FLUIDITY OF THE SURFACE OF CULTURED MUSCLE FIBERS

Rapid Lateral Diffusion of Marked Surface Antigens

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ABSTRACT

Fluorescent antibody fragments of anti-muscle plasma membrane antibody bound as small fluorescent spots when applied by micropipetting to cultured myotubes. The spots were observed to enlarge with time. The rate of enlargement of fluorescent spots was greater when fragments were applied than when divalent antibody was used. It was also greater at 23°-25°C than at 0°-4°C. With glutaraldehyde-fixed cells no increase in the size of the spots was seen. The observations are consistent with the spread of fluorescent spots due to diffusion of surface protein antigens within the plane of a fluid membrane. From measurements of spot size against time, a diffusion constant of 1-3 x 10^-9 cm^2 s^-1 can be calculated for muscle plasma membrane proteins of mol wt approximately 200,000. This value is consistent with other observations on the diffusion of surface antigens and of labeled lipid molecules in synthetic and natural membranes.

Several lines of evidence suggest that the proteins and lipids of a plasma membrane are free to move in the plane of the membrane. This evidence, obtained from both animal and bacterial cells, is corroborated by experiments on rotational motions of the principal rod outer segment protein, rhodopsin (2, 4), and by measurements of the mobility of spin-labeled lipid molecules in artificial lipid bilayer systems (6, 11, 24). In sum, the plasma membrane may be regarded as a fluid. This picture is useful in dealing with a variety of facts known about membrane proteins and membrane properties (22, 23); however, it is primarily a qualitative picture, since only a few experiments have estimated either the viscosity of the membrane (4, 9) or the lateral diffusion constants for some of its components (6, 13, 18, 24). We here present data, obtained from measurements on single cells, which give an estimate of the rate of diffusion of some membrane proteins. Cultured muscle fibers were marked with spots of antibody and the rates of spread of these spots were determined in order to derive this estimate.

When cells trypsinized from embryo muscle are plated on collagen in rich medium, the myoblasts in the cell population will fuse and differentiate to form multinucleated muscle fibers (25). These fibers may be 1 mm or more in length, and are typically 30 µm in diameter. Their shape allows fluorescent-labeled anti-membrane antibody to be applied to a small part of the surface area of a single fiber and then to be observed as it spreads from the point of application. The rate of this spread gives a direct estimate of rate of translation of surface protein molecules. The rate of patch spreading is greatest when monovalent antibody fragments (Fab) are used to make a patch. It decreases when cells are chilled to ice temperatures. These observations are consistent with a fluid state of myotube surfaces. The diffusion constants calculated from our data are within an order of magnitude of those derived for diffusion of spin-labeled phospholipids in artificial and natural membranes (6, 18, 24). They also agree with previous observations on the intermixing of heterokaryon surface antigens (9).
MATERIALS AND METHODS

Rat embryo muscle cultures were grown in collagen-coated plastic Petri dishes by methods previously described (8, 25).

Preparation of Antibody

Antibody to adult rat muscle surface was prepared by immunizing rabbits with purified rat muscle membranes (10). Crude membranes were prepared from rat leg muscle by the method of Peter (16) as modified by Hartzell and Fambrough (10), which involves treatment of crude membranes with collagenase and collection of plasma membrane vesicles by centrifugation. The purified membrane fraction largely consisted of 0.2-2.0 μm vesicles and had a highly active Na⁺K⁺-dependent ATPase (10). Membranes were suspended in phosphate-buffered saline (PBS) and the suspension was emulsified with Freund’s Complete Adjuvant (CFA). 1.5 mg dry weight of membranes was injected into each of two rabbits, distributed among the foot pads, toe pads, and multiple intracutaneous sites on both flanks. Animals were bled at 3 and 4 wk after immunization; this serum was of sufficiently high titer for indirect staining experiments. 6 months after primary immunization, each animal was injected intravenously with another 1 mg of membranes suspended in PBS. The animals were bled out 10 days after this booster and the serum was used to prepare Fab fragments. Characterization of the antisera by indirect immunofluorescence indicated that it reacted with other rat cells to some extent, but not with chicken myoblasts, fibroblasts, or myotubes, and that it reacted better with 1-2 wk old myotubes than with cells that had only been in culture for a few days. In contrast to this range of specificities, an antisera prepared against whole rat embryo muscle homogenate (administered in CFA) reacted extensively with rat collagen; hence it could not be used to stain cells growing on a collagen substrate.

