PROCION YELLOW M-4RS BINDING TO NEURONAL MEMBRANES

M. T. FLANAGAN, T. R. HESKETH AND S-H. CHUNG

National Institute for Medical Research, Mill Hill, London, NW7 1AA, England

Received for publication November 27, 1973

PROCION Yellow M-4RS, an intracellular neuronal marker, has been shown to bind covalently to the subcellular organelles of rat brain under physiologic conditions. Binding to purified proteins, nucleic acids and lipids also occurs under such conditions, the probable site of attachment being the primary amino group. Lipid membranes were highly impermeable to this dye. The fluorescence intensity of PROCION Yellow M-4RS was shown to be viscosity-dependent. These results are consistent with the behavior of the dye when it is used for mapping neurons.

In 1968 Stretton and Kravitz (28) demonstrated that several Procion dyes could be injected into nerve cell bodies of invertebrates by means of a microelectrode and that the dyes would diffuse throughout the neuron into the fine branches without leakage. They decided that the most satisfactory of these was Procion Yellow M-4RS, mainly on the basis of its high fluorescence intensity. They were able to determine the geometry of the neuron as the dye was not removed by fixation with glutaraldehyde and dehydration with alcohol and it fluoresced strongly in the ultraviolet light microscope.

Since then this dye has been used extensively in neuronal mapping work in both vertebrates (16, 18, 30) and invertebrates (25, 26) and it has also been observed to fluoresce strongly after injection but prior to fixation (22), although a solution of the dye in water is only very weakly fluorescent. The dye cannot cross nonjunctional axon membranes but has been used to measure the permeability of electrotonic synapses (19, 22). The penetration of the dye into the fine branches of the neuron may take several hours and extremely high electrode resistances have been encountered in iontophoresing the dye into cells (4). A dye with staining and fluorescence properties similar to Procion Yellow M-4RS but which could be more readily introduced into the neuron would therefore be of considerable use.

To design such a substance it must be determined why, of all the dyes screened by Stretton and Kravitz, only the Procion group penetrates neurons without leaking out and is unaffected by fixation. In addition, an explanation of the enhancement of fluorescence of Procion Yellow M-4RS on entering the neuron may provide information about the internal structure and composition of the cell.

The Procion dyes are all anionic compounds and all contain either a monochloro- or dichlorotriazinyl ring (10). Under alkaline conditions they are known to react with compounds containing an amino or hydroxyl group to form a covalent conjugate with the elimination of hydrogen chloride (10). In this paper we have examined the binding of Procion Yellow M-4RS to proteins, nucleic acids and lipids under physiologic conditions and to homogenates of immature rat brains which were subsequently fractionated to obtain the labeled subcellular organelles. The permeability of lipid membranes to this dye has also been studied as have the changes in the fluorescence of the dye on changing its environment.

METHODS AND MATERIALS

Fluorescence measurements: Fluorescence emissions from solutions were measured at 90° with respect to the exciting beam in an Amino-Bowman spectrofluorimeter. The emissions from labeled fibrous proteins were measured at 90° to the exciting beam with the fibers mounted on a quartz plate at an angle of 30° to the exciting beam. Measurements were performed at 25°C, except where otherwise stated, in a temperature-controlled cell holder. Procion Yellow M-4RS (Imperial Chemical Industries) was excited at 450 nm and the emission was observed at 550 nm when the total emission spectrum was not recorded.

Isolation and homogenization of cells: Cells from newborn rats were fractionated by the method of Hemminki and Suovaniemi (13) with minor modifications. Six cortices from 5-day-old rats were placed in a glass homogenizer in 10 ml of a buffer containing 3% Procion Yellow M-4RS, 140 mM sucrose, 5 mM glucose, 10 mM sodium phosphate and 1.25 mM
PROCION YELLOW BINDING TO NEURONAL MEMBRANES

Tris-HCl (pH 7.4) and homogenized with 15 strokes. For the unstained, control preparation the dispersing medium was 1 mM ethylenediaminetetraacetic acid, 280 mM sucrose, 10 mM glucose, 20 mM sodium phosphate and 10 mM Tris-HCl (pH 7.4). The suspensions were maintained at 25°C and at 0, 5 and 15 min were sucked into large pipettes to assist dispersion. After 30 min, 20 ml Eagle's minimal essential medium were added and the dispersions were centrifuged for 5 min at 900 x g. The supernatants were discarded and the pellets were resuspended in 20 ml fetal calf serum and centrifuged as above. The supernatants were again discarded and the pellets resuspended in 30 ml of the dispersing medium and centrifuged for 5 min at 900 x g.

