blood. The liver was homogenized in 1 mM $NaHCO_3$ buffer (pH 7.5) using a Dounce homogenizer. The homogenates were centrifuged at $1100 \times g$ for 20 min. The supernatant was removed, and the precipitate fraction was rehomogenized in the same buffer and centrifuged again at $1100 \times g$. This process was repeated twice, which yielded pellets of plasma membrane plus nuclear fractions. These pellets were subjected to stepwise sucrose density gradient centrifugation at $95\,000 \times g$ for 60 min. The membrane layer at the sucrose density 1.18–1.16 interface was collected and washed three times with the bicarbonate buffer by centrifugation at $1100 \times g$ for 15 min.

The plasma membrane samples were characterized by assaying the following marker enzymes: 5′-nucleotidase for plasma membrane [6], glucose-6-phosphatase for microsomes [6] and succinate dehydrogenase for mitochondria [7].

In a separate experiment, a hydrogen peroxide solution was added to the initial liver homogenates (final concentration 10 mM) and the mixture was incubated at 37 °C for 10 min. All other steps for obtaining the liver cell plasma membranes were the same as those described above for preparations without hydrogen peroxide treatment.

Preparation of microsomes, mitochondria and the $100\,000 \times g$ supernatant. Rat liver was perfused with a cold saline solution and homogenized in 0.25 M sucrose. The homogenate was centrifuged at $600 \times g$ for 10 min. The upper layer was carefully separated from the precipitates on the bottom and recentrifuged at $5500 \times g$ for 20 min. The precipitates were suspended in a sucrose solution and washed by repeated centrifugation. The resulting precipitates were used as the mitochondrial fraction. The separated upper layer was centrifuged at $100\,000 \times g$ for 60 min. The supernatant was separated from the pellets, and is designated the '$100\,000 \times g$ supernatant'. The pellets were suspended in a sucrose solution and washed by centrifugation. The resulting pellets were used as the microsomal preparation.

Gel-filtration chromatography of liver cell plasma membranes. The plasma membrane preparation was solubilized with a 1% sodium dodecyl sulfate (SDS) solution buffered with 20 mM Tris-HCl (pH 7.4) and applied to a column of Sephadex G-200. The eluent was a 1% SDS solution at a flow rate of 0.28 ml/min. The protein concentration, phospholipid content and fluorescence intensity of each 4.5 ml fraction were measured. The fluorescence was monitored by the emission at 525 nm excited at 468 nm. Authentic riboflavin, FMN and FAD were also subjected to the column chromatography with the same SDS solution, and were used as marker substances.

Photolysis of the membrane extract and riboflavin in an alkaline medium. The membrane preparation was extracted with 5% acetic acid. The acid extract solution was alkalinized with 2.0 M NaOH and exposed to the light of a mercury lamp for 60 min. The solution was then acidified by the addition of glacial acetic acid, and extracted with chloroform. Lumiflavin was prepared from authentic riboflavin in exactly the same way [8]. The fluorescence pattern of the chloroform layer from membrane preparation was then compared with that of lumiflavin.

Preparation of isolated hepatocytes. Hepatocytes were isolated as previously described by Van Bezooijen et al. [9]. The Trypan blue exclusion test showed that the viability of the isolated hepatocytes was more than 90%. No non-parenchymal cells were observed by light microscopy. The plasma membranes of the isolated hepatocytes were obtained by the method described above for the whole liver.

Measurement of fluorescence. The fluorescence intensities were measured using an Hitachi 650-10S fluorescence spectrophotometer and were expressed relative to that of a quinine sulfate standard (0.1 μg/ml in 0.1 M H_2SO_4, excitation 350 nm, emission 450 nm) which was designated as 1 unit.

Reaction of malondialdehyde with glycine ethyl ester. In order to compare the fluorescence of the rat liver plasma membranes with that of adducts

of malondialdehyde and amino acids, the adducts were synthesized by the method of Kikugawa et al. [10]. An aliquot of the reaction mixture of malondialdehyde and glycine ethyl ester was diluted with 0.1 M phosphate buffer (pH 7.4) or 1% SDS solution buffered with 20 mM Tris-HCl (pH 7.4), and its fluorescence was examined.