Extraction of Muscle Plasma Membrane

In order to further characterize antigens detected by the antisera, approximately 2 mg protein dry weight of purified muscle membranes were suspended by sonication in 1 ml of PBS, and 0.3 ml portions were either (a) incubated for 2 h with 100 μg papain in PBS containing 0.01 M cysteine and 0.002 M EDTA or (b) incubated for 2 h in the above buffer without enzyme.

Treatment a follows the method of Shimada and Nathenson (21) for extraction of Histocompatibility-2 (H-2) glycoproteins from cell membranes, and is expected to yield soluble antigenically active protein fragments in solution, with reduction of the activity of insoluble membranes (15). Treatment b controls for nonenzymatic release or destruction of antigenic activity. After incubation, both samples were centrifuged at 10,000 rpm for 10 min and separated into pellet and supernatant fractions. Pellets were washed three times in PBS, while the supernatants were dialyzed against PBS for 24 h at 4°C.

Another portion of membrane suspension was extracted with 6 ml of chloroform/methanol (2:1) at room temperature (treatment c). The extracted residue was caught on a glass fiber filter and re-extracted with 3 ml of chloroform/methanol for 30 min at room temperature, after which the mixture was briefly heated to boiling. The residue was again filtered off and the filtrate combined with the initial lipid extract. The combined extracts, containing cell lipids and glycolipids, were backwashed with PBS and the chloroform phase was taken to dryness.

To test extracts for antigen activity 0.1 ml of serum diluted to 1/45 in Hank’s balanced salt solution was incubated with either pellet or supernatant fractions of a and b or with the lipid extract c.

Iodination of Myotubes

In an attempt to estimate the size of the detected antigens, the surfaces of myotubes were radiiodinated by an enzymatic method (1, 14) and the iodinated proteins were extracted from the cells with NP-40, following procedures for H-2 antigens and immunoglobulins (1, 19). After centrifugation, 0.2 ml portions of detergent extract were reacted with either 0.1 ml of anti-muscle membrane or 0.1 ml of a control normal rabbit serum used as negative control in staining experiments. After incubation at 37°C for 2.5 h, 1.0 ml of goat anti-rabbit Fc was added to each tube, and after a further hour of incubation at 37°C, the tubes were refrigerated for 18 h. The immune precipitates that formed were washed three times in PBS containing 5 mM KI. The precipitates were dissolved in 2% sodium dodecyl sulphate (SDS), reduced, and alkylated, and equal volumes of solution were electrophoresed in 5% polyacrylamide gel containing 0.1% SDS (20).