The cellular pellets obtained were suspended in 7 ml 90 mM sucrose, 1 mM MgCl₂, and 40 mM Tris-HCl (pH 7.6) for 10 min at 4°C before a 20-stroke homogenization. A further 3 ml suspending buffer were added and the homogenates were filtered through a 30-μm nylon mesh. The sucrose concentration in the homogenates was adjusted to 0.2 M and the suspensions were centrifuged at 3500 x g for 5 min. The pellets were resuspended in 10 ml 2.2 M sucrose containing 1 mM MgCl₂ and 2.5-mI layers of 1.3 M and 0.25 M sucrose were added before centrifugation in a Beckman SW 27 rotor at 63,000 x g for 60 min. The pellets contain the nuclei, the 2.2/1.3 M sucrose interphases contain the rough membranes and the 1.3/0.25 M sucrose interphases contain the undisturbed cell processes.

The supernatants from the 3500 x g spins were centrifuged at 20,000 x g for 20 min and the resulting crude mitochondrial pellets were suspended in 0.25 M sucrose and centrifuged at 20,000 x g for 20 min. The pellets thus formed were suspended in 15 ml 0.85 M sucrose and each half of this solution was layered onto 2.5 ml 1.2 M sucrose followed by a 5-ml layer of 0.25 M sucrose: centrifugation was at 63,000 x g for 60 min. Mitochondria formed the pellets, synaptosomes formed the 1.2/0.85 M sucrose interphases and the plasma membranes formed the 0.85/0.25 M sucrose interphases.

The postmitochondrial supernatants were made 15 mM with CaCl₂, layered on to 7.5 ml 1.3 M sucrose and centrifuged at 100,000 x g for 3 hr to separate rough and smooth endoplasmic reticulum.

Gel electrophoresis: Proteins in each fraction were solubilized by incubation for 2 hr at 37°C in 0.01 M sodium phosphate, 1% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol (pH 7.9) and subjected to electrophoresis on 7% polyacrylamide gels containing 0.1% SDS for 12-14 hr using a constant current of 8 mA/gel. The method was essentially that of Weber and Osborn (32).

Usually 200 μg of the control samples were applied per gel; up to 500 μg of the Procion Yellow M-4RS-labeled fractions were used to enable the bands to be more easily visualized. The control sample gels were stained with Coomassie blue for 6 hr at 56°C and destained in a solution of 5% methanol and 7.5% acetic acid in water. The Procion Yellow M-4RS gels were photographed immediately after electrophoresis without further staining on a Kodak cold light illuminator using Ilford N4.E.50 film.

Protein molecular weights were obtained by comparison with bovine serum albumin, ovalbumin, tryptsin, lysozyme and hemoglobin.

Protein and Procion Yellow M-4RS estimations: The protein content of each subcellular fraction was determined according to Lowry et al. (17) and the amount of bound Procion Yellow M-4RS was measured by absorbance at 425 nm.

Nucleic acids: Deoxyribonucleic acid (DNA) (sodium salt, from calf thymus) and ribonucleic acid (RNA) (from yeast) were obtained from Sigma. After ethanol precipitation, the levels of protein contamination, as estimated by the method of Lowry et al. (17), were 0.5% for DNA and 3.1% for RNA. Samples of each were incubated overnight in 6% Procion Yellow M-4RS (5 mg/ml) at pH 7.4 and then dialyzed for 24 hr. The nucleic acid solutions were made 0.1 M with NaCl and then precipitated with 2 volumes of ethanol at 4°C: the precipitate was resuspended at 5 mg/ml in water, dialyzed for 3 days at 0°C and reprecipitated. After again resuspending at 5 mg/ml the absorbance due to bound Procion Yellow M-4RS was measured.

