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LABELING OF MEMBRANES FROM ERYTHROCYTES AND CORN WITH FLUORESCAMINE

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Summary

Fluorescamine was used as a fluorescent label for intact human erythrocytes and slices of corn coleoptile tissue. This reagent has a greater affinity for membranous than for soluble proteins, and also labels membrane lipids which contain primary amine groups. In addition, some membrane fractions from labeled coleoptiles have a higher affinity for fluorescamine than do others. The relative labeling of the various fractions can be altered by changing the pH of the external labeling medium. Because the pH of the medium determines the rate of hydrolysis of fluorescamine to an unreactive form, this result suggests that the specificity of this reagent towards different cellular structures is determined by the lifetime of the active reagent. Fluorescamine was not found to be a specific reagent for the cell surface.

Introduction

Fluorescamine has come into wide use as a sensitive fluorogenic reagent for primary amines, particularly proteins and amino acids [1,2,3]. It is logical therefore to consider its utility as a fluorescent label for supramolecular structures such as cells and cellular organelles. The short lifetime of fluorescamine in aqueous solution (seconds at slightly alkaline pH [4]) suggested that it might serve as a unique fluorescent reagent for sites on the plasma membrane. Its high reactivity and rapid hydrolysis in the extracellular medium might not allow the reagent sufficient time to diffuse into the cytosol before it was inactivated [5]. In addition, since the cytosol pH is not alkaline, its reactivity might be reduced for intra-cellular structures even if it did penetrate [2,5].

Hawkes et al. [6] have reported fluorescent surface labeling of avian fibroblasts on the basis of a similar argument. However, their evidence for surface

Abbreviation: MDPF, 2-methoxy-2,4-diphenyl-3(2H)-furanone.

labeling with fluorescamine was that known cytosol enzymes were not labeled under their conditions while certain undefined proteins and lipids were labeled. They did not measure the degree of labeling of different cellular fractions, however, so that the precise nature of the labeling remained unknown.

There are, moreover, reasons for doubting that fluorescamine could serve as a cell-surface label. First, fluorescamine is a highly lipophilic molecule which does not dissolve directly in aqueous solutions, but must be dispersed from an initial non-hydroxylic solvent into water. With such a lipophilic character, fluorescamine would be expected to permeate membranes rapidly. Fluorescamine is three to four times more reactive at pH 9 (the pH of the external labeling medium used by Hawkes et al. [6]) than at the pH of the cytosol [6,7]. However, the relative excess of amines found in the cytosol should compete strongly for available reagent. The resulting steep concentration gradient would be expected to favor diffusion of reagent into the cell.

Fluorescence microscopy of living cells stained with fluorescamine has been reported [6,7], but the authors described seeing uniform fluorescence over the cells. Hawkes et al. [6] also reported that mitotic cells were more brightly stained than interphase cells. Our own unpublished observations of higher plant and algal cells labeled with fluorescamine also revealed a uniform blue fluorescence, but fading of the label was too rapid to allow us to make any firm conclusions about its intracellular distribution.

In the experiments reported here fluorescamine was reacted with intact human erythrocytes or corn coleoptile tissue, and the patterns of membrane labeling compared with those obtained when the same cells were labeled after lysis or homogenization. The results show that fluorescamine has a higher relative affinity for membranes than for cytosol proteins, but is not a specific cell-surface label.

Methods

Labeling of erythrocytes

Human erythrocytes (type A, Rh (+)) were obtained from the Stanford University Hospital Blood Bank. They were diluted three-fold in phosphate-buffered saline solution (0.13 M NaCl, 0.01 M K_2HPO_4, 0.01 M KH_2PO_4, pH 7.0) at 0°C and washed by resuspension in this buffer four times. The cells were then resuspended in labeling medium (20 mM $Na_4P_2O_7$, 0.25 M sucrose, brought to pH 9.0 with HCl) at 21°C and centrifuged again. After cells were again resuspended in this medium they were given 25 µl acetone containing 1.5 mg fluorescamine per 10 ml of labeling medium while the tube was vortexed. After 5 min, the cells were again pelleted and resuspended twice in phosphate-buffered saline solution. Finally the cells were pelleted and resuspended in 2 ml water. After the cells lysed, they were diluted with 10 ml ghost washing buffer (20 mM potassium phosphate buffer, pH 7.0). The ghosts were pelleted at 10 000 × *g* for 5 min, and the upper portion of the supernatant fluid was sampled. The remainder of the supernatant fluid was aspirated and the loose pellet was resuspended in ghost washing buffer and washed until no visible hemoglobin remained in the pellet. A sample of the supernatant protein from the first centrifugation was diluted with 10 volumes of 1% HCl in acetone and

the precipitated protein centrifuged and resuspended in deionized water. The protein was extracted twice more in this fashion to remove residual heme.

