Lateral Transport of a Lipid Probe and Labeled Proteins on a Cell Membrane

Abstract. *Diffusion coefficients* (D) *of a lipid probe and labeled proteins on L-6 myoblast membranes have been measured giving* D(*protein*) $\sim 2 \times 10^{-10}$ *square centimeter per second and* D(*lipid probe*) $\sim 9 \times 10^{-9}$ *square centimeter per second. Some of the membrane proteins are immobile, but the lipid probe diffuses freely over macroscopic distances. Cytochalasin B slows protein but not lipid probe diffusion.*

It is now widely believed that membrane structure must be understood in dynamic terms (*1, 2*) and that lateral movements of cell surface constituents play an important role in controlling the response of a cell to its external environment (*3–4*). New procedures for measuring macroscopic lateral transport of fluorescent labeled molecules (*2, 5–8*) can define the structural constraints and test the physiological significance of cell surface dynamics.

Before investigating the transport of specific plasma membrane receptors and antigens, we deemed it useful to measure the diffusion of a probe of the lipid phase of the membrane and of a "cross section" of membrane proteins. The former indicates the degree to which the lipid bilayer fluidity can influence the transport of molecules embedded in it. The latter provides surveys of the behavior of membrane proteins in general.

We report here quantitative measurements of the lateral diffusion of a lipid probe and of nonspecifically labeled proteins on the plasma membrane of L-6 (rat) myoblasts. We compare the diffusion of the lipid probe and the proteins and the effects on them of a metabolic poison and agents that disrupt cytoskeletal elements. Measurements of the lateral transport of concanavalin A (Con A) binding sites (*2, 6, 7*) and of a carbocyanine lipid probe (*2*) in plasma membranes have been reported. This report pursues questions raised by Edidin (*5*) and by ourselves (*2, 7*).

We used fluorescence photobleaching recovery (FPR) (*2, 7–9*) to measure lateral transport. An intense laser light pulse irreversibly bleaches the fluorophore in a small region of the membrane. Transport coefficients are calculated from rates of recovery of fluorescence in the bleached region due to entry of unbleached fluorophores from adjacent parts of the membrane. In principle, FPR can be used to distinguish among diffusion and mechanisms of systematic driven transport (*8*). Diffusion is characterized by lateral diffusion coefficients D (square centimeters per second) deduced from half times for fluorescent recovery, $\tau_{1/2}$(seconds). This $\tau_{1/2}$ represents approximately the time for diffusion of the fluorophore over a distance w of a few micrometers (*8, 10*). Incomplete recovery is interpreted to indicate the presence of labeled particles which are immobile on the time scale of our measurements (*2, 7*).

We have used 3,3-dioctadecylindocarbocyanine iodide (diI) dissolved in the lipid portion of the plasma membrane as a fluorescent analog to indicate lipid behavior. Incorporation of the diI in the plasma membrane of these cells is indicated by detailed studies of peripheral fluorescence of various labeled cells (*11*). The diI does mimic the diffusion of phospholipids in model lipid bilayers (*12*) but may differ from natural lipids in its interaction with membrane proteins. Figure 1a presents a typical FPR recovery curve for diI. It coincides with theory (*8*) for diffusion with a single coefficient $D = 9.2 \times 10^{-9}$ cm²/sec. The mean value (± the standard deviation) obtained from measurements on 18 cells is $D = (9.0 \pm 4.0) \times 10^{-9}$ cm²/sec. This result was confirmed by the value $D = (8.0 \pm 3.0) \times 10^{-9}$ cm²/sec obtained by a different method, namely, fluorescence correlation spectroscopy (*13*). The diI diffusion coefficients in lipid bilayers are more than ten times larger (*12*). All measurements of diI diffusion in L-6 show ~ 100 percent recovery of the fluorescence after bleaching. This result suggests that the lipid bilayer exists as a continuous membrane matrix over distances >> 4 μm (the beam radius in these measurements) without being significantly partitioned into closed regions by membrane proteins. Furthermore, cross-linking of membrane glycoproteins (and possibly glycolipids) by Con A (66 μg/ml at 23°C) or by Con A and antibodies to Con A (100 μg/ml) did not affect the rate and extent of diI fluorescence recovery. Fixation of the cells with 5 percent glutaraldehyde for 2 hours did not affect the extent of recovery, but did reduce the apparent diffusion coefficient to $D = (1.5 \pm 0.5) \times 10^{-9}$ cm²/sec. Finally, sequential labeling first with Con A, and then with antibody to Con A, and then fixation with glutaraldehyde reduces both the rate, to $D = (1.5 \pm 0.4) \times 10^{-9}$ cm²/sec, and the extent, to 30 to 70 percent, of the fluorescence recovery. Presumably some of the lipid probe was trapped by this most drastic treatment. [Glutaraldehyde treatment completely inhibits protein diffusion (*2, 5, 14,* and this work).]