Lipids: Dipalmitoyl lecithin, phosphatidyl ethanolamine (from bovine brain) and cerebrosides (from beef brain) were obtained from Koch-Light Laboratories, Buckinghamshire, England; lecithin (from egg) and phosphatidyl serine from Lipid Products, Redhill, England, and cholesterol from Fluka; sphingomyelin was extracted from pigs kidneys and each lipid was checked for purity as described previously (14). Total human erythrocyte lipids were obtained by chloroform-methanol extraction from fresh red cells.

For Procion Yellow M-4RS binding experiments, 2.5 mg of each lipid were dissolved in chloroform in a 10-ml tube and the solvent was evaporated to give a thin film of lipid to which 1 ml of a 6% solution of Procion Yellow M-4RS (pH 7.2) was added, together with some small glass beads. The lipids were dispersed by vortex mixing under nitrogen and allowed to stand overnight at 3°C. Lipid and free Procion Yellow M-4RS were separated by repeated sonication and centrifugation, the pellet being finally resuspended in 3 ml water and both the absorbance and fluorescence spectra of bound dye measured as described above.

To estimate the permeability of a lipid membrane to the dye, lipid components which had been shown by the above method not to bind Procion Yellow M-4RS were required. Accordingly, egg lecithin-cholesterol-dicetyl phosphate and egg lecithin-cholesterol-dipalmitoyl lecithin and hemoglobin.
terol-stearolamine mixtures (2:1.5:0.2 M) were dispersed as described above (10 μmoles phospholipid/ml Procion Yellow M-4RS solution). To ensure that covalent binding to stearylamine did not occur the dye solution used in these experiments was maintained at 3°C for 3 days prior to use to remove the reactive chlorine. Free dye was separated from that trapped in the lipid vesicles by centrifugation at 25,000 × g for 20 min. After washing and further centrifugation the pellet was resuspended in 40 ml water or 0.075 M NaCl (the maximal salt concentration at which 6% Procion Yellow M-4RS is soluble) and incubated under nitrogen at 37°C. Fractions were removed at intervals and, after centrifugation to separate the membranes, the absorbance of the supernatant at 425 nm was measured to obtain an estimate of the Procion Yellow M-4RS which had diffused from the lipid vesicles.

Lipid chromatography: Procion Yellow M-4RS-phospholipid conjugates were separated from whole brain homogenates by paper chromatography after the protein had been precipitated by the addition of chloroform-methanol (2:1 v/v). The solvent system used was butan-1-ol-acetic acid-water (5:2:3 v/v/v). Conjugates of phosphatidyl serine and phosphatidyl ethanolamine were run as standards.

RESULTS

Fluorescence properties of Procion Yellow M-4RS: Procion Yellow M-4RS fluoresces extremely weakly in water with an uncorrected emission maximum at 550 nm when excited at 450 nm. Change of solvent dielectric constant from that of water to ethanol, for example, did not change the position of the emission maximum and increased the fluorescence intensity only by a factor of two.

The position of the emission maximum was also unaffected by solvent viscosity but the fluorescence intensity increased 11-fold in 90% glycerol from that in water. Oster and Nishijima (21) have measured a strong dependence of fluorescent yield on solvent viscosity for several compounds and have associated this with the ability of two aromatic rings within the fluorophor to rotate, so that they are in conjugation for part of the lifetime of fluorescence. They have shown that such a rotation leads to a radiationless deactivation of the first excited singlet state, characterized by a rate constant that is proportional to the absolute temperature of the solvent (T) divided by its viscosity (η). When this rate constant is substituted into the equation relating fluorescence efficiency to the rate constants of the processes deactivating the first excited singlet state, one obtains the equation

\[ \frac{1}{Q} = 1 + \frac{r}{(k + a \cdot T/\eta)}, \]

where Q is the fluorescence yield, r the intrinsic lifetime of fluorescence, k the sum of the rate constants of the deactivation processes other than fluorescence emission and diffusional quenching and a is a constant. Figure 1 shows the linear dependence of the reciprocal of the fluorescence intensity of Procion Yellow M-4RS on T/η.