Chemical analyses. The protein concentration was determined by the method of Lowry et al. [11] using bovine serum albumin as the standard. Lipid phosphorus was determined by the method of Bartlett [12].

Chemicals. SDS, FMN, FAD, glycine ethyl ester and quinine sulfate were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and riboflavin was from Sigma Chemical Co. (St. Louis, MO). Sodium hydrosulfite was from Koso Chemical Co. Ltd. (Tokyo, Japan). Malonaldehyde bis(dimethyl)acetal was from Aldrich Chemical Company, Inc. (Milwaukee, WI). Sephadex G-200 was from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were of analytical grade.

Results

Characterization of isolated liver plasma membranes

Table I shows activities of marker enzymes of subcellular fractions. These data suggest that the plasma membrane was highly purified, with minimal contamination by other subcellular fractions.

Fluorescence of the liver cell plasma membranes

Fig. 1A shows the fluorescence spectra of SDS-solubilized rat liver cell plasma membranes. When the emission was recorded by the excitation wavelength of 476.5 nm (that used for FRAP method [1–3]) the fluorescence peaked at 525 nm (dotted line). When the emission intensity was recorded at 525 nm against a wide range of excitation wavelengths, several peaks and shoulders were observed. However, the highest peak was at 468 nm – quite close to the 476.5 nm laser beam. When 468 nm was used as an excitation wavelength, the fluorescence pattern was very similar to that with 476.5 nm excitation, but had a higher peak at 525 nm.

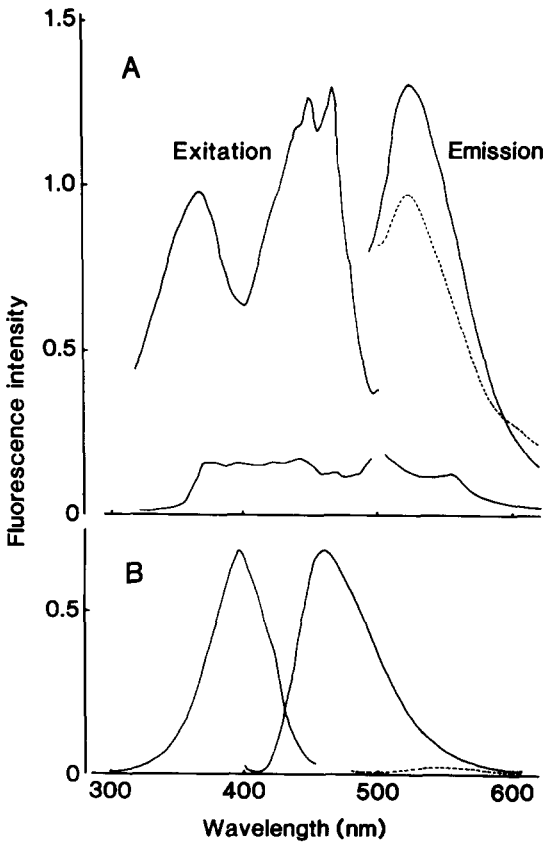

Fig. 1. Fluorescence spectra of SDS-solubilized rat liver plasma membranes (A) and of a synthetic adduct of malondialdehyde and glycine ethyl ester (B). (A) Dotted line is emission spectrum when excited at 476 nm: Upper solid lines are emission excited at 468 nm and excitation spectra monitored at 525 nm. Lower solid line is fluorescence spectra of membrane preparations after an addition of hydrosulfite. (B) Solid lines are a fluorescence emission spectrum of a reaction mixture of malondialdehyde and glycine ethyl ester diluted with a 1% SDS solution (pH 7.4) and excited at 397 nm, and an excitation spectrum monitored at 460 nm. The dotted line is emission spectrum excited at 476 nm.

TABLE I

SPECIFIC ACTIVITIES OF MARKER ENZYMES IN RAT LIVER PLASMA MEMBRANE

Values are mean ± S.D. (in μmol/mg protein per h) of three separate measurements.