Labeling of coleoptile tissue

Hybrid corn seed (*Zea mays* L. WF9 X Bear 38) were obtained from the Bear Hybrid Corn Company, Decatur, Ill. Seedlings were grown in the dark with a daily red light treatment as previously described [8].

In preparation for labeling, coleoptiles free of primary leaf were removed from the seedlings [8], and sliced into 2-mm segments [5]. The segments were washed free of the contents of cut cells at the ends by stirring slowly in four changes (30 min each) of 20 mM potassium phosphate buffer, pH 7 at 21°C. The washed slices were placed into the same labeling buffer used for erythrocytes, 5 ml per g tissue at 21°C. This was immediately aspirated and replaced with twice the volume of the same medium. The fluorescamine (dissolved in a non-hydroxylic solvent, usually acetone) was added as the slices were rapidly stirred. After 5 min, the slices were rinsed three times with 20 mM potassium phosphate buffer.

Fractionation of coleoptile tissue

Labeled coleoptile tissue was fractionated by differential sedimentation as previously described [5,8]. Labeled sections were flooded with ice-cold homogenization buffer (50 mM Tris/acetic acid, 1 mM disodium EDTA, 14 mM 2-mercaptoethanol, 0.25 M sucrose, pH 8.0 at 21°C), and mashed with a chilled mortar and pestle. The mash was then filtered through nylon parachute cloth (2.7 × 4 threads/mm). This filtrate was the basis for further fractionation by sequential sedimentation at $500 \times g$, 15 min; $9000 \times g$, 15 min; $21000 \times g$, 15 min and $48000 \times g$ for 60 min. The pellets from each of these fractions were resuspended in a small volume of the homogenization buffer. For experiments in which K^+-stimulated ATPase was measured, the particulate fractions were washed by repelleting at the same centrifugal force.

Density gradient centrifugation

The supernatant from the $9000 \times g$ centrifugation described above was gently layered over gradients and centrifuged. In some experiments the $9000 \times g$ supernatant was washed free of soluble components by centrifugation onto a cushion of 45% sucrose (w/w) at 25000 rev./min for 2 h in the SW27 rotor. The membranes were resuspended in buffer adjusted to 9% sucrose (w/w) and this step was repeated. Then a 15%—45% (w/w) sucrose gradient was formed atop the cushion membranes. This cushioning process avoided the formation of membrane aggregates which formed when the coleoptile membranes were pelleted against the wall of a tube. Gradient buffers contained (besides sucrose): 10 mM Tris/acetic acid, 1 mM disodium EDTA and either 0.1 mM or 10 mM $MgCl_2$ as indicated, pH 8.0 at 21°C. Gradients were centrifuged at least 4 h at 25000 rev./min, 4°C.

Measurement of fluorescence

To avoid light scattering in particulate fractions, samples were diluted in 0.25% sodium dodecyl sulfate and centrifuged in a clinical centrifuge. Relative

fluorescence was then determined with a Perkin-Elmer PE-MPF-3L spectrofluorimeter. Fluorescence emission was measured at 475 nm with 390 nm excitation (10 nm slits for both) [2]. The relative fluorescence of each labeled sample was corrected for protein content and for the intrinsic fluorescence of the corresponding unlabeled sample.

Analytic methods

Protein was measured after precipitation with 10% trichloroacetic acid by the method of Lowry et al. [9] with bovine serum albumin as the standard. Potassium-stimulated ATPase was assayed by the method of Hodges et al. [10] using the Tris salt of ATP prepared by their method. Cytochrome *c* oxidase and NADH-dependent cytochrome *c* reductase were assayed by the methods of Jesaitis et al. [8]. Magnesium-independent glucan synthetase (glucan synthetase II) was assayed by the method of Ray [14].

