Submitted to Biochemical and Biophysical
Research Communications

LBL-4631
Preprint

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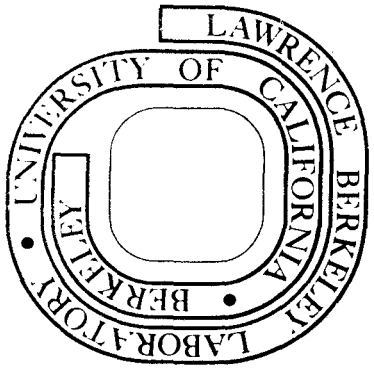
November 1975

Prepared for the U. S. Energy Research and
Development Administration under Contract W-7405-ENG-48



DISCLAIMER

THE USE OF FLUORESCAMINE AS A PROBE FOR LABELING THE OUTER SURFACE OF THE PLASMA MEMBRANE

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SUMMARY

A rapid method was developed to label the outer surface of chick embryo fibroblasts with fluorescamine without disruption of the cell monolayer. Polyacrylamide gel electrophoresis resolved two distinct areas of fluorescence: a group of high molecular weight polypeptides and several rapidly migrating species. The latter were demonstrated by tlc to be phospholipids. Fluorescamine did not label internal components of the cell as evidenced by two intracellular proteins which were found to be non-fluorescent. Intact normal cells were labeled 3-fold more than transformed cells, indicating a possible loss of exposed sites at the surface, while disrupted cells, subsequently labeled, yielded similar amounts of fluorescence.

INTRODUCTION

Selective labeling of plasma membrane components of whole cells should be rapid, physiological and minimize proteolysis and structural rearrangement. Furthermore, internal components should not be labeled. These conditions were met by use of a fluorescent probe to label proteins exposed specifically at the cell surface and a subsequent method of analysis of sufficient resolution to eliminate the necessity of fractionating cells and purifying their membranes.

Fluorescamine is an attractive candidate for use as a membrane label for many reasons. It reacts with primary amines to form a fluorescent product at an optimum pH 9 and with a half-time of a fraction of a second. Excess reagent is hydrolyzed to a non-fluorescent form with a half-time of several seconds. Theoretically, then, fluorescamine should react with all accessible primary amines on the cell surface; for example, with N-terminal amino acids of proteins, epsilon amino groups of lysine residues and free amino groups of lipids. Furthermore, any fluorescamine passing through the membrane would in all likelihood be hydrolyzed to an unreactive form. Thus, all fluorescent products should be located on the outer part of the cell membrane.

MATERIALS AND METHODS

Growth of cell cultures and labeling procedure: Cultures of chick embryo fibroblasts were prepared and transformed with the Schmidt-Ruppin strain of Rous sarcoma virus, subgroup A, as described (1).

Normal and transformed chick embryo fibroblasts were labeled 48 hours after secondary seeding. After removal of the medium from the monolayer, the cells were washed with warm Hanks and borate buffer.[1] A solution of fluorescamine in acetone was added to buffer to a final concentration of 0.5% acetone and immediately applied to the cells. After thirty seconds, the cells were washed and solubilized in 2% SDS.

Gel electrophoresis: Samples of total protein from chick embryo fibroblasts were subjected to electrophoresis on polyacrylamide using the dissociating system of Laemli (2). A method (Hawkes, S. P., Reinhardt, J., unpublished) was developed for the separation of polypeptides on a linear gradient of 10-20% acrylamide. Gels were examined for fluorescence with a uv hand lamp and then fixed in acetic acid/isopropanol, stained with Coomassie Blue and destained sequentially (3).

Cell sonicates were analyzed by starch gel electrophoresis using the discontinuous tris-citrate buffer system and staining procedures described by Selander *et al.* (4).

Lipid analysis: Lipids were extracted from fluorescamine labeled cells by the method described by Radin (5), and analyzed by tlc on pre-coated silica gel plates (adsorbisol-5, Applied Sciences). Samples were applied in chloroform and the plates developed in a solvent system of chloroform : methanol : glacial acetic acid : water (25:15:2:1 or 80:20:4:2). Spots were visualized with iodine vapor, phosphate was detected with molybdate spray (6) and fluorescence located with a uv hand lamp.

Analytical procedures: Emission of fluorescent compounds was monitored at 470 nm with the exciting wavelength set at 390 nm and relative fluorescence was determined by comparison with a standard solution of quinine sulfate in IN H_2SO_4. Protein concentrations were determined by the method of Lowry (7).