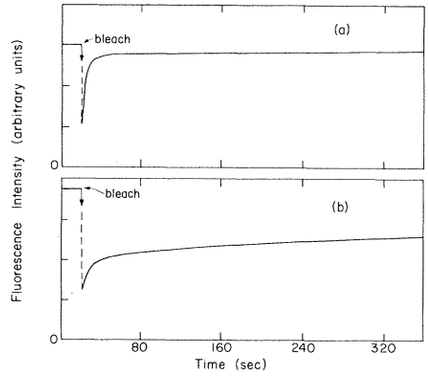

Fig. 1. Photobleaching recovery curves of L-6 myoblasts labeled with (a) diI, $D = 9.2 \times 10^{-9}$ cm²/sec (for beam radius $w = 4$ μm) with a fluorescence recovery of 95 percent; (b) TNBS and rhodamine-labeled antibodies to DNP, $D = 1.9 \times 10^{-10}$ cm²/sec (for beam radius $w = 1.1$ μm) with a fluorescence recovery of 54 percent. Both types of recovery curves fit the theory (*8*) for diffusion with a single diffusion coefficient D within their experimental accuracy. [Note that the fluorescence recovery is *not* expected to be described by simple exponential curves (*8*).] Experimental uncertainties preclude analysis sufficiently detailed to determine the spread of values of D contributing to recovery. The observed variation of D by a factor of 3 with position on the cell surface suggests the range over which D varies because of the heterogeneity of the labeled proteins.

Treating the cells with azide (which poisons oxidative metabolism), with colchicine (which disrupts microtubule structure), or with cytochalasin B (which, among other effects, disrupts some microfilaments) had no effect on the diffusion of diI—all under the same conditions as used in studies of protein diffusion.

The lateral transport of fluorescent labeled proteins on L-6 plasma membranes is both far slower and more heterogeneous than that of the lipid probe. Proteins labeled with fluorescein isothiocyanate (FITC) should be unselected with respect to function or antigenic specificity. Edidin *et al.* (*5*) showed that most labeled molecules were indeed proteins. They have found that membrane proteins of mouse fibroblasts in dense culture labeled with FITC at *p*H 9.5 are effectively immobile for 2 hours after labeling. After this period, photobleaching recovery methods gave a mean value of $D = (2.6 \pm 1.0) \times 10^{-10}$ cm²/sec. We have labeled L-6 plasma membrane with FITC at *p*H 7.6 without noticeable cell damage (*15, 16*). Measurements by FPR promptly after labeling and 3 hours later yielded diffusion recovery curves with a

Table 1. Measurements by FPR at three different positions on a single cell surface labeled with TNBS and rhodamine-marked antibodies to DNP.