Chemicals

Fluorescamine and 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF), were obtained from Hoffman-La Roche, Inc., Nutley, N.J. Disodium ATP, 2-mercaptoethanol, Folin phenol reagent, bovine serum albumin, equine cytochrome *c* and butylated hydroxyanisole were obtained from Sigma Chemical Co. UDP-glucose was from PL Laboratories. Schwarz/Mann Enzyme Grade sucrose was used. All other chemicals were reagent grade.

Results

To test the specificity of fluorescamine for the cell surface, we initially labeled human erythrocytes under several different conditions of buffer and of initial solvent for the fluorescamine. In the first experiments there was an excess of cells to fluorescamine. The labeled cells were then hypotonically lysed, and the ghosts and soluble protein were separated. Heme was extracted from the soluble protein with acid acetone before the fluorescence was mea-

TABLE I

EXTRACTION OF FLUORESCAMINE-LABELED GHOSTS OR SOLUBLE FRACTIONS WITH NON-AQUEOUS SOLVENTS

I. Erythrocytes were labeled with fluorescamine and ghosts prepared from them as described in Methods. The ghosts were suspended in 20 mM potassium phosphate buffer, pH 7.0 and twice extracted with chloroform/methanol (2 : 1, v/v). The organic phases were combined, taken to drynes under a stream of air, and the residue was taken up in 0.25% sodium dodecylsulfate. The total fluorescence of the aqueous and organic phases was then measured. II. Soluble erythrocyte protein (60 μg) was extracted with acid acetone before (1) or after (2) labeling with an excess of fluorescamine (1.5 mg) and the fluorescence was then measured as described in text.

I Extraction of labeled ghosts with chloroform/methanol (2 : 1, v/v)			II Extraction of soluble protein with acid/acetone	
Phase	Relative fluorescence	% of original fluorescence	Extraction before or after labeling	Relative fluorescence
1 Aqueous	1329	58	1 Before	4514
2 Organic	1198	52	2 After	4566
Total	2527	110		

sured. When the cells were suspended in 0.15 M NaCl + 20 mM $Na_4P_2O_7$ buffer (pH 9.0) we found that the ghosts acquired 84 times the fluorescence per mg of protein as did the soluble protein. The initial solvent for the fluorescamine did not appear to affect the distribution of label greatly, since the ratios of ghost to soluble protein fluorescence were approximately the same for acetone and *p*-dioxane, although the rate of hydrolysis of fluorescamine is two orders of magnitude faster when initially dissolved in dioxane than when dissolved in acetone [4]. Borate and pyrophosphate buffers gave similar results, so pyrophosphate was used in subsequent experiments. In this experiment the osmoticum was NaCl, but similar results were obtained with sucrose, which was used in later experiments.

This apparent specificity for membranes could be caused by an excess of primary amines on the membranes rather than by lack of free permeation of the reagent into the cell. Therefore we labeled small, equal amounts of ghost protein and heme-free soluble protein with a saturating amount of fluorescamine. Table I shows that the ghosts became labeled twice as well per mg of protein as did the soluble protein. However, half of the fluorescence in the ghosts could be extracted into chloroform/methanol (2 : 1, v/v). Thus, the ghost protein itself became labeled no more than the heme-free soluble protein. Furthermore (Table I), hemoglobin that was extracted with acid-acetone after fluorescamine labeling was as fluorescent as that which was extracted before applying the label. Therefore, acid-acetone extraction does not itself remove the label from soluble protein. These results indicate that ghost protein and cytosol protein are about equal in their content of fluorescamine-reactive sites.

If fluorescamine is truly an external label it should lose its specificity for ghosts when the red blood cells are lysed before labeling (Table II). To test this, equal volumes of packed cells were labeled intact, lysed in distilled water prior to labeling, or first separated into ghost and soluble fractions which were then labeled separately. In these experiments there was an excess of cells over fluorescamine. We see that fluorescamine prefers the ghosts of pre-lysed cells

TABLE II

LABELING OF ERYTHROCYTES BEFORE AND AFTER LYSIS

Intact erythrocytes (1.0 ml packed cells) were either resuspended in pH 9.0 labeling buffer as in Methods (A), or lysed with distilled water and brought to pH 9.0 with crystals of $Na_4P_2O_7 \cdot 10\ H_2O$ (B), or lysed with distilled water and separated into ghost and soluble protein fractions as described in Methods (C and D). The ghost fraction (C) was resuspended into labeling buffer (pH 9.0), but the soluble fraction (D) was left at pH 7. Each sample was then labeled and the fluorescence in the ghosts and heme-free soluble protein was then determined.

