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Parameters Affecting Fusion between Liposomes and Synaptosomes. Role of Proteins, Lipid Peroxidation, pH and Temperature

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Abstract. We investigated the effect of several parameters, such as temperature, pH and proteins, on the fusion between synaptosomes, freshly isolated from rat brain cortex, and large unilamellar phosphatidylserine liposomes. These studies were carried out in both peroxidized and nonperoxidized synaptosomes. Mixing of membrane lipids was monitored using a fluorescence resonance energy transfer assay. Ascorbate (0.8 mM)/Fe^{2+} (2.5 μM)-induced peroxidation of synaptosomes enhanced the fusion process (twofold) which may reflect an increase in synaptosomal protein hydrophobicity and hence a facilitation of intermembrane aggregation. The fusion process was shown to be temperature sensitive, a reduction in the extent being observed (twofold) as the temperature was lowered from 37 to 25°C. This effect may be due to changes in membrane fluidity. The fusion process is pH dependent, an increase in both kinetics and extent being observed when the pH was lowered from 7.4 to 5.5. A significant inhibition (92% at pH 7.4; 35% at pH 5.5) of the interaction between synaptosomes and liposomes by trypsin pretreatment of synaptosomes was found, thus indicating that the fusion reaction is a protein-mediated process. The inhibitory effect of trypsin at pH 5.5 is not so strong as that at physiological pH. These results suggest that, in addition to the involvement of proteins, nonspecific interactions between the synaptosomal and liposomal membranes under acidic conditions may also play a role in the fusion process. The investigation of binding of synaptosomes to liposomes under several experimental conditions provided evidence for the participation of proteins in membrane aggregation, as well as for the role of electrostatic forces in this process, at mild acidic pH.

Key words: Membrane fusion — Lipid peroxidation — Liposomes — Synaptosomes

Introduction

To understand the molecular mechanisms of membrane fusion in a defined model system, it is important to establish the involvement of specific membrane components, such as proteins, as well as of medium characteristics, such as pH and temperature (Düzgünes, 1985).

We have investigated the effect of several parameters on the fusion between synaptosomes, isolated from rat brain cortex, and large unilamellar phosphatidylserine (PS) liposomes, prepared by reverse phase evaporation. Synaptosomes appear to be a valid model for studying the functional properties of nerve terminals. Therefore, synaptosomes offer the opportunity to study the mechanism of neurotransmitter release that occurs by an exocytotic process. This exocytotic process involves fusion between storage neurotransmitter vesicles and the plasma membrane of the synaptic terminal. Liposomes containing PS were selected since this phospholipid is one of the most relevant lipids of the inner leaflet of the plasma membrane and is also highly fusogenic. To demonstrate the role of proteins in the fusion reaction, we examined the extent of fusion following trypsin pretreatment of the synaptosomes. The nervous tissue is particularly susceptible to free radical induced injury, due to its high content of phospholipids with polyunsaturated fatty acids (Halliwell & Gutteridge, 1984) and a high rate of oxygen consumption (Zaleska & Floyd, 1985). Since

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lipid peroxidation has been suggested to be one of the mechanisms through which cellular damage occurs during the aging process and in several neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease (Ben-Shachar, Riederer & Youdim, 1991; Viani et al, 1991), we were interested in studying how this process affects fusion in our experimental model system.

The overall process of membrane fusion can be separated into two distinct stages that are coupled kinetically. The first step is the close approach or aggregation of the membranes of the fusing vesicles. The following step is the fusion reaction, involving the local and transient loss of structural integrity of the intervening membranes and resulting in coalescence of the internal aqueous contents with concomitant intermixing of membrane components (Düzgünes, 1985). For this reason, it is important to establish the way in which the parameters under investigation influence each of these stages. Accordingly, we carried out binding studies to investigate the behavior of aggregation in this model system under various experimental conditions.

Fusion has been monitored using a fluorescence resonance energy transfer (RET) assay that measures intermixing of membrane lipids (Struck, Hoekstra & Pagano, 1981). This assay allows continuous measurement of the decrease in energy transfer efficiency between two nonexchangeable fluorescent lipid analogues, *N*-(-7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine (*N*-NBD-PE), the donor, and *N*-Rh-PE, *N*-(Lissamine rhodamine B sulfonyl) phosphatidylethanolamine (*N*-Rh-PE), the acceptor, as they dilute into a nonlabeled membrane. The two fluorophores were incorporated in the liposomal membrane at a concentration such that the relative increase of the *N*-NBD-PE fluorescence intensity is proportional to the dilution of the probe in the synaptosomal membrane, and hence to the extent of fusion (Struck et al., 1981).