RESULTS AND DISCUSSION

The stability of the fluorophor and hence the suitability of fluorescamine as a potential membrane probe was examined by the use of a standard protein, ovalbumin. Solutions of ovalbumin were reacted with fluorescamine adsorbed to celite in order to eliminate interference by acetone in subsequent procedures. Under these conditions, ovalbumin could easily be detected in the picomole range. The addition of 2% SDS and 5% 2-mercaptoethanol had minimal effect on fluorescence yield. Furthermore, the binding of fluorescamine to ovalbumin did not interfere with protein determination by the Lowry procedure. Solutions

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[1] Abbreviations; buffer - 0.2 M H_3BO_4 (pH 9.0), SDS - sodium dodecyl sulfate.

of labeled ovalbumin were maintained in the dark at -70°, -20°, 4° and 22° for 24 hours. Fluorescence was stable over this period of time. Similar stability was observed with the fluorescent derivatives of cellular components, despite the report by Bohlen et al. (8) that some proteins were relatively unstable under the conditions of their assay.

It was desirable to maintain cells in as close a physiological environment as possible during treatment with fluorescamine. Labeling occurs at physiological tonicity and temperature. The integrity of the membrane at pH 9.0 was examined by the trypan blue exclusion test and the degree of leakage of radioactively labeled proteins from the cells. In both tests no difference was observed between cells at pH 7.4 and pH 9.0 for at least 10 minutes, for both normal and transformed cells. Therefore, under the conditions described in the labeling procedure, the membrane appeared to be intact. Finally, acetone was shown to have little effect on the labeling of cells since the extent of labeling and the profile of separated polypeptides was no different when acetone was omitted by the use of fluorescamine adsorbed to celite particles.

The time required to label chick embryo fibroblasts was approximately one minute. The cells and all solutions were maintained at 37° until the removal of fluorescamine at which time all subsequent operations were carried out at 4°. Monolayers examined by fluorescence microscopy were uniformly labeled, with the exception of mitotic cells which fluoresced more intensely.

In order to eliminate cell fractionation and membrane purification procedures, a method of gradient polyacrylamide gel electrophoresis, which gave good resolution in separations of complex mixtures of proteins from 2000 to 200,000 daltons, was developed. Figure 1 shows a typical separation as visualized by Coomassie blue staining. In a similar separation of proteins from cells prelabeled with fluorescamine, two major areas of fluorescence were observed (Fig. 1): a group of bands, around 200,000 daltons, and a

second group of rapidly migrating species. These were shown by tlc to be phospholipids with R_f's corresponding to fluorescamine-treated phosphatidylethanolamine and phosphatidylserine, which are constituents of membranes. There were one or two bands in the intermediate region which were difficult to locate precisely with the instrumentation available.

In order to determine whether fluorescamine penetrated the cells in a reactive form, two intracellular proteins were examined to determine whether they were labeled under the conditions described previously. Total protein from fluorescamine-labeled cells was subjected to starch gel electrophoresis. The gel was sliced horizontally into three replicates, two of which were stained for lactate dehydrogenase--an abundant enzyme in chick embryo fibroblasts (9), and phosphoglucose isomerase. The third was examined for fluorescence. In no case were the fluorescent bands coincident with the stained proteins, thus supporting the contention that intracellular proteins were not labeled.

A comparison of the fluorescence of intact normal and transformed cells is presented in Figure 2. Transformed cells saturated around 250 ug fluorescamine/ml buffer whereas normal cells were not saturated at 500 ug/ml. At this point the extent of labeling was 3-fold greater than the fluorescence of transformed cells. That this is a surface phenomenon is demonstrated in Figure 3. Cells disrupted by sonication and then reacted with fluorescamine were labeled to almost the same extent at all ratios of fluorescamine/protein examined. Indeed, transformed cell sonicates may have been labeled more.

Why the difference? Firstly, it could reflect a difference in the quantity of sites on the surface. For example, many workers (10,11) have observed the disappearance of the so called '250K' protein from the plasma membranes of cells after transformation, as can be seen by the absence of one (or more) bands of molecular weight > 200,000 daltons in Fig. 1. Whether this could account for such a difference is questionable. Secondly, it could

reflect differences in the identity of the reactive sites and their local environments which would affect fluorescence yield. Finally, it could be a question of accessibility of the fluorescamine, as normal and transformed cells undoubtedly have different geometries. The answer probably lies in a combination of several or all of these.