Preparation of Fab

Fab was prepared from our highest titered serum by the method of Porter (17). 50 ml of 24% sodium sulfate (pH 6.4) was slowly added to 25 ml of serum at room temperature with constant stirring. The precipitate that formed was washed twice in 16% sodium sulfate, dissolved in 30 ml of borate-buffered 0.3 M NaCl (pH 8.1), and reprecipitated with 60 ml of 18% sodium sulfate. The precipitate was dissolved in 10 ml of 0.1 M phosphate buffer (pH 7) containing 0.01 M cysteine and 0.002 M EDTA. The solution contained a total of 100 mg of protein,
to which was added 1 mg of papain (PAP, Worthington Biochemical Corp., Freehold, N. J.). The solution was incubated for 9 h at 37°C; at the end of this incubation the solution was turbid. It was dialyzed in the cold against 1 liter of distilled water and then three times against 1 liter of PBS. The turbid solution was clarified by centrifugation at 100,000 g for 45 min. The clear supernatant reacted with both goat anti-rabbit Fc and goat anti-rabbit Fab. 60 mg of supernatant protein were dialyzed against 0.01 M acetate buffer, pH 5.5, and the solution was loaded onto a 55 ml carboxymethyl cellulose column. After washing the column with 60 ml of starting buffer, protein was eluted with a 700 ml gradient of acetate buffer, pH 5.5, from 0.01 to 0.90 M. Three protein peaks were obtained (Fig. 1). Pooled fractions were tested by gel diffusion against goat anti-rabbit Fc and anti-Fab. Pools I and II reacted with anti-Fab, but not with anti-Fc, while pool III reacted only with anti-Fc.

Conjugation of Fab Fragments

Labeled Fab was prepared from samples of pool I or pool II, essentially by the method of Cebra and Goldstein (3), but four times the usual amount of tetramethylrhodamine (TMR) isothiocyanate was added, 200 µg/mg protein. The reaction products were chromatographed on Sephadex G-25 to remove free TMR, and material made with pool II Fab was refractionated on CM-cellulose. The conjugate eluting in 0.01 M acetate was refractionated on G-25 to remove the last traces of free TMR. The G-25 eluate contained 2.88 mol of TMR per mol of Fab. It was concentrated 20-fold to give a final protein concentration of approximately 2.5 mg/ml. Two similar preparations were made of pool I material, but the CM-cellulose step was omitted. The preparations did not stain chick myotubes and would not stain rat myotubes treated for 15 min with intact antibody before reaction with labeled Fab.

Fiber Marking

Fibers cultured in 35-mm plastic dishes for 1–2 wk were selected under the phase-contrast microscope, photographed for reference, and the plastic dish was marked with a sharp point for further reference. Marking (Fig. 3) and observation were done with cells in culture media containing 18 mM HEPES buffer.

A fiber selected for marking was labeled by bringing it close to a micropipette containing 1–2 µl of TMR-Fab. Fab was ejected from the micropipette by gentle pressure from a micrometer syringe. An empty micropipette, of larger bore than the delivery pipette, was positioned opposite the delivery pipette. This empty pipette sucked unbound Fab away from the fiber by capillary action, and prevented excessive lateral spread of the reagent. Most marking was done at room temperature, but in two series of experiments marking was done in cold medium, in an effort to prevent lateral spread of label. When intact unlabeled antibody was applied in a patch, only this was micropipetted; a fluorescent antiglobulin was applied afterward over the entire dish. The fiber-marking procedure was observed with an inverted phase-contrast microscope and the micropipettes were mounted on Leitz micromanipulators. The Fab delivery pipette had a tip diameter of 2–3 µm while the larger empty pipette typically had a diameter of 10–20 µm. Fiber marking time was 5–10 min; 5–10 fibers could be marked with 2 µl of Fab. In two experiments fibers were fixed in freshly prepared 5% glutaraldehyde-PBS for 30 min on ice. The fixed fibers were extensively washed in medium before marking.

**125I-Labeled α-Bungarotoxin**

125I-labeled α-bungarotoxin (prepared and characterized as described in reference 7), which reacts with acetylcholine receptors, was mixed with the Fab in one series of experiments; it was visualized by autoradiography after the fluorescence of the fibers had been photographed.

**TMR Goat Anti-Rabbit Light Chain**

TMR goat anti-rabbit light chain (anti-allotype b4) was used in a few experiments to visualize the extent of spread of Fab fragments.