	Specific activity	
	Homogenate	Plasma membrane
5′-Nucleotidase	2.48 ± 0.14	104.1 ± 1.5
Glucose-6-phosphatase	4.65 ± 0.42	1.85 ± 0.08
Succinate dehydrogenase	3.12 ± 0.28	0.29 ± 0.01

The liver cell plasma membrane preparations were fractionated into three subfractions by extraction with chloroform (Fig. 2): the water-soluble protein fraction, the protein fraction that precipitates at the phase interface, and the lipid fraction in the chloroform phase. The fluorescence spectra of the first two fractions were very similar to that of the SDS-solubilized membranes, whereas the chloroform fraction showed practically no fluorescence at 525 nm when excited at 468 nm (Fig. 2).

Effect of hydrosulfite

Fig. 1A also demonstrates the fluorescence recorded after the addition of sodium hydrosulfite crystals to the cuvette. The hydrosulfite decreased the fluorescence intensity very strongly. When the same sample was shaken for several minutes in air, or when a hydrogen peroxide solution was added under buffered conditions, the intensity recovered to the initial level (before the addition of hydrosulfite). If the hydrogen peroxide solution was added under unbuffered conditions, the recovery of the fluorescence was incomplete, achieving only 40–60% of the initial level.

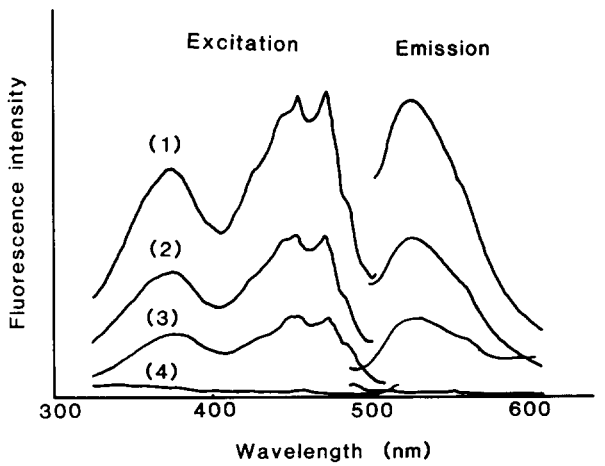

Fig. 2. Fluorescence spectra of the subfractions of the liver membrane preparation extracted with chloroform. (1) SDS-solubilized total plasma membrane fraction; (2) the protein fraction precipitated at the chloroform-water interface (resolubilized in SDS); (3) the water-soluble fraction; (4) the chloroform-soluble fraction.

Fluorescence of the malondialdehyde-glycine ethyl ester adducts

Fig. 1B shows the fluorescence spectra of the reaction mixture of malondialdehyde and glycine ethyl ester. The spectrum had an excitation maximum at 397 nm and an emission maximum at 460 nm. This fluorescence pattern did not change with the presence or absence of SDS. Excitation at 476 nm gave no appreciable fluorescence.

The effect of hydrogen peroxide pretreatment on membrane fluorescence

The fluorescence emissions of the hydrogen peroxide-pretreated membrane fraction did not differ from those of untreated preparations, nor was the intensity of fluorescence enhanced. We, therefore, continued to investigate the autofluorescence of the untreated membrane fraction only.

Sephadex G-200 column chromatography of membrane fluorescence

Fig. 3 shows a typical distribution of fluorescence and of protein and phospholipid contents in the eluted fractions obtained from Sephadex G-200 column chromatography of the SDS-solubilized membrane fraction. Fluorescence at 525 nm appeared over a wide range of fractions, some containing protein and some not. The highest fluorescence intensity, however, was found after fraction

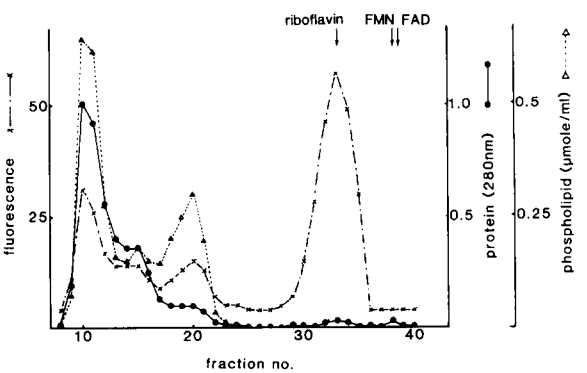

Fig. 3. Sephadex G-200 column chromatography of SDS-solubilized total plasma membrane fraction. Fluorescence was monitored by emission at 525 nm with 468 nm excitation. The protein concentration is expressed as the absorption at 280 nm. Phospholipid concentration is expressed as the concentration of inorganic phosphorus. Elution positions of riboflavin, FAD and FMN are shown.