Material labeled (pH)	Sample	Fluorescence per mg protein	Ratio of fluorescence (ghost/soluble)
A. Intact cells (pH 9.0)	Ghost	10999	120
	Soluble protein	92	
B. Lysed cells (pH 9.0)	Ghost	12961	79
	Soluble protein	164	
C. Isolated ghosts (pH 9.0)	Ghost	32048	
D. Soluble protein (pH 7.0)	Soluble protein	316	101 (C/D)

almost as well as those of intact cells, and that ghosts labeled separately are still preferentially labeled. Because of the excess of protein over fluorescamine the kinetics of reaction of fluorescamine must favor membranous sites.

As was mentioned, 50% of the fluorescence of the ghosts was associated with lipid. We chromatographed the chloroform/methanol (2 : 1, v/v) extract on thin layers of silica gel G (0.25 mm thickness) using chloroform/methanol/ammonia (46 : 18 : 3, by vol.) along with authentic standards of fluorescamine-labeled phosphatidylserine (R_F 0.03) and phosphatidylethanolamine (R_F 0.49). The extract resolved into two major fluorescent spots which correspond in R_F to the spots of authentic, fluorescamine-labeled phosphatidylserine and phosphatidylethanolamine. Hawkes et al. [6] have also observed these lipids to be labeled. When extracted with chloroform, these spots showed fluorescence axcitation and emission spectra characteristic of fluorescamine derivatives. As no fluorescence remained at the origin, we saw no evidence for the labeling of chloroform-soluble protein. Chromatography in an acidic solvent system did not reveal any further spots.

Bretscher [12] has shown that these two membrane lipids face the inner surface of the erythrocyte plasma membrane. Therefore it is clear that fluorescamine can permeate this membrane in intact cells.

Fluorescamine labeling of corn coleoptile tissue

Although it is clear that fluorescamine permeates the plasma membrane of erythrocytes, the preceding data do not tell us anything about the pattern of labeling in more typical cells which contain organelles. In the following experiments, therefore, slices of tissue from dark-grown corn coleoptiles were labeled and then fractionated by differential sedimentation or by isopycnic equilibrium sedimentation in sucrose gradients.

We previously reported [5] that the highest fluorescamine specific activity co-sedimented with the highest specific activity of K^+-stimulated Mg^{2+}-dependent ATPase in differential sedimentation experiments. Table III shows the results of such an experiment: the distribution of fluorescamine labeling most resembles that of the K^+-stimulated ATPase. The fraction showing the highest specific activity (the $21000 \times g$ pellet) shows even greater relative activity of the Mg^{2+}-independent glucan synthetase (glucan synthetase II) [11]. This enzyme is thought by Van Der Woude et al. [11] to reside in the plasma membrane by virtue of its coincidence with fractions exhibiting specific binding of the auxin transport inhibitor, naphthylphthalamic acid [5]. The distribution of fluorescence differs somewhat from the cytochrome *c* oxidase activity, a mitochondrial marker, but there is not a clear difference between the pattern of labeling and the pattern of NADH-dependent cytochrome *c* reductase, a marker for endoplasmic reticulum. None of these enzymatic activities were reduced in the labeled samples as compared to unlabeled controls (not shown). Thus the pattern of fluorescence does not rule out association of the label with any of these enzymatic activities.

The pattern of fluorescence does not, however, coincide with the overall distribution of protein. This is particularly clear in the soluble protein fraction, which labeled only 0.7 times as well as the total homogenate, although it contained 75% of the total protein.

TABLE III

FLUORESCAMINE LABELING OF CORN COLEOPTILES AND ENZYMATIC ANALYSIS: DIFFERENTIAL SEDIMENTATION

Washed corn coleoptile slices were labeled, homogenized and fractionated by differential sedimentation as described in Methods. Each fraction was washed by repelleting in the homogenization medium. Fluorescence and enzymatic activities per mg of protein have been normalized to the values for the total homogenate. Fluorescamine labeling in vivo had no effect on any of the measured activities. Enzymatic activities for the homogenate were: K^+-stimulated ATPase, 178 nmol · min^{-1} · mg^{-1} protein; cytochrome *c* oxidase, 62 μmol · min^{-1} · mg^{-1} protein; NADH-dependent cytochrome *c* reductase, 5.5 mmol · min^{-1} · mg^{-1} protein.