The iron/ascorbate oxidation pair was used to induce membrane lipid peroxidation, the extent of the peroxidative process being measured by malondialdehyde (MDA) production (Buege & Aust, 1978).

Materials and Methods

Chemicals

N-Rh-PE was obtained from Avanti Polar-Lipids (Birmingham, AL). *N*-NBD-PE and PS were purchased from Avanti Polar-Lipids (Pelham, AL). L-Ascorbic acid, 2-thiobarbituric acid (TBA) and bovine serum albumin (BSA) were obtained from Sigma Chemical (St. Louis, MO).

Isolation of Rat Brain Synaptosomes

Rat brain cortex was homogenized at 9% (w/v) in 0.32 M sucrose, 10 mM HEPES using a Potter-Elvehjam homogenizer rotating at 400 rpm/min (10 strokes). The homogenate was centrifuged at 1,500 × *g* for 10 min and the supernatant was recentrifuged at 9,000 × *g* for 20 min. The pellet (crude mitochondrial pellet) was resuspended in 0.32 M sucrose, 10 mM HEPES, layered on top of a sucrose gradient, composed of 25 ml of 0.8 M sucrose, and centrifuged at 9,000 × *g* for 30 min. The synaptosomal fraction was diluted with distilled water to 0.4 M sucrose and centrifuged at 20,000 × *g* for 30 min (Hajós, 1975). The synaptosomal pellet obtained was resuspended in 10 mM HEPES, 145 mM NaCl, pH 7.4, at 4°C, at a protein concentration of about 12.8 ± 3.1 mg/ml, as determined by the biuret method (Layne, 1957).

Preparation of Liposomes

Phosphatidylserine large unilamellar vesicles, (LUV), incorporating 0.6 mol% each of *N*-NBD-PE and *N*-Rh-PE, were prepared in 10 mM HEPES, 145 mM NaCl, pH 7.4 buffer by reverse phase evaporation (Szoka & Papahadjopoulos, 1978). Liposomes were sized to 0.1 μm by extrusion through Unipore polycarbonate membranes and analyzed for total lipid phosphorous (Barttlet, 1959).

Peroxidation Reaction System

Synaptosomal membranes at a protein concentration of 1.0 mg/ml in 10 mM HEPES, 145 mM NaCl, pH 7.4 (4 ml final volume) were incubated at 37°C, for 15 min, in the presence of 0.8 mM ascorbic acid and 2.5 μM $FeSO_4$, with continuous stirring. Peroxidation was terminated by plunging the samples into ice. The use of peroxidized synaptosomes in fusion experiments required a centrifugation of the peroxidation mixtures at 20,000 × *g* for 30 min to remove the iron/ascorbate oxidation pair. The synaptosomal pellet was resuspended in 10 mM HEPES, 145 mM NaCl, pH 7.4.

Measurement of Lipid Peroxidation

Lipid peroxidation was measured by using the TBA reaction (Buege & Aust, 1978). A 0.5 ml aliquot of both control and peroxidation mixtures was taken and 0.5 ml of 40% (w/v) trichloroacetic acid (TCA) was added. The color reaction was initiated by treating the acidified peroxidation mixture with 2 ml of 0.67% (w/v) TBA and incubating the mixture in a boiling-water bath for 15 min. The samples were then plunged into ice and centrifuged at 3,000 rpm for 10 min. The optical density of the supernatants was measured at 530 nm and the amount of MDA formed was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (Wills, 1969). The results are expressed as nanomoles of MDA produced per milligram of membrane protein.

Trypsinization Reaction System

Aliquots of 1.2 mg of synaptosomal membranes were incubated at 37°C in the presence of trypsin, at a ratio of 1 mg of trypsin/10 mg of protein. Following 30 min incubation, a twofold concentration of trypsin-inhibitor was added to the mixture. Control samples were treated under the same conditions, except that trypsin-inhibitor was added prior to trypsin.