**Examination of the Fibers**

Fiber examination was done with a Leitz Ortholux microscope fitted with HB0200W mercury burner. Petri dishes were mounted on a metal holder and

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**Figure 1.** CM-cellulose chromatography of papain-digested rabbit anti-rat plasma membrane gamma globulin. Details of elution are given in the text.
oiled to the standard dark-field condenser. Exciting light was filtered through either Schott and Gen. (Mainz) or Corion (Corion Instrument Corp., Waltham, Mass.) interference filters to isolate the 5461 mercury line. Emitted light was viewed through the Schott RG-1 glass filter. Fibers were photographed on Kodak 2475 or 2485 high-speed black and white film; exposure times were 30 s for 2475 and 20 s for 2485. A X 10 dry lens, numerical aperture 0.25, was used for most of this work, but X 10 and X 22 water immersion lenses were used for some experiments.

**Fab Patch Spreading**

Fab patch spreading was followed by photographing the fibers in cold medium, replacing the medium with room temperature medium with the dish containing the fiber remaining on the microscope stage, and then changing the medium back to ice temperature before each photograph. Warming the medium caused considerable fading of fluorescence intensity, which was reversible by cooling. On the other hand, fading over time, documented by photographing the chilled fiber, seemed to be irreversible, and we interpret it as being due to spread of the Fab patch and diminution of the peak concentration of TMR on the fiber. Measurements of patch lengths were made on X 330 enlargements of the fibers. Both authors made independent measurements of the patches. These estimates varied for many fibers; the agreement was best for well-marked fibers, and for all fibers relative rankings of patch spread were fairly consistent.

**RESULTS**

**Characterization of Membrane Antigens**

Antigenic activity of extracts was evaluated in terms of their ability to inhibit the binding of antimuscle membrane antibody to cultured myotubes. If an extract contained muscle membrane antigens, there should be no staining of myotubes by the absorbed serum. Only two extracts did indeed reduce the staining of the serum when compared to controls, the membrane pellet after incubation in buffer lacking papain and the papain supernatant. The papain-digested membrane residue and the lipid extract of membranes failed to reduce antisemum staining. These observations suggest that the antigens detected are proteins.

The electrophoretic profiles of radioactivity for total reduced and alkylated detergent extract of radiiodinated myotubes, for material specifically precipitated by antiserum (solid line), and for material nonspecifically bound to a precipitate of anti-Fc and normal rabbit serum are shown in Fig. 2. Comparing the three profiles, it appears that the serum reacts specifically with several antigens running slowly on the gels. From calibration runs on the gel and comparison of specific *serum* control precipitates, it appears that the antigens detected on muscle cell surfaces by our antisemur are of at least 200,000 mol wt. A globular molecule of this size should have a diameter of approximately 100 Å. Variations of several-fold in molecular weight will affect diffusion constants only slightly, however, as diffusion is a function of the cube root of the molecular weight of the diffusing molecule.

**Fibers Marked with Polyvalent Antibody and Fluorescence Antiglobulin**

Of some 30 fibers marked by an indirect technique, and hence bearing patches of cross-linked antibody, only three clearly showed spread of the marked patch with time. The other fibers either broke during culture, or changed shape so drastically that comparison of patch lengths with time was meaningless. In one instance, an entire patch, of 100 μm length, appeared to have moved considerably along the cell surface. Observations in this series were made over 20 h duration, and cells were held at 37°C in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES)-buffered medium between observations. Measurements of those cells that showed spreading patches are given in Table I. It will be seen that the rates are appreciable, though low. They are consistent either with the spread of 100-Å particles in a medium of viscosity 10-50 poise or with the spread of very large diameter aggregates in a medium 1/10-1/100 as viscous. Aggregates of antigen are observed when antiglobulins are applied to cells previously treated with antibodies to the plasma membrane (5).