Fraction	Percentage of total protein	Fluorescence	Relative enzymatic activities			
			K^+-stimulated ATPase	Glucan II synthetase	Cytochrome *c* oxidase	NADH cytochrome *c* reductase
Homogenate	100	1.0	1.0	1.0	1.0	1.0
500 × *g*, 15 min pellet	4	2.2	0.2	2.4	0.9	3.1
$9 \cdot 10^3 \times g$, 15 min pellet	11	1.5	1.4	4.8	3.4	1.1
$21 \cdot 10^3 \times g$, 15 min pellet	3	3.0	4.2	11.2	3.8	10.9
$148 \cdot 10^3 \times g$, 60 min pellet	6	2.9	3.2	3.9	0.9	4.3
Supernatant	75	0.7	1.9	1.8	0	2.3

We find, however, that the pattern of labeling can be altered by the pH of the external medium (Fig. 1). Since the pH of the medium determines the rate of hydrolysis of the reagent (this rate is 100 times higher at pH 9 than at pH 7) [4], it appears that the time allowed for penetration of the active reagent can influence the result. In Fig. 1 we see that increasing pH results in enhanced labeling of most particulate fractions, while reactivity of the supernatant amines was relatively unaffected.

In order to study this phenomenon further we made use of the analogous fluorogenic reagent, MDPF. MDPF is more lipophilic than fluorescamine, reacts more slowly with amines, and has a slower rate of hydrolysis [13]. When its pattern of reaction was tested as a function of pH (Fig. 1) we found two differences from fluorescamine: (1) the label appears predominantly in fractions other than the 21000 × *g* pellet and (2) as the pH of the medium is raised, its reactivity towards the particulate fractions (other than the 500 × *g* pellet) increases. However, the increase is less than with fluorescamine. From the effect of pH on the pattern of labeling we infer that this pattern is influenced by the degree of penetration of active reagent from the external medium.

To gain more information about the distribution of label in the post-mitochondrial fraction we employed sedimentation to isopycnic equilibrium on sucrose density gradients. We have used both low Mg^{2+} (0.1 mM) and high Mg^{2+} gradients, since low Mg^{2+} characteristically allows ribosomes to become detached from rough endoplasmic reticulum and causes it to sediment to lower density. Fig. 2 shows the result of such analysis. When both the homogenization buffer and gradient contained 10 mM Mg^{2+} and 1 mM EDTA, the major par-

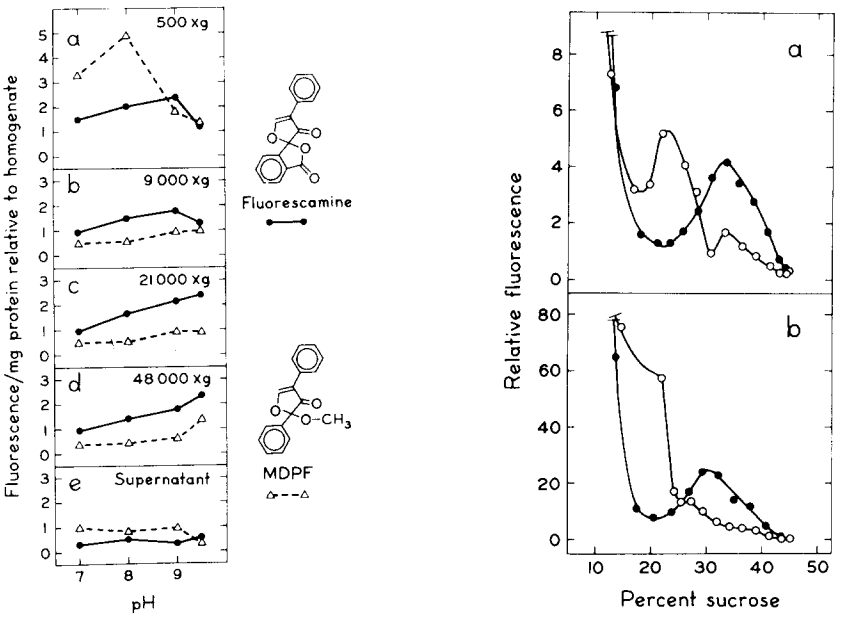

Fig. 1. Labeling of coleoptile fractions by fluorescamine and MDPF as a function of pH. Washed coleoptile fractions were labeled and fractionated as described in the text except that labeling buffer was adjusted with HCl to the indicated pH at 21°C before use. Results are plotted as fluorescence at 475 nm per mg protein in the indicated fraction, divided by the fluorescence in the homogenate at that pH. a, 500 × *g* pellet; b, 9000 × *g* pellet; c, 21000 × *g* pellet; d, 48000 × *g* pellet; e, supernatant. Circles, fluorescamine; triangles, MDPF.

