The reversal potential obtained here (–4 mv) is some 10 mv more positive than that obtained at the vertebrate neuromuscular junction (*2*). This difference may or may not indicate a difference in the specificity of the synaptic conductance channels. Assuming, as at the neuromuscular junction, that the major conductance change is to Na and K, the ratio of the two ionic conductances is given by the relation $\Delta g_{Na}/\Delta g_K = -(E_K - E_r)/(E_{Na} - E_r)$, where Δg_{Na} and Δg_K are the drug-induced conductance changes in the postsynaptic membrane and E_{Na} and E_K are the equilibrium potentials for Na and K. For frog muscle, the conductance ratio is about 1.3 (*2*). If we take E_K for the electroplaque as –90 mv and E_{Na} as +140 mv (*4*), then the conductance ratio $\Delta g_{Na}/\Delta g_K$ is 0.60, slightly less than half that at the neuromuscular junction. However, the estimate of E_{Na} is somewhat uncertain, and if we take instead a value of +62 mv the synaptic conductance ratio would be the same in the electroplaque as at the neuromuscular junction. Consequently, until a value for E_{Na} is established accurately, there is no overwhelming reason to suppose that the electrophysiological characteristics of the ACh receptors in the two preparations are different.

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Measurement of Membrane Protein Lateral Diffusion in Single Cells

Abstract. *Fluorescence rapidly returns to spots bleached by a laser beam in the continuous fluorescence of cultured cells labeled on the surface with fluorescein isothiocyanate. The rate of recovery of fluorescence after bleaching can be interpreted as a measure of the lateral diffusion of integral membrane proteins labeled with fluorescein.*

Cell membranes are fluids whose constituent molecules move freely in two dimensions (*1*). This lateral mobility may be important to the action of cell surface receptors in transmitting signals across membranes. Also, changes in relative mobility of membrane proteins have been associated with malignant transformation of cultured cells. However, convincing values for diffusion have been derived only for lipids (*2*). Quantitative data on protein movements in cell membranes are scant. There are rough estimates of diffusion times for antigens of cultured cells (*3*), but the only precise measurements on membrane protein mobility are those by Cone (*4*) and Poo and Cone (*5*) on rotational and translational diffusion of rhodopsin in outer segment disk membranes. Poo and Cone (*5*) measured the return of native rhodopsin to a portion of the disk in which rhodopsin has been bleached. The value calculated for D was about 4×10^{-9} cm^2 sec^{-1} in concordance with previous estimates of lipid viscosity in disk membranes.

Peters *et al.* (*6*) performed an experiment, similar in principle to that of Poo and Cone (*5*), which may be applied to a variety of cell surface membranes. A fluorophore, fluorescein, was introduced into membranes by permitting fluorescein isothiocyanate to react with intact erythrocytes. The cells, covalently labeled largely in their membrane proteins, were made into ghosts and an attempt was made to follow diffusion of the proteins labeled with fluorescein from an untreated hemisphere of each ghost into a hemisphere in which the photolabile fluorescein had been bleached. Recovery of fluorescence in the bleached hemisphere, accompanied by loss of fluorescence from the intact hemisphere, would have indicated diffusion of label from one hemisphere to the other, but in this experiment no diffusion was observed. Nevertheless, the method is attractive because it should be applicable to any cell that can withstand the labeling procedure. We present data on the movement of fluorescein-labeled proteins of cultured mouse fibroblasts (L cells). Our observations on the return of fluorescence to bleached spots on the cell membrane, together with a solution of the diffusion equation applicable to the geometry of the cells, allow calculation of constants for the lateral diffusion of membrane protein.

We labeled cells of L cell–derived line Cl ld (*7*) by brief exposure to fluorescein isothiocyanate at *p*H 9.5 (*8*), at room temperature, approximately 24°C. All cells became labeled at the surface as judged by a ring of fluorescence observable at their periphery. Damaged cells fluoresced throughout their interiors. Labeling in ice in the presence of millimolar concentrations of NaCN and NaF did not affect the distribution or intensity. Freshly prepared reagents and dense healthy cultures were required for successful labeling. When old or sparse cultures were labeled, all cells were damaged by the procedure.