Interaction of Procion Yellow M-4RS with detergents: When interacting with subcellular structures, Procion Yellow M-4RS, being a large, aromatic, negatively charged molecule, is likely to bind at hydrophobic sites on lipids or proteins that are adjacent to positively charged groups. Information about the ability of fluorescence probes to partition in this way has been obtained by examining their binding to detergent micelles (11). Consequently, we have measured the fluorescence intensity of Procion Yellow M-4RS in the presence of concentrations of SDS, polyoxyethylene lauryl ether (Brij 35) and cetyltrimethylammonium bromide (CTAB) exceeding their critical micelle concentration. The fluorescence intensity was unaffected by 2% SDS and was enhanced 2-fold by 4% Brij 35. CTAB greatly enhanced the fluorescence intensity, as shown in Figure 2, which also includes the determination of critical micelle concentration from the increase in the fluorescence intensity of dichlorofluorescein with micelle formation (8). As micelle formation begins, the fluorescence intensity of the Procion Yellow M-4RS increases dramatically and then falls to a plateau value once this process is complete. The interiors of CTAB and SDS micelles are known to be more viscous than water (24, 27) and this should lead to an increase in the fluorescence efficiency of any bound dye. The very high degree of enhancement observed at the onset of micelle formation may be due to very high internal viscosity in small micelles where the hydrocarbon chains are constrained in a small volume. To test this hypothesis we added potassium bromide to a solution of Procion Yellow M-4RS in 0.03% CTAB (w/v), a concentration at which the fluorescence intensity is above the plateau level. Potassium bromide increases the size of cationic micelles (1, 2) so one would expect the fluorescence intensity...
Concentration of Cetyltrimethylammonium Bromide (w/v)

**FIG. 1.** The reciprocal of the fluorescence intensity of Procion Yellow M-4RS in water-glycerol mixtures versus the absolute temperature divided by the viscosity. The glycerol concentration was varied from 0 to 100% and the Procion Yellow M-4RS concentration was 25 μg/ml. The fluorescence excitation wavelength was 450 nm and the intensity units, which were obtained by measuring the area under the emission envelope, are relative.

**FIG. 2.** The fluorescence intensity of procion yellow M-4RS (●) (2 μg/ml) and dichlorofluorescein (▲) (10⁻⁷ M) in solutions of increasing concentration of CTAB. The Procion Yellow M-4RS and dichlorofluorescein were excited at 450 and 300 nm, respectively, and their emissions were measured at 550 and 520 nm, respectively. The intensity units are arbitrary. Inset, the effect on the fluorescence intensity of Procion Yellow M-4RS in a solution of 0.03% w/v CTAB of added potassium bromide.

to fall to a constant level as the micelles increase to a maximal size with increasing salt concentration. The inset in Figure 2 shows that this does occur.

The strong interaction of the dye with the positively charged CTAB, the weak interaction with the neutral Brij 35 and the absence of any interaction with the negatively charged SDS are consistent with the partitioning of the dye at a hydrophobic interface, this partitioning being
enhanced by the presence of adjacent cationic groups but prevented by adjacent anionic groups. This suggests that the presence of anionic groups on the dye may seriously affect its solubility in a lipid membrane. To investigate this further we examined the partitioning of the dye between water and butan-1-ol. The dye was highly insoluble in the butan-1-ol phase, the partition coefficient being 0.003.

This insolubility is due in part to the anionic groups as the partition coefficient rises to 0.33 (Fig. 3) on adding stearylamine to the butan-1-ol phase, thus allowing a salt link to neutralize the anionic groups in the hydrophobic phase.