30, where the protein content was minimal. This position coincided with that of an authentic riboflavin.

Phospholipid was not detected in any of the fractions after fraction 25. When the supernatant obtained after centrifugation of SDS-solubilized membrane suspension at $300000 \times g$ for 10 min was subjected to the same gel chromatography, most of the fluorescence observed with proteins (fractions 10–22 in Fig. 3) was not found. The fluorescence of a protein-rich fraction (fraction 10) was practically identical to that of a protein-free fraction (fraction 33) as well as to that of riboflavin.

Light sensitivity of the fluorescence

Membrane fractions were solubilized with a 1% SDS solution and exposed to the light of a 100-W mercury lamp for various lengths of time. The fluorescence intensities were recorded at an excitation of 468 nm. The fluorescence intensity at 525 nm decreased in a time-dependent fashion up to 20 min, after which the rate of decrease lowered (Fig. 4).

Fluorescence of lumiflavin and photolyzed membrane extract

Fig. 5 compares the fluorescence pattern of the membrane acid extract that was photolyzed in an alkaline solution with the fluorescence spectrum of lumiflavin obtained by the photolysis of authentic riboflavin. These fluorescence spectra were practically identical. Fig. 5 also shows fluorescence spectra of authentic samples of ribofla-

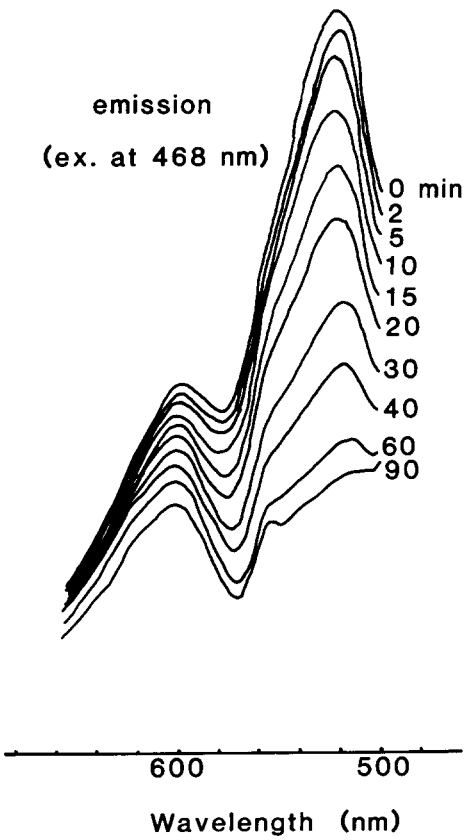

Fig. 4. The light sensitivity (fluorescence fading) of the autofluorescence in the total liver cell plasma membrane fraction. The curves show the emissions recorded with an excitation at 468 nm after exposure to light (mercury lamp, 100 W) for various time intervals (min).

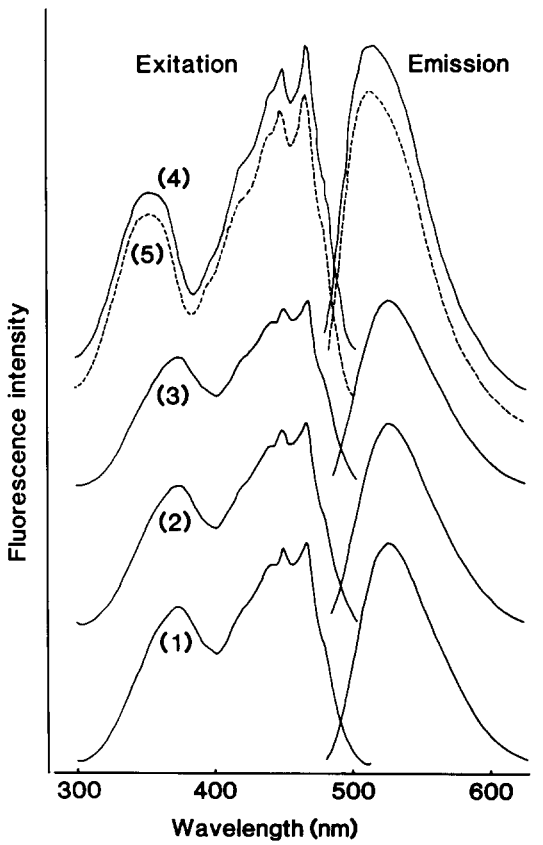

Fig. 5. Excitation and emission spectra of several flavin compounds and photolyzed membrane extract. (1), FAD; (2), FMN; (3), riboflavin; (4), lumiflavin; (5), chloroform extract after photolysis of membrane extract.