Fluorescence was measured on a Leitz microscope, with excitation from above the specimen. The microscope also has a side window through which the 441-nm beam of a Liconix HeCd laser was introduced, via a ×90 objective with a numerical aperture of 1.32, to bleach a spot about 5 μm in diameter at the cell periphery. The cells, although adhering to cover slips, were nearly spherical; their diameters ranged from 12 to 17 μm (with most being 12 to 15 μm). The laser beam was directed at a tangent to the sphere to form the spot; the half angle θ subtended by the bleached spot was approximately 20°. An image of the center of the spot was passed through a limiting diaphragm to a photomultiplier (*9*). All measurements were made at 22° to 24°C.

Immediately after labeling, the cells appeared to contain some fluorescein in their interiors, as well as being prominently outlined by a ring of fluorescence. When such cells were bleached and examined no recovery of fluorescence was observed within the first 2 or 3 minutes after bleaching, and the recording of fluorescence intensity was a horizontal line which did not shift during the time of observation. Such data were obtained for 42 cells.

In contrast to cells examined immediately after labeling, those cultured for at least 2 hours, or trypsinized and allowed to reattach to cover slips (minimum of 20 hours, maximum of 48 hours), were fluorescent only at the periphery, with no internal stain. All cells in untrypsinized cultures appeared to have shed labeled material collected in aggregates that were often stuck to cells. Bleaching measurements were never made at or near aggregates, but rather at regions of cell periphery that were clearly ring-stained.

When cells that had been cultured for at least 2 hours after labeling were bleached, striking recovery of fluorescence of the bleached spot could be observed (Fig. 1). During bleaching and between measurements the shutter to the photomultiplier was closed so that the trace dropped to baseline. Recovery of fluorescence, measured at intervals during the first few minutes after bleaching, is evident. In particular, immediately after bleaching, a sharp rise in intensity occurs (this is not a recorder artifact since it is not found in cells ex-

amined immediately after labeling, even when the shutter was closed during bleaching), and this rise is seen even if the shutter to the photomultiplier is left open during bleaching.

Data for one of the cells of Fig. 1 and for three other cells whose actual recordings are not shown are plotted in Fig. 2 in terms of corrected intensity

$$\hat{I} = \frac{I_t - I_0}{I_i - I_0} \quad (1)$$

where I_i is the initial intensity before bleaching, I_t is the intensity of the spot fluorescence at time t after bleaching, and I_0 is an estimate of the intensity immediately after bleaching. The value of I_0 is determined by extrapolation from the recorder trace. For most cells, the error in extrapolation is ≤ 5 percent; this could lead to a twofold error in the value of D. In general, corrected intensities recover to about 60 percent of initial intensity during the times observed. Recovery was not inhibited by prior fixing of cells in 0.5 percent paraformaldehyde or by bleaching cells in the presence of $2.5 \times 10^{-3}M$ NaCN and $2.5 \times 10^{-3}M$ NaF. Recovery of fluorescence was completely prevented by fixation of labeled cells in 5.0 percent paraformaldehyde (*10*).

In order to compare the amount of fluorescein bound to protein with that bound to or dissolved in lipid, cells labeled several hours previously and found to be ring-stained were divided into two portions. One was dissolved in 0.5 percent NP-40 detergent in phosphate-buffered saline. The other was extracted with a mixture of chloroform and methanol (2 : 1). Fluorescence emission of the extraction was scanned over the range 500 to 600 nm, exciting at 485 nm. After adjusting for extract volume and the proportion of the total cells in each extract, we found that the ratio of fluorescence in the detergent extract (protein plus lipid) to that in the chloroform-methanol extract (lipid) was 15 : 1.