Fluorescence intensity* (arbitrary units)	First bleach		Second bleach	
	D (cm²/sec)	Fractional recovery (%)	D (cm²/sec)	Fractional recovery† (%)
22.5	6.6×10^{-10}	31	6.6×10^{-10}	90
27.0‡	3.3×10^{-10}	62	3.3×10^{-10}	84
17.0	2.0×10^{-10}	54	2.3×10^{-10}	66

*The fluorescence intensity was measured from an illuminated spot radius ~ 1.1 μm. †Note that fractional recovery on second bleach remains < 100 percent because bleaching is always incomplete; ultimately subsequent bleaches approach 100 percent recovery. ‡In this experiment the bleached region was above the nucleus.

representative value, $D = (2.2 \pm 1.0) \times 10^{-10}$ cm²/sec for 11 cells with the degree of fluorescence recovery in the range 30 to 50 percent. Repeated measurements at the same position yield reproducible values of D. However the value of D and fractional fluorescence recovery vary from position to position on the cell. The substantial spatial variation of apparent diffusion coefficient is probably due to heterogeneity of the composition or arrangement of proteins labeled by FITC.

To test the generality of these results and discover whether FITC was selectively labeling proteins with exceptional diffusion coefficients, we have applied a distinctly different labeling procedure. Plasma membrane components of L-6 were conjugated with 2,4,6-trinitrobenzene sulfonate (TNBS) and marked with rhodamine-labeled antibody to dinitrophenol (DNP) (*16, 17*). Figure 1b presents a recovery curve (typical of 15 cells) from a cell labeled in this way; $D = 1.9 \times 10^{-10}$ cm²/sec, with a fluorescence recovery of 54 percent. In general, results obtained with TNBS-labeled components closely parallel those obtained with FITC. Hence our results seem to reflect the behavior of a representative collection of membrane components (expected to be mostly proteins) able to bind FITC or TNBS (for example, with exposed lysine amine groups). The components labeled with TNBS and then antibody to DNP components also show substantial variation of D and fractional recovery at different positions in the same cell (Table 1).

The existence of an immobile fraction of the labeled proteins and known drug effects on transport of Con A receptors (*2, 3*) suggest that factors other than fluidity of the lipid bilayer determine the rate of transport of the proteins embedded in it. We have therefore attempted to assess the role of cytoskeletal structures and metabolism in the lateral transport. Neither colchicine ($10^{-5}M$ for 45 minutes) nor azide ($10^{-2}M$, 30 minutes) affect the rate or extent of recovery with either protein label. Hence neither oxidative phosphorylation nor microtubules seem to be strongly involved in the observed protein mobility. Cytochalasin B (10 μg/ml, 45 minutes), however, dramatically decreases the apparent diffusion coefficient of the labeled proteins by a factor of 10 into the range $1.9 \times 10^{-11} \leq D \leq 3.3 \times 10^{-11}$ cm²/sec without noticeably affecting the extent of recovery. We had observed a similar effect on Con A receptors (*2*). Furthermore, the mobility of the proteins labeled with TNBS and antibody to DNP is reduced to $D \sim 2 \times 10^{-11}$ cm²/sec by Con A (33 μg/ml). This result could derive either from a direct cross-linking of the labeled proteins by the lectin or by an anchorage-dependent modulation as proposed by Edelman (*3*).

We have attempted to assess the contribution of systematic cell surface flow (*18*) to the observed recovery curves; we used curve-fitting procedures already described (*8*). In all curves tested, only diffusive transport was detected. This was expected because the reported flow rates are too slow by about a factor of 10 to affect our results. Our analysis indicates that the flow velocity of dil and labeled proteins must be less than 2×10^{-5} cm/sec and 2.4×10^{-6} cm/sec, respectively.