Fusion of NBD-PE/Rh-PE-labeled PS Liposomes with Unlabeled Rat Brain Synaptosomes

Fusion, monitored continuously with the RET assay (Struck et al., 1981), was initiated by rapid injection of NBD/Rh-labeled liposomes, at a final concentration of 16.5 μM, into a cuvette containing the synaptosomes at a final concentration of 275 μg/ml. The final incubation volume was always 2 ml. The fluorescence scale was calibrated such that the initial fluorescence of synaptosomes and labeled liposomes was

set at 0% fluorescence. The value obtained by lyzing the liposomal and synaptosomal membranes at the end of each experiment was set at 100% fluorescence, with the detergent polyoxyethylene 6 lauryl ether ($C_{24}H_{50}O_7$) at a final concentration of 1 mM. Addition of detergent at higher final concentrations did not induce further dilution of fluorescent lipids. The buffer solution did not contribute to the fluorescence signal at the wavelengths used. It has been shown in several systems that the probes NBD-PE/Rh-PE do not exchange between membranes in a fusion-independent fashion (Pedroso de Lima & Hoekstra, 1994).

Fluorescence measurements were performed in a Perkin-Elmer LS-50 luminescence spectrometer with excitation at 465 nm and emission at 530 nm, using 5 and 20 nm slits, respectively, in the excitation and emission monochromators. The sample chamber was equipped with a magnetic stirring device, and the temperature was controlled with a thermostated circulating water bath.

Measurement of the Percentages of Binding between Labeled PS Liposomes and Unlabeled Rat Brain Synaptosomes

Liposomes and synaptosomes, at final concentrations of 16.5 μM and 275 μg/ml respectively, were incubated in an appropriate volume of buffer (2 ml final volume), at 0–4°C (on ice), for 60 min. Under these conditions the fusion between synaptosomes and liposomes is inhibited although the aggregation process occurs. The samples were then centrifuged at 20,000 × *g* for 30 min. Prior to the incubation of synaptosomes with liposomes, the latter were centrifuged at the same speed and for the same time, and liposomes contained in the supernatant were used in aggregation experiments. Upon centrifugation, aggregated membranes pellet while nonaggregated material remains in the supernatant. Binding percentages were calculated from fluorescence values after addition of $C_{24}H_{50}O_7$, at a final concentration of 1 mM, both for the supernatant and the pellet (resuspended in 2 ml buffer). Fluorescence measurements were performed as previously described.

The time dependence of the aggregation process was analyzed by incubating synaptosomes with liposomes in the cold for different times. At 20 min incubation a saturation value was obtained and further incubation up to 80 min did not produce any increase in the percentage of aggregation. Preincubation of liposomes with synaptosomes in the cold did not exhibit any effect on the rate of fusion. We tested the possibility of occurrence of probe transfer in our system and confirmed that under aggregation conditions (incubation of PS liposomes with synaptosomes at 4°C) no increase of NDB fluorescence was observed. That under these conditions the pellet is essentially quenched supports the absence of probe transfer via a fusion-independent process and confirms the validity of the fusion assay.

Hydrophobicity Measurements

For hydrophobicity measurements, fluorescence emission scans of 1-anilinonaphthalene-8-sulfonate (ANS) were taken between 450–560 nm with the fixed excitation set at 350 nm using a SPEX Fluorolog fluorometer and a 46 μM ANS solution in buffer containing 150 mM NaCl and 10 mM HEPES, pH 7.4. All experiments were carried out at 37°C in a final volume of 2 ml. Following an initial control scan (ANS in solution only) 200 μg of synaptosomal protein (either peroxidized or nonperoxidized) was added to the cuvette and, after a 5 min incubation period, another emission scan was recorded. Similar experiments were also performed at pH 5.5, using an appropriate buffer containing 150 mM NaCl and 10 mM sodium acetate.

Increases in hydrophobicity were assessed by an increase in ANS fluorescence and a simultaneous blue shift in the emission maximum of the fluorescent probe (Stryer, 1968; Yoshimura, Maezawa & Hong, 1987). It should be noted that ANS fluorescence in solution is the same at pH 7.4 and 5.5 (*not shown*).