It appears, then, that the bulk of fluorescence detected entering a spot after bleaching was due to dye bound to protein rather than to lipid. Return of fluorescence was probably not due to spontaneous recovery of bleached dye, since whole cells that were totally bleached immediately after labeling, although brightly ring-stained, showed little if any return of fluorescence in the bleached spot.

The proteins labeled are at the cell surface and accessible to macromolecules in the medium. Antibody to fluorescein binds specifically to labeled cells and, after addition of antibody to globulin, aggregates the label into patches. Also, papain digests fluorescein label from the cells, as judged

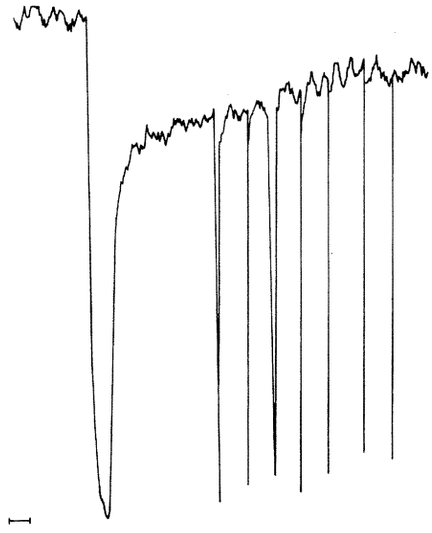

Fig. 1. Bleaching of a spot on a C1 ld fibroblast 4 hours after labeling. The intensity trace before bleaching is at the left. Recovery of fluorescence was followed for 3.5 minutes; the initial period of continuous recording was 30 seconds, and subsequent portions of the trace were taken of 45, 60, 90, 120, 150, 180, and 210 seconds. The vertical spikes were made when the recorder was turned off between measurements. The bar indicates 1 cm = 6 seconds. Time of bleaching was 3 seconds.

both by release of fluorescence into the medium and by the fluorescence of the cells treated with papain. The location of the labeled proteins in the membrane remains a problem. If fluorescein is attached to peripheral proteins that are merely bound at the membrane surface (*1*), then the movements described need not reflect diffusion within the plan of the bilayer. If, however, integral proteins are labeled, then changes in dye intensity within a bleached spot may be used to calculate average diffusion rates for membrane proteins of a single cell. Both integral and peripheral proteins must be defined operationally when their compositions or structures are unknown. In general, high salt concentrations of chelating agents and excesses of soluble proteins ought to displace peripheral proteins. These operational tests left about 70 percent of the fluorescein associated with protein that was bound to cells (*11*).

It is most likely that labeled protein moved into the bleached spot by diffusion, in which case the rate of recovery of fluorescence should be described by a diffusion equation with boundary conditions appropriate for our experiment. The description of diffusion into a spot on a sphere may be approximated in terms of the intensity of the spot as evaluated in Eq. 1 by

$$\hat{I} = \frac{\sin\theta}{1 - \cos\theta} \sqrt{\frac{\tau}{3}} \quad (2)$$

where $\tau = Dt/r^2$, r is the radius of the spherical cell, and θ is the half angle of the spot (*12*). The solution is limited to $\hat{I} \leq 0.292$. In Fig. 2 experimental data for four cells are shown as points plotted along the theoretical curves calculated from Eq.