The main conclusions of this study of L-6 myoblasts are: (i) The rate ($D \sim 9 \times 10^{-9}$ cm²/sec) and completeness of the fluorescence recovery of the lipid probe dil suggest that the plasma membrane contains a fluid lipid bilayer matrix that is continuous over distances much larger than 4 μm. (ii) Cross-linking of membrane glycoproteins by Con A and treatment with azide, colchicine, or cytochalasin B does not affect the diffusion of the lipid probe. (iii) Proteins on the plasma membrane labeled with FITC and TNBS show similar rates, $D \sim 2 \times 10^{-10}$ cm²/sec, and extents of fluorescence recovery. (iv) The mobility of the labeled proteins is probably not determined by the viscosity of the lipid bilayer alone (some of these proteins are immobile on the experimental time scale) (*2*). (v) The temporary immobility of protein after FITC labeling is avoided by labeling at *p*H 7.6. (vi) The rate of lateral transport of mobile proteins and the proportion of "immobile" proteins is spatially heterogeneous on the plasma membrane of a single cell. Perhaps this is related to observed patterns of ultrastructural organization (*19*). (vii) The reduction of protein mobility by cytochalasin B and the absence of any effect on the mobility of the lipid probe suggests that constituents of microfilaments influence transport of the proteins.

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References and Notes

1. M. Edidin, *Annu. Rev. Biophys. Bioeng.* **3**, 179 (1974); L. D. Frye and M. Edidin, *J. Cell Sci.* **7**, 319 (1970); M. Edidin and D. Fambrough, *ibid.* **57**, 27 (1975); M.-m. Poo and R. A. Cone, *Nature (London)* **247**, 438 (1974); I. Yahara and G. M. Edelman, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 608 (1972).
2. J. Schlessinger *et al., Proc. Natl. Acad. Sci. U.S.A.* **73**, 2409 (1976).
3. G. M. Edelman, *Science* **192**, 218 (1976).
4. M. S. Bretcher and M. C. Raff, *Nature (London)* **258**, 43 (1975).
5. M. Edidin, Y. Zagyansky, T. J. Lardner, *Science* **191**, 466 (1976).
6. Y. Zagyansky and M. Edidin, *Biochim. Biophys. Acta* **433**, 209 (1976); K. Jacobson, J. Wu, G. Poste, *ibid.*, p. 215.
7. E. L. Elson, J. Schlessinger, D. E. Koppel, D. Axelrod, W. W. Webb, Proceedings of the National Cancer Institute Conference on New Approaches to the Role of Membranes in Neoplasia, Keystone, Colo., 1976.
8. D. Axelrod, D. E. Koppel, J. Schlessinger, E. L. Elson, W. W. Webb, *Biophys. J.* **16**, 1055 (1976).
9. D. E. Koppel, D. Axelrod, J. Schlessinger, E. L. Elson, W. W. Webb, *ibid.*, p. 1315.
10. A krypton laser beam at 520.8 nm was focused on a small area on the surface of a labeled cell attached to a petri dish. The small illuminated spot was exposed for 0.5 second at a laser power of ~ 10 mw (*2, 9*). Bleached areas are only a negligible part of cell surface, ≤ 1 percent. The course of the fluorescence recovery is monitored with the laser intensity attenuated a thousandfold until it reaches a stable plateau. The apparent diffusion coefficient D equals $(w^2/4\tau_{1/2})\gamma$, where w is the e^{-2} radius of the focused Gaussian laser beam and γ is a factor to account for the amount of bleaching and the beam profile (*8*). For these experiments, $\gamma \simeq 1.3$ (*8*).
11. DiI was incorporated into the plasma membrane by incubating the cells with 1 ml of dye (3.0 to 6.6 μg/ml) in Hanks balanced salt solution (BSS) containing 1 percent ethanol for 15 minutes at 37°C. In the L-6 cells used for our diffusion experiments, only peripheral dil fluorescence was visible by fluorescence microscopy. We believe that only this peripheral fluorescence contributed significantly to the observed diffusion. Round mitotic L-6 cells showed the characteristic "fluorescent ring." The laser beam focus could be scanned from the top to the bottom of the cells to see that virtually all of the fluorescence was confined to the cell membrane. In cells with membranes that contain different proportions of various phospholipids, a noticeable fraction of dil was internalized, and the nucleus and cytoplasmic granules were visible by fluorescence microscopy. Fluorescence recovery in FPR experiments on these cells was incomplete, indicating that some of the probe was trapped presumably within organelles smaller than the laser beam diameter. In macrophages, internalization of plasma membrane substantially reduces the fluorescence recovery of dil. Prevention of the internalization by inhib-