The advantages of the technique of fluorescamine labeling and subsequent analysis by high resolution polyacrylamide gel electrophoresis can be summarized:

1. The labeling procedure is rapid and minimizes the possibility of the cell responding to experimental manipulations.
2. In comparison to other methods, the reaction conditions are mild.
3. Proteins can be detected at least to the picomole range.
4. The method does not rely on the occurrence of specific amino acid residues and should therefore be a general label for all proteins and some lipids.
5. It appears to label only those components exposed on the outer surface of the cell.
6. The separation of a complex mixture of proteins with good resolution allows cells to be labeled, solubilized and analyzed without complicating the result by the use of proteases, chelating agents, and long purification procedures.

Finally, the method should have wide application in the study of outer surfaces, not only of cells, but also of subcellular organelles such as nuclei and mitochondria.

ACKNOWLEDGEMENTS

We should like to thank Dr. S. Y. Yang for help with the starch gel electrophoresis and Jill Hatie for indispensable technical assistance. This work was supported by an American Association of University Women Fellowship and the Elsa U. Pardee Fellowship to S. H., by NIH Fellowship to T. M., No. 1 F22 CA0 1837-01, and by NCI Grant No. 1-RO-1-CA14828-2.

An abstract of this paper was published in Proceedings of the Fifteenth Annual Meeting of the American Society for Cell Biology (12).

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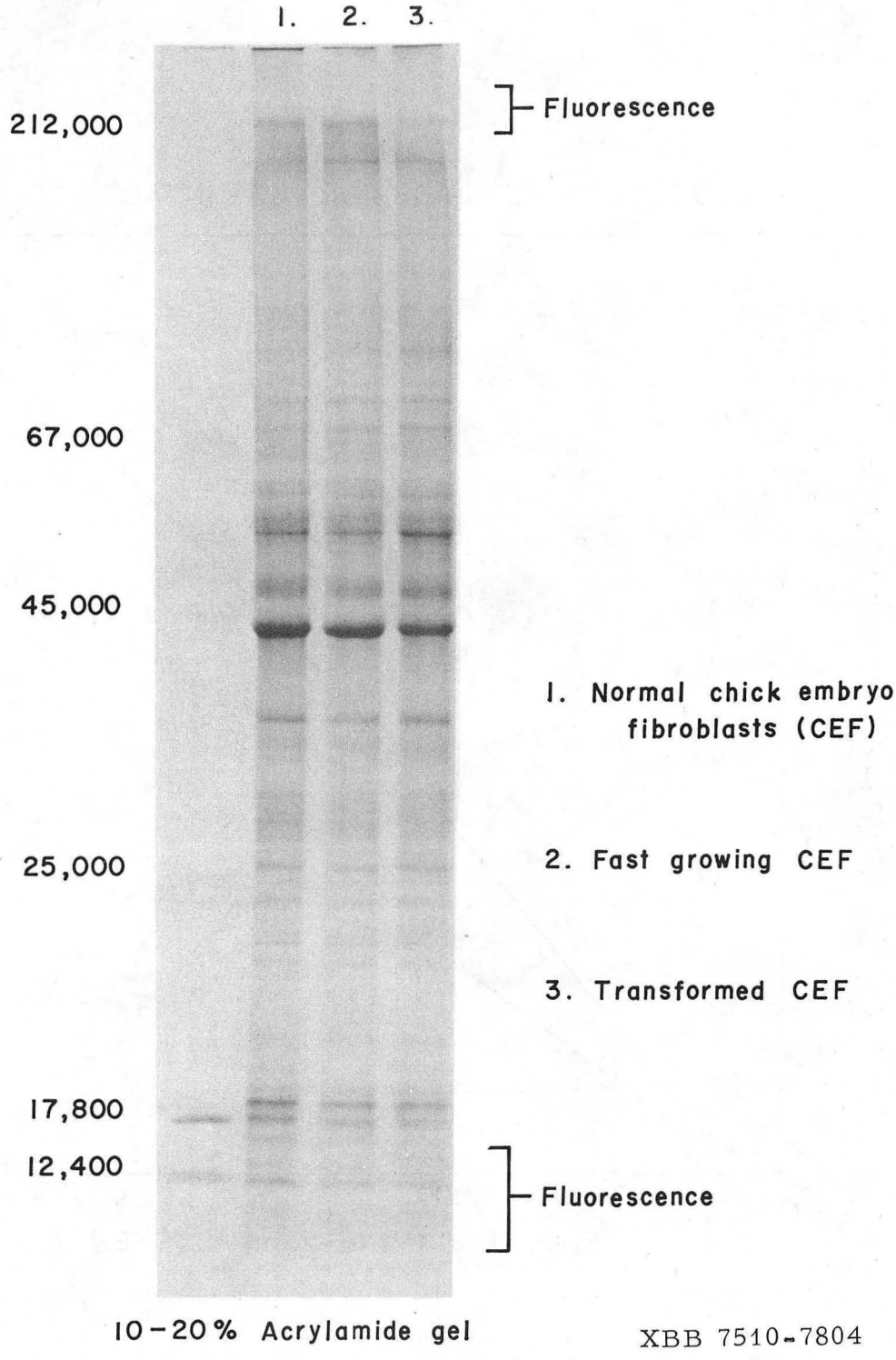