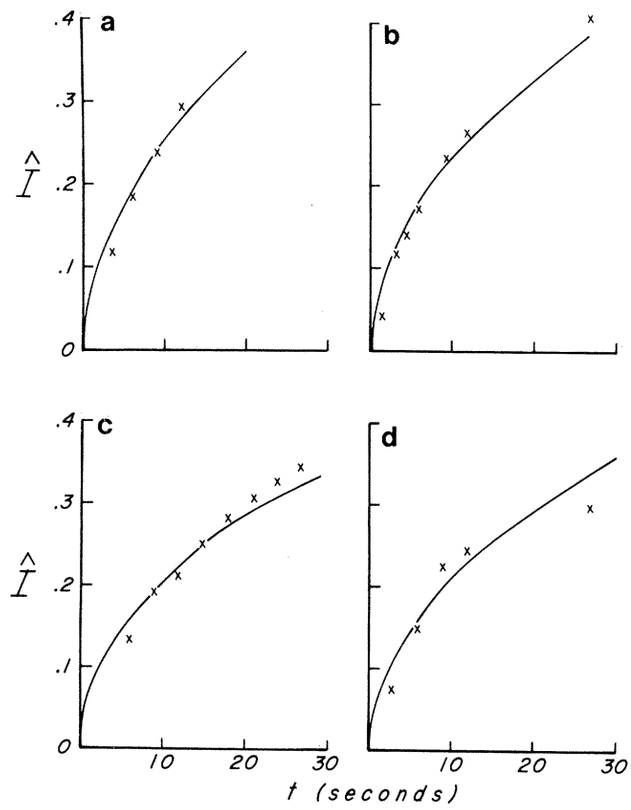

Fig. 2. Intensity ($\hat{I}$) plotted for the cell of Fig. 1 and for three other cells. (x) Experimental points; (——) theoretical curves. Diffusion constants used for these curves are (cm^2 sec^{-1}):
(a) 2.7×10^{-10}
(b) 1.4×10^{-10}
(c) 3.3×10^{-10}
(d) 2.8×10^{-10}

2. Diffusion constants estimated for these cells range from 1.4×10^{-10} to 3.3×10^{-10} cm^2 sec^{-1}. The data scatter about the line but systematic deviation only occurs for $\hat{I} > 0.292$. Data have been evaluated from 46 cells and give a range of values for D from approximately 5×10^{-11} to 5×10^{-10} cm^2 sec^{-1}, with a mean ($\pm$ standard error) of $2.6 \pm 0.16 \times 10^{-10}$. This is about an order of magnitude slower than the value of D for rhodopsin in disks (*5*). It is consistent with the mobilities inferred from other data on protein diffusion in heterokaryons (*3*).

The general application of our method remains to be tested. Preliminary work indicates that many cell types are severely damaged by labeling and cannot be used for direct conjugation with fluorescein. Another approach to such cells is to prepare monovalent labeled ligands such as Fab fragments of antibody, or succinylated concanavalin A. Experiments in our laboratory and those of others (*13*) indicate that this approach is feasible with concanavalin A, although the labeled ligand may itself cause cross-linking of receptors, or may trigger their cross-linking from within the cell.

Whatever the label, our method should be useful for evaluating lateral motions of membrane proteins in a wide variety of cells.

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8. Cells to be labeled were plated at high density either on cover slips or into 35-mm petri dishes and cultured overnight in Eagle's minimal essential medium containing 5 percent fetal calf serum and antibiotics. To label the cells, medium was removed and the plates or cover slips were washed three times with Hanks solution free of serum and buffered with HEPES to *p*H 7.2. After the washing, cells were covered with Hanks solution adjusted to *p*H 9.5 with NaOH immediately before use. Fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratories) at 1 mg/ml was added to the dishes or slips to give a final FITC concentration of 0.6 to 1.0 mg/ml. Cells were permitted to react with FITC for 10 to 20 minutes at room temperature. They were then washed three times with Hanks solution, *p*H 7.5, and three more times with same solution containing 5 percent fetal calf serum.
9. A 1P28 photomultiplier (RCA) was operated in the photon counting mode as part of an SSR model 1140 (Princeton Applied Research) photon counting system. The discriminator output of the system was fed to a digital counter and could be monitored continuously. The voltage output of the 1140 was fed to a chart recorder (Houston, Omnigraphic) with adequate response time for the measurements. Both measuring light and light for initially scanning the field were supplied by a 200-watt mercury arc, operated from an Oriel d-c power supply. Lamp intensity was reduced to 0.5 to 1 percent for measurement to avoid further bleaching.
10. Freshly prepared 0.5 percent to 5.0 percent paraformaldehyde in phosphate-buffered saline, *p*H 7.4, was applied to cells at 0°C for 1.5 hours.
11. The operational tests were as follows. (i) Isotonic NaCl did not leach fluorescence from labeled cells. (ii) Excess calf serum added during labeling reduced rather than augmented the degree of labeling. Calf serum added after labeling did not displace fluorescence from labeled cells. (iii) Both 1*M* NaCl and 1*M* NaCl plus 0.001*M* EDTA removed about 30 percent of fluorescence from labeled cells previously washed in isotonic saline (four experiments). (iv) Trypsin did not remove label, while papain removed almost all label from the cells.
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Characterization of a Cell-Lethal Product from the Photooxidation of Tryptophan: Hydrogen Peroxide