iting endocytosis permitted complete recovery (J. Reidler, unpublished data). It should be noted that diI exists in water only as micelles with low fluorescence quantum efficiency; only from our dilute alcohol solution could the diI be induced to label the cell membrane.

12. P. F. Fahey, D. E. Koppel, L. S. Barak, D. E. Wolf, E. L. Elson, W. W. Webb [*Science* **195**, 305 (1977)] show that diI diffusion in planar phospholipid bilayers is representative of lipid self-diffusion.
13. E. L. Elson and W. W. Webb, *Annu. Rev. Biophys. Bioeng.* **4**, 311 (1975).
14. P. K. Brown, *Nature New Biol.* **236**, 35 (1972).
15. The cells were incubated with 1 ml of FITC (25 to 200 μg/ml) in phosphate-buffered saline (PBS), *p*H 7.6, for 10 minutes at 37°C and then washed at least five times with Hanks BSS.
16. The L-6 cells were labeled with FITC under mild conditions (*15*). The FPR measurements were performed only on cells stained peripherally and not on cells in which the cytoplasm was stained. Sparse cultures yielded a greater fraction of damaged cells than confluent populations (*5*). All cells stained with TNBS and rhodamine-labeled antibodies to DNP were stained peripherally. Similar diffusion coefficients were obtained for cells labeled with TNBS and rhodamine-labeled Fab antibodies to DNP indicating that cross-linking between surface proteins via the intact antibody is unlikely.
17. The cells were incubated with 1 ml of TNBS (10 m*M*) in Hanks BSS for 15 minutes at 37°C, then washed three times and incubated for 15 minutes at 37°C with 1 ml (25 μg/ml) of rhodamine-labeled antibodies (sheep, immunoglobulin G) to DNP.
18. M. S. Bretscher, *Nature (London)* **260**, 21 (1976).
19. K. T. Porter, *Biophys. J.* **16** (part 2), 115a (1976).
20. We thank R. Buchsbaum for growing the cells. Supported by NIH grant GM-21661, a grant from NSF Division of Condensed Matter, an EMBO travel grant (to J.S.), an NIH postdoctoral fellowship NS-00432A (to D.A.), an NIH career development award (to E.E.), a Guggenheim fellowship (to W.W.W.), and a grant from the Research Corporation.

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Developmental Dyslexia: Two Right Hemispheres and None Left

Abstract. *Developmental dyslexia may be associated with (i) bi-hemisphere representation of spatial functions, in contrast to the right-hemisphere specialization observed in normal children, and (ii) typical left-hemisphere representation of linguistic functions, as is observed in normal children. The bilateral neural involvement in spatial processing may interfere with the left hemisphere's processing of its own specialized functions and result in deficient linguistic, sequential cognitive processing and in overuse of the spatial, holistic cognitive mode. This pattern of cognitive deficits and biases may lead dyslexics to read predominantly with a spatial-holistic cognitive strategy and neglect the phonetic-sequential strategy. Such an approach in learning to read phonetically coded languages, such as English, may be inefficient and limited.*

Developmental dyslexia, or specific reading disability, refers to the clinical syndrome of difficulty in reading in intellectually, emotionally, and medically normal individuals. Such a deficit is particularly incapacitating in modern, highly literate societies and frequently results in secondary behavioral and emotional difficulties. Estimates of the incidence of the disorder are as high as 5 percent of school-age children, which makes it a prevalent as well as a serious disorder (*1*).