TABLE II

SPECIFIC INTENSITY OF THE FLUORESCENCE IN SUBCELLULAR FRACTIONS OF RAT LIVER

	Specific fluorescence intensity (U/mg protein [a])	Flavin content (pmol/mg protein)		
		Riboflavin [b]	FAD [c]	FAD calculated from the literature [13,14]
Plasma membrane	0.119 ± 0.004	0.019	0.401	–
Mitochondria	0.254 ± 0.019	0.041	0.857	0.561
Microsomes	0.155 ± 0.007	0.025	0.523	0.317
100000 × *g* supernatant	0.064 ± 0.005	0.010	0.216	0.245

[a] Mean of four experiments ± S.D.
[b] Values of specific fluorescence intensity expressed as riboflavin concentrations.
[c] Values of specific fluorescence intensity expressed as FAD concentrations.

vin, FMN and FAD. They all closely resemble that of the hepatocyte plasma membrane fractions.

Subcellular distribution of the fluorescence

Table II shows the specific fluorescence intensities of SDS-solubilized subcellular fractions determined at 525 nm with the excitation at 468 nm. The mitochondrial fraction had the highest value, almost twice that of the membrane fraction. Fluorescence intensities were also expressed as concentrations of either riboflavin or FAD. Although no directly comparable values were found in the literature, riboflavin and FAD contents that were calculated from the values found in several reports are also shown in Table II for comparison. When fluorescence intensities were expressed as FAD content, which is the most abundant form of flavin compounds, our values were fairly comparable with values calculated from past literature [13,14].

Fluorescence of membranes obtained from isolated hepatocytes

The fluorescence spectra for the membranes isolated from isolated hepatocytes were practically identical to those for membrane preparations obtained directly from the whole liver.

Discussion

The results of the present study demonstrate the presence of autofluorescence in rat liver plasma membranes excited at 476 nm. This wavelength was used in our FRAP study using an argon laser [1–3]. The fluorescence of the isolated membranes closely resembled that of a flavin in terms of fluorescence spectra under oxidized and reduced conditions, movement in SDS gel chromatography, and photolysis in an alkaline medium. Although there is no direct proof, these results strongly suggest that the autofluorescence of isolated liver plasma membranes is derived from a flavin(s) in the membranes.

It has been postulated that PIAF is located at the cell surface [2]. When excited at 476 nm, it appears as a yellowish-green fluorescence [1–3]. The fluorescence found in the rat liver plasma membranes had excitation maxima at 365, 450 and 468 nm, the latter being very close to the wavelength of the argon laser used for our FRAP study. The emission maximum at 525 nm also agrees with the yellowish-green color observed with the PIAF in our FRAP study.

The fluorescence of the membranes isolated from hydrogen peroxide-pretreated liver homogenate did not differ from that of the control membrane in either the pattern or the intensity of fluorescence. However, treating the membrane preparation with hydrosulfite greatly reduced the fluorescence intensity. Furthermore, the fluorescence recovered completely when hydrogen peroxide was added to the hydrosulfite-treated membrane preparation at physiologic pH, presumably due to reoxidation of the fluorophore. When this reoxidation was performed unbuffered, hydrogen

peroxide induced only partial fluorescence recovery. These phenomena also support the contention that the fluorescence in the membrane was due to a flavin(s), since the fluorescence of flavins is developed in their oxidized form, and its intensity depends on the pH value [15,16]. Hydrosulfite is known to react with hydrogen peroxide, yielding sulfuric acid. The incomplete recovery of the fluorescence in an unbuffered solution was probably due to a reduction of the pH value caused by sulfuric acid. In fact, the intensity of fluorescence recovered fully when the pH was adjusted.