Fig. 2. Equilibrium sedimentation of labeled corn coleoptile membranes on sucrose gradients. a. Membranes from tissue labeled before homogenization. 4 g of tissue were labeled and homogenized as described in the text. The supernatant after the 9000 × *g* centrifugation was layered onto a 15—45% linear sucrose gradient prepared in low Mg^{2+} (○———○) or high Mg^{2+} (●———●) as described in the text, and centrifuged in the SW27.1 rotor at 25000 rev./min for 4 h. The gradients were fractionated with an Isco density gradient fractionator. b. Membranes from tissue labeled after homogenization. Tissue was flooded with buffer (20 mM $Na_2P_2O_7$/HCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.25 M sucrose, 14 mM 2-mercaptoethanol, pH 8.0) and homogenized. The homogenate was vortexed at room temperature with the same amount of fluorescamine as in a, and processed exactly as above. ○———○, low Mg^{2+}; ●———●, high Mg^{2+}.

ticulate peak of fluorescence was found at about 35% sucrose (ρ = 1.15 g · cm^{-3} at 4°C). If the gradient only contained 0.1 mM Mg^{2+} and 1 mM EDTA, then the major peak sedimented to about 22% sucrose (ρ = 1.09 g · cm^{-3}) instead, but there was still a minor peak at 35% sucrose.

This shift of membrane label from higher to lower density is probably due to the stripping of ribosomes from rough endoplasmic reticulum in the low Mg^{2+} medium. However, the greater density in the presence of Mg^{2+} may be partly due to membrane aggregation, since aggregates can be seen by eye at this density. Also the material at 35—45% sucrose only partially resedimented at 22% sucrose when it was recentrifuged in a gradient containing EDTA without Mg^{2+} (not shown).

To discover if the pattern of labeling was determined by the access of fluorescamine to intracellular structures, tissue was first homogenized and then the homogenate labeled. Pyrophosphate buffer was substituted for Tris in the