**Covalent binding to proteins, nucleic acids and lipids:** Under alkaline conditions Procion dyes will react covalently with hydroxyl or amino groups, losing a chlorine from the chlorinated triazinyl ring (10). We have studied the covalent binding of Procion Yellow M-4RS to proteins, nucleic acids and lipids under the milder condition of pH 7.4. Fibers of jute, casein, zein and nylon were incubated for 2 hr in a 6% solution of dye at 25°C and then washed with water to remove any unbound dye. Fibers were chosen as they provide a solid support for the bound dye which will fluoresce strongly in such a rigid environment. The relative fluorescence intensity of labeled casein, zein, jute and nylon is shown in Table I and it is clear that jute and casein are heavily labeled but very little dye is bound to zein or nylon. Although differential labeling of the fibers due to variation in the penetrability of the dye may occur, these results are fully consistent with labeling via primary amino groups, as shown by the data of Table I, which indicate the deficiency of zein in lysine by comparison with casein (7). Nylon was included as it is, in effect, a protein with no side chains. As with zein there is very little labeling, the small amount occurring possibly being to the peptide groups (10). The high binding to jute shows that the dye will react with sugar hydroxyls under these conditions as this fiber is predominantly cellulose (20).

The binding of Procion Yellow M-4RS to soluble proteins was demonstrated by incubating bovine serum albumin and ovalbumin with the dye as above. The labeled proteins were separated from unreacted Procion Yellow M-4RS by polyacrylamide gel electrophoresis. The protein samples contained both dimer and trimer as well as monomer and comparison of the gels of labeled and unlabeled protein showed that Procion Yellow M-4RS binding did not perturb the electrophoretic properties of these proteins in such a system.

It was found that Procion Yellow M-4RS bound covalently to DNA and RNA to the extent of 0.54 mg and 0.55 mg/mg of nucleic acid, respectively.

The lipids phosphatidyl serine, phosphatidyl ethanolamine, egg lecithin, dipalmitoylphosphatidylcholine, sphingomyelin, cerebroside and cholesterol were incubated with 6% dye.

![Graph showing partition coefficient of Procion Yellow M-4RS](image)

**Figure 3.** The partition coefficient of Procion Yellow M-4RS in water and butan-1-ol as a function of the concentration of stearylamine added to the butan-1-ol phase. The partition coefficient is defined as the concentration of dye in the butan-1-ol phase divided by the concentration of dye in the water phase. The total dye concentration was 1 mg/ml and the experiment was carried out at 25°C.

### Table I

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Casein</th>
<th>Zein</th>
<th>Nylon</th>
<th>Jute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>16.1</td>
<td>5.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>21.4</td>
<td>9.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>42.8</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative fluorescence intensity</td>
<td>54.0</td>
<td>15.0</td>
<td>10.0</td>
<td>145.0</td>
</tr>
</tbody>
</table>
solution as described previously. Only the first two lipids formed a yellow conjugate with the dye. This again indicates a reaction via a primary amino group as these are the only lipids among those tested that possess such a group. The reaction with hydroxyl groups in cerebroside and cholesterol does not appear to take place at this pH.

**Binding to brain homogenates:** The results of the Lowry estimation and the absorbance measurement of protein-associated Procion Yellow M-4RS are summarized in Table II in which the amount of bound dye is expressed in milligrams per milligram of protein for each subcellular fraction. With the exception of the nuclei and the rough membranes and free polysomes, these figures diverge from a mean value by a factor of less than 2.2. The two fractions that contain protein normally associated with nucleic acid, however, show an order of magnitude greater affinity for Procion Yellow M-4RS.

In Figure 4 the polyacrylamide gels of each subcellular protein fraction are compared with those of the corresponding Procion Yellow M-4RS-labeled fraction. The numbering system of each pair of gels refers to the same fractions as in Table II. It is clear that most protein components of each fraction are able to bind the dye. There is particularly strong Procion Yellow M-4RS binding by the high molecular weight components of the undisrupted cell processes (fraction 1) and by both the low molecular weight (below 40,000) mitochondrial (fraction 6) and (approximately 15,000) nucleic acid-associated proteins (fraction 3). The low molecular weight (15,000) rough membranes and nuclei components do not show Procion Yellow M-4RS binding (fraction 2) and the rough membranes and free polysomes (fraction 8) show strong dye affinity over the middle of the molecular weight range, although there is insufficient protein for visualization by the Coomassie blue stain.