Assuming that PIAF is due to the oxidized flavin, the rapid development of the autofluorescence after exposure of the liver smear to hydrogen peroxide may be explained. Hydrogen peroxide may oxidize the surface layer of the smear, resulting in rapid development of the fluorescence. In contrast, isolated membrane preparations very likely become fully oxidized during processing, so that the addition of hydrogen peroxide would have no further enhancing effect on the intensity of fluorescence.

It is well known that polyunsaturated fatty acids are peroxidized and form malondialdehyde under oxidizing conditions. Malondialdehyde was thought to react with free amino groups, forming conjugated Schiff bases with high fluorescence intensities [17]. Such a mechanism has been suggested as the basis for the fluorescence of so-called age pigments [18]. It is also conceivable that such a reaction could occur in the plasma membranes of hepatocytes, when treated with hydrogen peroxide, since all the other elements for such a reaction (polyunsaturated fatty acids, Fe^{2+}, amino acids) are believed to be present in the membrane fraction. Indeed, we ourselves suggested previously that the PIAF may be due to such a Schiff base fluorophore [2]. Recently, however, Kikugawa et al. [10,19] suggested that the fluorescence of the adducts of malondialdehyde and amine was not due to conjugated Schiff bases but, rather, to 1,4-dihydropyridine-3,5-dicarboaldehyde, which was postulated to be a Hantzsch-type adduct of malondialdehyde and amine. Whatever the real fluorophore for such a complex, the reaction mixture of malondialdehyde and glycine ethyl ester showed a fluorescence which was totally different from that of either the liver plasma membrane or the flavin(s) in the present study. The fluorescence of the adducts of malondialdehyde and amine reported in literatures all had an excitation maximum at about 320–420 nm, and an emission maximum at about 350–470 nm, i.e., they are blue fluorescences. So far, there is no Schiff-base-type fluorescence reported in the literature emitting with a peak wavelength above 500 nm [10,17–19]. In agreement with this, our reaction mixture of malondialdehyde and glycine ethyl ester did not emit any appreciable fluorescence when excited at 476 nm, suggesting that the fluorescence of liver plasma membranes is totally different from a Schiff-base-type fluorescence.

We know of no report of the presence of flavoproteins in the hepatocyte plasma membrane except for one study that demonstrated the uptake of riboflavin by isolated hepatocytes [20]. However, in recent studies, a flavoprotein in the plasma membrane of leukocytes was found [21,22] and attributed to a role in the formation of superoxide anion radicals. Our hepatocyte plasma membrane preparations retained fluorescence even though they were washed several times with aqueous solutions during preparation. Only when the membranes were solubilized with SDS or extracted with chloroform was most of the fluorescence released into the aqueous phase. Furthermore, a previous study demonstrated that the uptake of riboflavin by hepatocytes takes places by means of a carrier-mediated process, suggesting the presence of a specific carrier protein for riboflavin [20]. Although there is no direct proof for the binding of riboflavin with membrane proteins, our results coupled with the latter report [20] suggest that the this fluorophore (presumably flavin(s)) forms a complex with macromolecular membrane components, probably proteins. If the PIAF we used for our FRAP experiments is due to flavin(s) bound to surface membrane proteins, as the present results suggest, it represents an ideal fluorophore, since the fluorescence of flavins is very sensitive to light, (i.e., it is bleachable) and has its major part of fluorescence above the 500 nm wavelength and, thus, can be excited by an argon laser beam and since none of the external fluorescent labeling materials we have tried have proved suitable for FRAP studies on liver plasma membranes [23].

As discussed above, it seems very likely that the fluorescence of isolated plasma membranes is due to a flavin(s) located in the plasma membranes. Although this hypothesis may explain the nature of the autofluorescence which we used for FRAP, one should not rule out the possibility that some special types of protein-bound conjugated Schiff base may be involved in the PIAF, since there is no direct proof that the PIAF contains nothing else but membrane autofluorescence examined in vitro in the present study. Although no Schiff base complex with an emission spectrum above 500 nm (yellowish-green fluorescence) is known, one may exist. A similar argument has widely been used to explain the conflict between the blue (Schiff base) fluorophore originally postulated as the source of so-called age-pigment fluorescence and the actual yellow color observed under a fluorescence microscope [24,25]. Further work is needed to solve this problem.

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