Figure 1. Samples of total protein from unlabeled chick embryo fibroblasts were electrophoresed under dissociating conditions on a polyacrylamide gradient gel. Polypeptide bands are visualized by Coomassie Blue staining. Numerical values represent the molecular weight (in daltons) of standard proteins. Areas of fluorescent bands observed in a similar separation of protein from fluorescamine labeled cells are indicated.

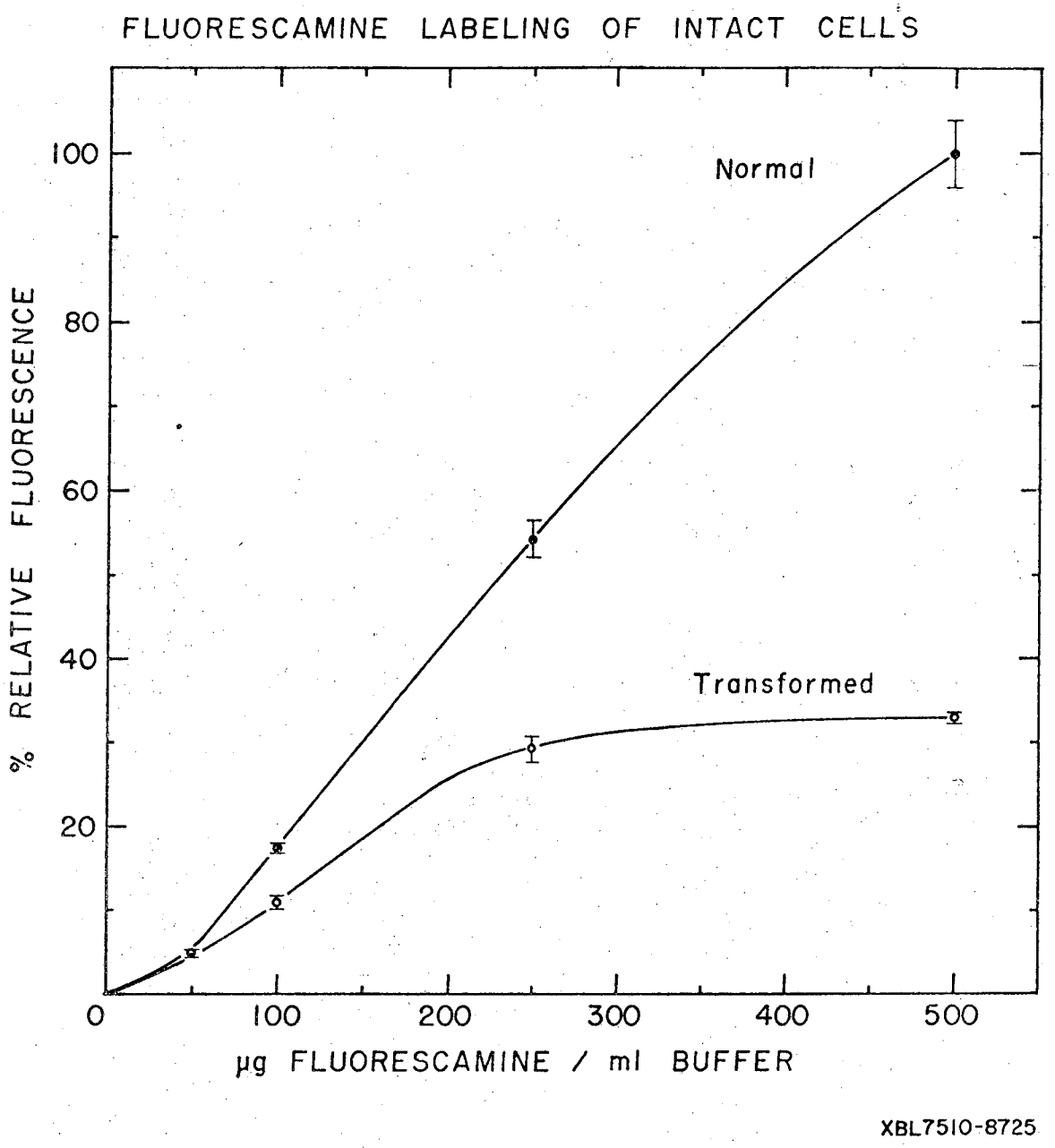

Figure 2. Monolayers of chick embryo fibroblasts were labeled with fluorescamine for 30 seconds and solubilized in SDS. Fluorescence yields were compared on the basis on protein content and with reference to a quinine sulfate standard. Percentage relative fluorescence values were calculated by placing the fluorescence yield for normal cells at 100%.