**Fibers Marked with TMR Fab**

Over 50 fibers were labeled with TMR Fab (Fig. 3). Some of these were so lightly labeled that their marks could not be followed with time. Some patches faded without ever showing an increase in length. This fading we interpret as due to dilution of a low initial concentration of Fab. We expect that the Fab concentration at the ends of a patch of stain may be so low as to be undetectable, and that the true extent of a patch may be under-
estimated for this reason. Indeed, when cells were co-labeled with Fab and 125I-labeled α-bungarotoxin, the extent of α-bungarotoxin binding (determined autoradiographically) was greater than the extent of fluorescence (Table II). Hence, if spread of an initially faint Fab mark occurred, concentrations of TMR at the edges of a patch would rapidly fall below the level detectable by our techniques. A few other patches showed no detectable spread over the short times that they were examined (5 min). A time series on one fiber is shown in Fig. 4.

Data on 20 fibers in which spread was observed or which served as controls are listed in Table III. Six other fibers fixed in glutaraldehyde before marking showed no spread when photographed after 5, 10, 15, 20, 30, and 45 min at room temperature and are omitted from the table. Values independently measured by each of us (ME, DF) are given. They are in good agreement for many

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fibers, though one of us has, on average, estimated greater rates of spread than has the other. In Table IV, diffusion constants \((D)\) are tabulated for the surface antigens detected by our Fab anti-rat plasma membrane. The fluorescent spots on cells appear to be roughly circular, hence \(D\) for two-dimensional diffusion is taken \(\sim X^2/4t\), where \(X = \) change in radius of the patch (one-half the length of the patch) in centimeters, and \(t\) is the time over which the change occurs (in seconds) \((12)\). A typical value for \(D\) is \(1 \times 10^{-9}\) cm\(^2\)/sec, though values 10-fold greater and 5-fold less were also calculated for some cells. The square term tends to exaggerate differences in measurements, and in some instances \(D\) for a given mark varies by an order of magnitude. Because of the uncertainty in determining the true extent of the fluorescent spot (see above), we feel that these variations are due to technique and do not represent measurements on cells with differing states of the membranes.

The average value for \(D\) \((\bar{D})\) calculated from measurements on the fibers examined in the warm without further treatment is \(2.67 \times 10^{-9}\) cm\(^2\) sec\(^{-1}\) (SE = 0.68) for ME data and \(1.1 \times 10^{-9}\) cm\(^2\) sec\(^{-1}\) (SE = 0.58) for DF data. \(\bar{D}\) for fibers treated with unlabeled antibody after spotting with Fab \((14-16)\) is about \(0.15 \times 10^{-9}\) cm\(^2\) sec\(^{-1}\), while \(\bar{D}\) for iced fibers \((18-20)\) is \(\leq 0.01 \times 10^{-9}\) cm\(^2\) sec\(^{-1}\).

Though calculation of membrane viscosities from these data requires many assumptions, the magnitude of the value is of interest. Using the equation for diffusion of a sphere in an isotropic medium, we find a range of apparent viscosities for the myotube membrane of from 0.2 to 3 poise.

### Table I

| Fiber | Time observed (h) | Spread min \((X 500)\) | Spread cm \(X 10^{-4}\) | \(D\) cm\(^2\) sec\(^{-1}\) \(X 10^{-9}\) |
|-------|------------------|-------------------------|------------------------|----------------------------------|
| 1. 23-12-1 | 9 | 10 | 20 | 3.1 |
| 2. 14-6 | 8 | 18 | 36 | 11.2 |

![Figure 3](image)

**Figure 3** Marking a fiber. The smaller pipette delivers labeled Fab, which is seen as a dark area in the solution adjacent to the fiber.
This is consistent with a lipid-rich plasma membrane, as are previous estimates (4, 9).

DISCUSSION

Patches of fluorescent Fab anti-plasma membrane antibody made on cultured myotubes have been observed to enlarge with time. This spread was inhibited, though not completely, by lowered temperature and by the presence of excess unlabeled anti-surface antibody. The data are consistent with the migration of surface antigens in the plane of a fluid myotube plasma membrane. Several observations serve as controls for the system. The fluorescent Fab preparations did not stain cultured chick myotubes and did not membrane-stain rat myotubes pretreated with unlabeled intact antibody. This indicates that binding to the rat myotubes was specific. Addition of labeled anti-light chain antibody to previously marked fibers intensified the strain observed. The intact antibody does not penetrate cells, and this indicates that the Fab was bound to the cell surface and not lodged in the cytoplasm.