Numerous etiological hypotheses of dyslexia have implicated various neurological, social, and educational factors (*2*). None, however, has received strong or consistent support. One long-standing neural hypothesis, originally suggested by Orton (*3*), implicates abnormal cerebral dominance or functional asymmetry of the hemispheres. Testing this hypothesis has become possible only within the last decade with the development of a number of experimental techniques; for example, tasks requiring the perception of lateralized stimuli allow inferences about hemisphere specialization in non-brain-damaged individuals (*4, 5*). Numerous studies using these techniques, particularly dichotic (auditory) stimulation (*6*) and, to a lesser extent, tachistoscopic stimulation in the lateral visual fields (*7*), have been reported with variously defined groups of poor readers. All these studies used linguistic stimuli and addressed themselves to the question of whether the left hemisphere is specialized for linguistic processing in such children; the implicit assumption has been that specialization of the left hemisphere is impaired in dyslexia. This assumption probably arose from the well-established clinical knowledge that acquired alexia or dyslexia is usually associated with lesions in the left (speech dominant) hemisphere (*8*) and from the fact that reading has traditionally been conceptualized as a language skill. The results of these studies (*6, 7*) have consistently indicated right-ear and right-visual-field superiorities and, by inference, specialization of the left hemisphere for linguistic processing in poor readers, as is the case in normal individuals. However, in spite of the data, many of these reports contain unfounded suggestions of a lack of, or less strong, specialization of the left hemisphere in dyslexia.

In contrast, I have investigated (i) specialization of the right hemisphere for spatial processing, (ii) specialization of the left hemisphere for linguistic processing, and (iii) the relative participation of the two hemispheres on a task that requires the specialized functions of both. Performance on the last task may be particularly illuminating for, like reading, it involves both types of cognitive processing (*9*).

The results indicate that in dyslexics, spatial functions are represented in both hemispheres in contrast to the specialization of the right hemisphere in normal children. In addition, and consistent with the previous studies, dyslexics have the typical pattern of left-hemisphere representation of linguistic functions. Although the left hemisphere may mediate the typical cognitive functions, the results suggest that left-hemisphere processing may be deficient in dyslexics. These two possible neural correlates may result in a cognitive pattern of deficits and biases in dyslexia; specifically, a deficiency in the linguistic, sequential, analytic cognitive mode of information processing, and an intact or even overdeveloped use of the spatial, parallel, holistic mode.

A group of 85 right-handed boys (6 to 14 years of age, $\bar{X}$ = 10.6), selected as cases of developmental dyslexia on the basis of extensive pediatric, psychiatric, and clinical psychological assessments, were given a battery of four tests considered to reflect hemisphere specialization. Two tests are considered to be indices of right-hemisphere specialization for spatial processing: (i) "dichhaptic stimulation" with meaningless shapes, a relatively new task, in which two different shapes are simultaneously presented one to each hand, to be perceived by active touch alone (*5, 10*), and (ii) a tachistoscopic task, adapted from the test procedures originally developed with adults, in which pairs of identical or different figures of people were presented in either the right or left visual half-field and had to be identified as "same" or "different." Specialization of the left hemisphere for linguistic processing was assessed with a typical dichotic stimulation test that used free recall of series of pairs of digits. The final test involved dichhaptic presentation of letters that were to be named by the subject (*5*). The performance of the dyslexic group on these tests was compared to that of a group of 156 normal, right-handed boys who were matched for age ($\bar{X}$ = 10.5 years) and socioeconomic class, who had no history of academic or behavioral difficulty, and who obtained age-appropriate scores on reading and spelling achievement tests.

On the dichhaptic shapes test, the dyslexic group showed no difference in accuracy in recognizing shapes presented to their left and right hands ($\bar{X}$ = 5.1 and 5.5, respectively, t = 1.43, d.f. = 61), in contrast to the normal group, who ob-