Abstract. *Near-ultraviolet (300 to 400 nanometers) irradiation of saturated, oxygenated solutions of tryptophan in the absence of added sensitizer gives rise to substances that have various biological effects on isolated cells, including mutagenicity and selective lethality to recombination-deficient bacterial mutants. One of these biologically active products has been identified as H_2O_2, on the basis of spectrometric, chromatographic, chemical, and biological properties. Now H_2O_2 has been shown to account for the biological activities mentioned above.*

Near-ultraviolet to visible irradiation of tryptophan gives rise to substances that have various biological effects on isolated cells, including mutagenicity (*1, 2*), selective lethality to recombination-deficient (*recA*) bacterial mutants (*2, 3*), inhibition of repair of DNA single-strand breaks and of DNA replication gap closure in *Escherichia coli* (*4*), sensitization of bacteria to near-ultraviolet induced DNA strand breaks (*5*), inhibition of growth of cultured mouse embryonic fibroblasts and of fertilized sea urchin eggs (*6*), lethality to cultured mammalian cells (*7*), and binding to a lens γ-crystallin (*8*).

At the same time, several groups (*9*) have examined the physical photochemistry of tryptophan with flash photolysis

Table 1. Purification of tryptophan photoproduct by column chromatography. The purification was begun with 100 ml of a crude photomixture generated by photolysis for 24 hours with four RUL-3500 lamps (Rayonet RPR-202 reactor; Southern New England Ultraviolet Company). The eluents indicated were aqueous solutions. Column chromatography was followed by a selective bacterial killing assay, with *Salmonella typhimurium* strains KSU 2480 (*recA* 7) and the isogenic *recA*$^+$ KSU 9557.

Column*	Dimension	Eluent	Detection method	Elution volume of active peaks (ml)
Sephadex G-10	53 by 2.5 cm	H_2O	Ultraviolet starch-iodide	230–240
Sephadex QAE	15 by 0.9 cm	NH_3/NH_4Cl†	Ultraviolet	10–20
Sephadex G-10	90 by 1.5 cm	$(NH_4)_2SO_4$‡	Refractive index starch-iodide	112–114§

*Listed in sequence used. †0.05*N* Cl. ‡0.1*N*. §Elution volumes of blue dextran and D_2O were 46 and 99 ml, respectively.

Table 2. Thin-layer chromatography of tryptophan photoproduct (TP) and H_2O_2.

Stationary phase	Solvent system	Detection method	R_fTP	$R_f H_2O_2$
Silica gel	Methanol, toluene (3 : 7)	DMDAB*	0.36	0.36
Cellulose	Ether †	DMDAB; $NH_4SCN/FeSO_4$	0.25	0.25
Cellulose	Water, ether, *n*-butanol (1 : 10 : 10)	DMDAB; $NH_4SCN/FeSO_4$	0.53	0.53

*DMDAB, *p*-*N*,*N*-dimethyldiaminobenzene. †Diethyl ether.