A third artifact possible in our system is patch spreading due to dissociation of Fab fragments, which, since they are monovalent, have apparent binding constants equal to the square root of the binding constants of intact antibodies. If Fab fragments diffused short distances and then reassociated with the membrane, spread of a marked patch would be seen. We have performed two experiments that indicate that the fragments do not dissociate during the course of our measurements. In the first, fibers were labeled in the usual manner, and photographed cold at \( t = 0 \). Then, instead of returning the fibers to warm medium, they were incubated at room temperature in 1 ml of undiluted intact antibody. For later photographs during the time course (fibers 14–16 in Table III), fibers were returned to cold medium. Fiber 16, which was followed over several hours, received fresh antiserum after each photograph. If Fab fragments had dissociated in the presence of unlabeled intact antibody, we would have expected the spots to fade within the 5-165 min period of observation, as labeled fragments were replaced by excess unlabeled antibody. In fact, spot intensity was maintained, and the spots showed spreading, although at a somewhat lower than usual rate.

Our second approach to the possibility of Fab dissociation was to fix the fibers, before labeling, in 5% glutaraldehyde in PBS. This fixation has been shown (2, 4) to prevent rotational diffusion of rhodopsin in rod outer segment membranes. After fixation, fibers still stained well with our reagent, and could be readily marked. None of the patches on fixed fibers showed any increase in length over the times up to 45 min observed. It seems that labeled Fab specifically bound to muscle surface does not dissociate from the surface during the course of our experiments.

The estimated diffusion constants for antigen movement for untreated fibers is \( 1-2 \times 10^{-9} \text{ cm}^2 \text{ sec}^{-1} \), about an order of magnitude lower than that found for diffusion of labeled phospholipids in synthetic lipid multilayers and in sarcoplasmic reticulum (6, 18, 24). While this difference may reflect greater restrictions on molecular movement in natural membranes than in artificial membranes, it may be that most of the difference is due to the size of the molecules followed. The cross-sectional area of a phospholipid molecule is given as 70 \( \text{A}^2 \); the area of a protein molecule of 100-200,000 mol wt may be estimated as 1,500-3,000 \( \text{A}^2 \), accounting for much of the discrepancy in diffusion constants. Since, for technical reasons cited above, we tended to underestimate diffusion, there is a further systematic error tending to lower our value for \( D \). If the concentration of fluorescent Fab could be measured at a single point over time, a more accurate estimate of the mobility of the surface antigens of cultured muscle cells could be made.

The data presented here support observations made in another system (9) on the rapid intermixing of surface antigens of newly formed somatic cell heterokaryons. The apparent diffusion constant calculated from observations on heterokaryons is \( 0.2 \times 10^{-9} \text{ cm}^2 \text{ sec}^{-1} \). This
FIGURE 4  Increase in size of a spot of TMR-Fab anti-muscle plasma membrane. (a) A cultured fiber before marking $\sim \times 200$. The same fiber after marking (no. 16, Table III) and warming for: (b) 0 min, (c) 6 min, (d) 12 min, (e) 165 min. The fiber was cooled before each photograph. Final magnification, 650.
constant is based on single time point measurements on cells that were exposed to Sendai virus, and whose surface antigens were not visualized until after diffusion or mixing had occurred. Thus in the heterokaryon system it was possible to attribute antigen mixing either to changes in the membrane caused by the inactivated virus, or to migration of antigen molecules from membrane sites through the cytoplasm to reemerge on the surface elsewhere. Neither of these possibilities arises in the present system. The myotubes are not virus treated, and the antibody marker is applied before measuring diffusion. It appears that the plasma membranes of several cell types are fluid in nature, allowing intermixing of surface proteins and lipids. How general this aspect of