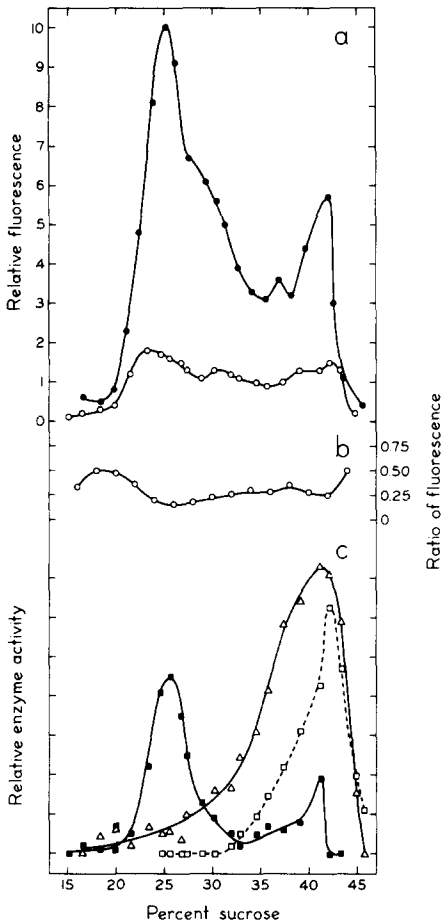

Fig. 3. Equilibrium sedimentation of labeled coleoptile membranes before or after homogenization. Three 10-g samples of corn coleoptiles were prepared for labeling as described. One sample was left unlabeled, the second was labeled in vivo as described, and the third was labeled after grinding in the mortar and pestle with homogenization buffer. For this experiment, labeling buffer was 50 mM Tricine/KOH, 1 mM disodium EDTA, pH 8.0 at 21°C. Homogenization buffer was the same plus 0.25 M sucrose and 14 mM 2-mercaptoethanol. The supernatant membranes from the sedimentation at 9000 × *g* were twice collected on a cushion as described and floated upwards on a 15—45% sucrose gradient for 12 h at 25000 rev./min in the SW27 rotor. a. Fluorescence from two separate gradients of membranes from tissue labeled before (○———○) or after (●———●) homogenization. The autofluorescence of the unlabeled sample was insignificant and this gradient is not shown. b. Ratios of fluorescences from tissue labeled before grinding to that labeled after grinding. Ratios were computed at intervals from the smooth curves (a) drawn through the points. c. Enzymatic activities from the gradient labeled before grinding. ■———■, NADH-dependent cytochrome *c* reductase; □———□, cytochrome *c* oxidase; △———△, glucan II synthetase. Peak activities were; NADH-dependent cytochrome *c* reductase, 2.6 mmol · min^{-1} · ml^{-1}; cytochrome *c* oxidase, 36 μmol · min^{-1} · ml^{-1}; glucan II synthetase, 380 pmol · min^{-1} · ml^{-1}.

homogenization medium because Tris is a primary amine. As is seen (Fig. 2b) relatively more of the label is found at the top of the gradient, but the major peak is still at about 22% sucrose in 1 mM EDTA (here not completely resolved from the massive labeling at the top of the gradient). Again, 10 mM Mg^{2+} shifts the peak to greater density, here about 31% sucrose.

The high fluorescence at 25% sucrose in 0.1 mM Mg^{2+}, 1 mM EDTA suggests

strongly that the endoplasmic reticulum is being labeled. Fig. 3 shows the results of a further isopycnic density gradient fractionation and enzymatic analysis of the postmitochondrial supernatant. There is, in fact, a good correlation between one of the peaks of fluorescence and the peak of NADH-dependent cytochrome *c* reductase, an endoplasmic reticulum marker. However, label appears additionally over a broad range of densities, including fractions which contain cytochrome *c* oxidase and glucan synthetase II [11] activities.

Shown on the same graph (Fig. 3a) is the fluorescence pattern from a gradient analysis of a similar preparation which had been labeled after homogenization in a medium containing Tricine (a secondary amine) instead of Tris. Here we can see clearly that the pattern of labeling is nearly the same. In Fig. 3b is plotted the ratio of labeling when tissue is labeled before homogenization to labeling after homogenization. Except for a certain amount of noise at the top and bottom of the gradient, this plot is nearly flat over the range of densities. Thus fluorescamine permeation is not a significant factor in determining the relative labeling of different postmitochondrial fractions.

Discussion

Although fluorescamine exhibits an apparent specificity for labeling the plasma membranes of erythrocytes (when not used in excess), we conclude that it does so because of an apparently greater reactivity for membranes per se, rather than because of any inability to permeate the plasma membrane. This increased reactivity may be due either to favorable partitioning of fluorescamine into the membrane phase or a longer lifetime of the active reagent in the aprotic regions of the lipid. The relative affinity of this reagent for different cellular structures can be altered by adjusting the pH of the external medium. It is not the time for diffusion into the tissue which is important, however, but rather the relative binding to lipophilic and hydrophilic structures. This conclusion is supported by the similarity of the patterns of labeling when the reagent is introduced either before or after homogenization, both with erythrocytes and with corn tissue.

Since fluorescamine is commonly used as a reagent for protein determination, caution is advised to insure that the amine sites being assayed are being saturated. In the case of a membrane preparation, the degree of labeling of lipids must be ascertained as well, since the protein in lipophilic structures may otherwise be considerably over-estimated.

The higher affinity of fluorescamine for membranes may make this reagent a useful fluorescent probe of membranous structures. We, and others [6], have observed that labeled cells are not killed, and that several membranous enzymes (K^+-stimulated ATPase, cytochrome *c* oxidase, NADH-dependent cytochrome *c* reductase) are not inhibited in extracts of labeled cells (not shown). In addition to direct labeling of cells, membrane components like phospholipids could be labeled in vitro and then added to cells.

However, fluorescamine is not an external label for the plasma membrane of animal or plant cells, despite a report to the contrary [6]. Hawkes' finding that fluorescamine did not significantly label internal soluble enzymes is under-

standable on the basis of the preference of this reagent for lipophilic structures and the small amount of the label she used.

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