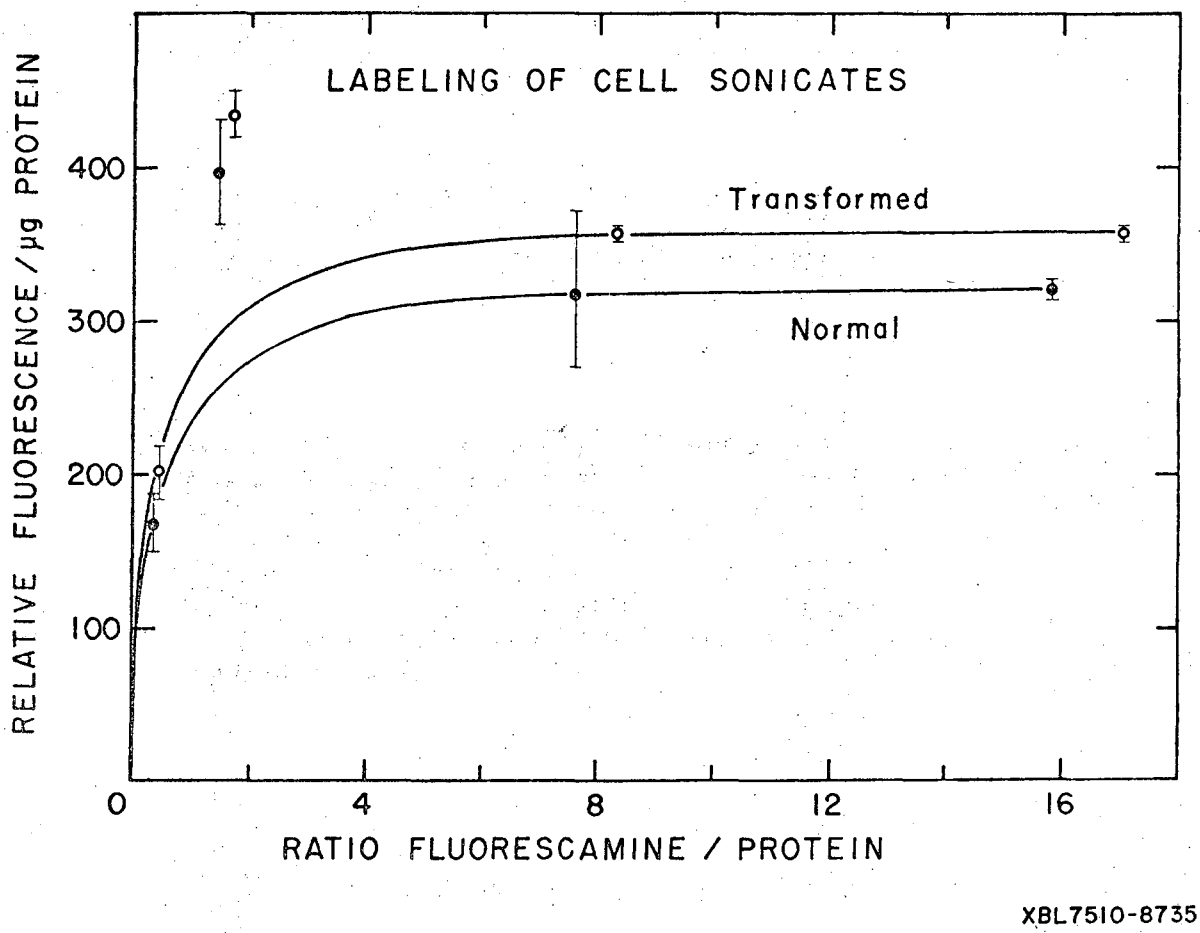

Figure 3. Monolayers of chick embryo fibroblasts were removed from culture dishes by scraping with a rubber policeman. The cells were suspended in borate buffer, transferred to a test tube and disrupted by sonication. The sonicates were labeled by the addition and rapid mixing of a solution of fluorescamine in acetone (final concentration, 0.5% acetone). Fluorescence yields were determined with reference to a quinine sulfate standard.

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