### Table III

**Spread of TMR-Fab Anti-Muscle Membrane Patches with Time**

| Fiber | Time observed (min) | Observed spread in mm ($\times 330$) | Calculated spread (cm $\times 10^{-4}$) |
|-------|---------------------|-------------------------------------|-------------------------------------|
|       | ME | DF | ME | DF | ME | DF |
| 1     | 5  | 3  | —  | 9.1 | —  | —  |
| 2     | 5  | 5  | 3.1| 15.0| 9.4| |
| 3     | 12 | 12 | 6.2| 36.0| 19.0| |
| 4     | 5  | 5  | 4.4| 15.0| 13.5| |
| 5     | 10 | 10/12| 11| 30/36| 33| |
| 6     | 6  | 6/23| 2.5| 9.1/6.1| 7.6| |
| 7     | 2  | 4  | 2.1| 12.1| 6.3| |
| 8     | 4  | 6  | 3.6| 18.1| 10.8| |
| 9     | 3  | 1.1| —  | 3.3 | —  | —  |
| 10    | 6  | 1.8| 1.7| 5.3 | —  | —  |
| 11    | 3  | 5  | —  | 15.1| —  | —  |
| 12    | 6  | 9  | 1.9| 27.2| 5.9| |
| 13    | 38 | 12 | 8.6| 36.0| 25.8| |
| 14    | 5  | 1.6‡| 1.0| 2.4 | 1.5| |
| 15    | 6  | 1.5‡| 3.6| 2.3 | 5.5| |
| 16    | 6  | 1.5‡| 3.2| 2.3 | 4.9| |
| 17    | 12 | 2.5‡| 5.3| 3.8 | 8.2| |
| 18    | 165| 3.1‡| 7.2| 4.8 | 11.1| |
| 19    | 90 | 12 | —  | 36.0| —  | —  |
| 20    | 5  | 1.0| —  | 3.0 | —  | —  |

* Fibers were treated with excess, intact, unlabeled, anti-surface antibody.
‡ Measurements made at X650.
§ Fibers were kept cold.
TABLE IV
Diffusion Constant, \( D = \frac{(X^2)}{4t} \) for the Fibers of Table III

| ME \( \times 10^{-9} \) | DF | ME \( \times 10^{-9} \) | DF |
|-------------------------|----|-------------------------|----|
| 1. 0.68, — | 11. 3.2, — |
| 2. 1.88, 0.73 | 5.1, 0.24 |
| 4.5, 1.2, 12. 1.2, — |
| 3. 0.95, 0.76, — | 0.06 |
| 4. 0.69, 0.70 | 13. 1.4, 0.7 |
| 0.95, 0.76 | 14. 0.04, 0.04 |
| 5. 7.6/11, 9.1 | 15. 0.04, 0.2 |
| 6. 0.57/0.26, 0.4 | 16. 0.04, 0.17 |
| 7. 3.1, 0.8 | 0.05, 0.23 |
| 3.4, 0.12 | 17. 0.61, — |
| 8. —, 0.15 | 18. 0.05, — |
| 9. —, 0.59 | 19. 0.01, 0.001 |
| 10. 1.0, 0.2 | 0.05, 0.01 |
| 20. —, — |

membrane structure is, and what restrictions are placed on the migration of membrane components are at the present unknown.

We thank Mr. C. Hartzell for membrane preparations, and Dr. John Cebra for fluorescent anti-44 antiseraum. Miss Nancy Joseph provided technical assistance and Ms. Laura Gordon made excellent photographic prints for this work.

This work was supported by National Institutes of Health Grant AM 11202 to M. Edidin.

This is contribution number 708 from the Department of Biology.

Received for publication 4 July 1972, and in revised form 20 November 1972.

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