**Identification of labeled brain lipids:** A brain homogenate and a total brain lipid extract were incubated with Procion Yellow M-4RS under the same conditions as those used prior to cell fractionation. The solutions were then centrifuged at 20,000 × g for 30 min and the pellets were suspended for 1 hr at 37°C in 0.01 M sodium phosphate, 1% SDS and 1% β-mercaptoethanol (pH 7.9). Procion Yellow M-4RS-phospholipid conjugates were then separated from these solutions by paper chromatography. The only visibly labeled component of the total lipid extract ran in a position almost identical to that of the labeled phosphatidyl ethanolamine. In the total homogenate, however, labeled lipid ran more slowly at a position between the labeled phosphatidyl ethanolamine and serine.

**Membrane permeability to Procion Yellow M-4RS:** The permeability of lipid membranes was measured by observing the rate of loss of hydrolyzed Procion Yellow M-4RS from lecithin-cholesterol-dicetyl phosphate and lecithin-cholesterol-stearylamine vesicles and the results are shown in Figure 5. This figure includes data for incubation of the vesicles both in water and in the presence of 0.075 M NaCl, the latter to ensure that the release of Procion Yellow M-4RS is not rate-limited by the absence of a counterion, these membranes having been shown to be highly permeable to chloride (3). Within experimental error, membranes with the same composition gave identical results in the two systems. As covalent binding of the hydrolyzed dye cannot be occurring, the results represent genuine trapping by the membrane and it is clear that the negatively charged vesicles are highly impermeable, exhibiting a linear loss of 80% of initially trapped dye over a period of 24 hr. After a rapid loss of approximately 22% of the Procion Yellow M-4RS the stearylamine vesicles are even less permeable, losing only a further 21% in 26 hr. Although these experiments were carried out with hydrolyzed dye, because of the presence of stearylamine, the leakage rate from dicetyl phosphate vesicles was
Fig. 4. These are 7% polyacrylamide gels of brain homogenate fractions. The numbers denote the same fractions as in Table II. The molecular weight markers are bovine serum albumin, ovalbumin, trypsin and lysozyme. a, Stained with Coomassie blue; b, Procion Yellow M-4RS-labeled.

Fig. 5. The rate of release of Procion Yellow M-4RS at 37°C from liposomes consisting of egg lecithin-cholesterol-dicetyl phosphate (●) and egg lecithin-cholesterol-stearylamine (▲) in water. ○ and △, incubation in 0.075 M NaCl.
unchanged when fresh, reactive Procion Yellow M-4RS was trapped.

DISCUSSION

The experiments described in this paper show that, under physiologic conditions, Procion Yellow M-4RS readily forms conjugates with a wide range of purified biologic molecules, reacting with the primary amino groups of proteins, nucleic acids and lipids. The formation of such conjugates within the iontophoresed neuron would clearly offer an explanation for the resistance of Procion Yellow M-4RS to fixation procedures.

Five-day-old rats were used in the preparation method of Hemminki and Suovaniemi (13) as the homogenate obtained from the immature brain provides a reasonable model for the iontophoresis of Procion Yellow M-4RS into neurons. The results of the brain homogenate incubation demonstrate that dye conjugates are formed in situ. A comparison of the corresponding Procion Yellow M-4RS-labeled and Coomassie blue-stained gels shows that although proteins in all subcellular fractions are labeled there is also a high degree of specificity for the dye. As the experiments with pure proteins indicated an alkylation of the amino groups, this specificity is probably directed toward the more basic proteins. The best example is in the ribosomal fraction (fraction 8) where a large amount of Procion Yellow M-4RS is bound to a quantity of protein too small to be visualized by Coomassie blue. The high degree of labeling of the nuclear and ribosomal fractions by comparison with the mitochondrial and plasma membrane fractions suggests the formation of both RNA and DNA conjugates as these molecules were shown to have a very high affinity for the dye. Most of the proteins of the ribosome are rich in lysine (23), however, and the demonstrated specificity of the dye for this residue is probably also involved in the heavy labeling of this fraction. The importance of protein-Procion Yellow M-4RS conjugates in tracing the geometry of the axon and fine dendritic structure is indicated by the uniformity of labeling of the membranes as shown by the relevant gels (fractions 1, 4 and 5 in Fig. 4).

In addition to the labeling of these membrane proteins we have shown that some lipid components are also labeled and that these must contain a primary amino group. The major problem in isolating the lipids is that conjugation with the anionic procion dye renders them highly insoluble in organic solvents. A partial separation was achieved by paper chromatography, however, although there was some overlap between the phosphatidyl ethanolamine and serine bands. In both the extracted lipid and the total homogenate it was found that phosphatidyl ethanolamine was the predominantly labeled lipid with a smaller amount of the phosphatidyl serine conjugate. An analysis by Breckenridge, Gombos and Morgan (5) has shown these to be the only major lipids in rat brain synaptosomal plasma membranes containing a primary amino group, occurring in concentrations of 34.2% (phosphatidyl ethanolamine) and 13.7% (phosphatidyl serine) of the total phospholipid.

The retention of Procion Yellow M-4RS within neurons is clearly related to the impermeability of lipid membranes to the dye. The partitioning experiment showed this impermeability to result from the negative charge of the dye and this parameter greatly affected the rate of release from the two liposomal systems studied. The phosphatidyl choline-cholesterol ratio of 2.0:1.5 was chosen in the light of the known lipid composition of rat brain synaptosomal plasma membranes in which the ratios of total phospholipid and phosphatidyl choline to cholesterol are 2.0:0.9 and 2.0:2.2, respectively (5). When the lipid membrane was negatively charged diffusion was very slow: with a positive membrane an initially high rate of leakage was followed by a constant, although almost negligible, release of dye. The extreme impermeability of the positively charged membrane is almost certainly due to the binding of the dye to the membrane, as has been previously shown to occur with other aromatic, anionic dyes (12) and is analogous to the strong binding to CTAB micelles described above. The initial, rapid release may be that of excess unbound dye, possibly passing through the membrane as an ion pair with stearylamine (15). The established phenomenon of decreasing liposome permeability to small molecules with increasing membrane cholesterol (9) suggests that the intact neuron membrane may be even

Downloaded from jhc.sagepub.com by guest on November 7, 2016
less permeable to Procion Yellow M-4RS than were the lipid membranes. This membrane impermeability explains the nonappearance of the dye in the extracellular space after infusion into ganglia. Payton, Bennett and Pappas (22) have observed, however, that Procion Yellow M-4RS can diffuse from cell to cell through the electrotonic synapses. This would require the existence of high permeability regions in junctional membranes and there is evidence for these both from electrical measurements (31) and from the visualization of transmembrane channels in the electron microscope (6).

The very strong dependence of the fluorescence intensity of Procion Yellow M-4RS on the viscosity of its environment explains the intense fluorescence intensity of Procion Yellow M-4RS on the channels in the electron microscope (6).

The immediate enhancement of the fluorescence that Payton et al. (22) have observed suggests that the dye rapidly enters a region of high viscosity on injection. This may occur if the dye is immobilized on the surface of the proteins to which it binds, but covalent reactions of this type are unlikely to be so rapid and it is more probable that this initial enhancement is brought about by the dye partitioning itself across the inner surface of the cell membrane in the manner described for the binding to detergent micelles.

Tasaki, Watanabe and Hallett (29) have introduced the fluorescent probe 2-p-toluidinyl-naphthalene-6-sulfonate, like Procion Yellow M-4RS a large, aromatic, anionic dye, into squid axon. They showed that it was bound at or near the inner surface of the nerve membrane. In addition, a study of the polarization of the fluorescence of this dye revealed this region of the nerve to be highly ordered, causing a marked immobilization of the dye after entry. If such a region also exists generally in neurons, Procion Yellow M-4RS would almost certainly become more fluorescent if it were bound there.

The results presented in this paper show that the binding of Procion Yellow M-4RS to subcellular organelles is nonspecific. Consequently, Procion Yellow M-4RS may prove to be a useful intracellular probe for the delineation of the cellular interconnections of other tissues.

ACKNOWLEDGMENT

We are grateful to Dr. G. H. Beaver for the gift of the pure protein fibers.

LITERATURE CITED

18. Matsumoto N, Naka K-I: Identification of intra-
19. Mulloney B: The structure of the giant fibres of earthworms, as disclosed by procion yellow injections. J Physiol (Lond) 210:P, 1960