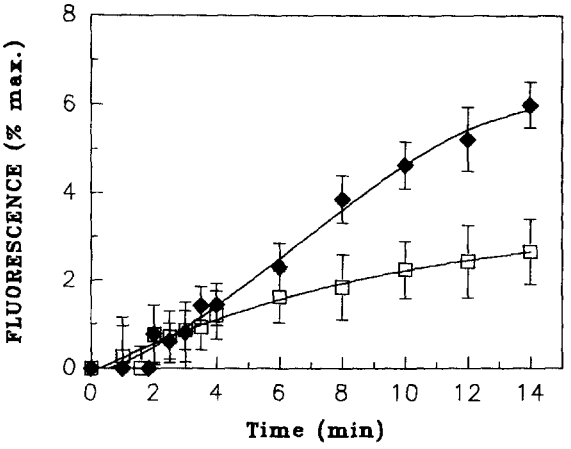

Fig. 1. Effect of lipid peroxidation on the fusion between *N*-NBD-PE/N-Rh-PE labeled liposomes and synaptosomes, at neutral pH. (♦) Peroxidized synaptosomes; (□) nonperoxidized synaptosomes. Fusion was monitored at 37°C in a final volume of 2 ml. Final liposome and synaptosome concentrations were 16.5 μM and 275 μg/ml, respectively. Lipid peroxidation produced 8.1 ± 3.6 nmol MDA/mg protein. Values represent means of four experiments ± SD.

Results and Discussion

Effect of Lipid Peroxidation on the Fusion Process

We have investigated the parameters affecting fusion between rat brain synaptosomes and phosphatidylserine liposomes as a model system for the exocytotic release of neurotransmitters. The synaptosomal preparation used in this work consists of a heterogeneous mixture of right-side-out and inside-out synaptosomes. In terms of physiological relevance, preparations containing only inside-out synaptosomes would be the appropriate model. Despite this, our results were very reproducible with different synaptosomal preparations, suggesting that the ratio of right-side-out to inside-out synaptosomes is kept constant.

Our studies show that in the model system used lipid peroxidation leads to an increase in the rate and extent of fusion (Fig. 1). This enhancing effect occurs both at neutral pH and mildly acidic conditions (*data not shown*).

Among the most widely described consequences of lipid peroxidation are the increase in the degree of order of membrane lipid acyl chains due to a decrease in the number of unsaturations of these chains (Gonçalves, Carvalho & Oliveira, 1991); a reduction in lateral and rotational mobility of membrane lipids and therefore a decrease in membrane fluidity (Richter, 1987; Palmeira &

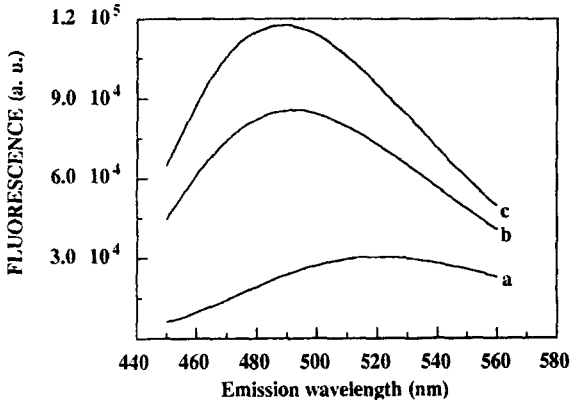

Fig. 2. Effect of peroxidized and nonperoxidized synaptosomes on ANS fluorescence. Synaptosomes (110 μg protein/ml) were added to buffer containing 46 μM ANS at pH 7.4 and at 37°C. After a 5 min incubation, fluorescence emission scans were taken between 450–560 nm, with the excitation set at 350 nm. (*b*) Nonperoxidized synaptosomes; (*c*) peroxidized synaptosomes. For comparison, an emission spectrum of ANS in the absence of any synaptosomal protein is also shown (*a*).

Oliveira, 1992). These changes would tend to render peroxidized synaptosomes less fusogenic since they would not favor the intermixing of synaptosomal and liposomal membrane lipids. Nevertheless, this membrane rigidification might result in drastic changes in the proteins embedded in the membrane, since peroxidation has been shown to induce structural changes in membrane proteins (Dean et al., 1991).

Experiments using the fluorescent probe ANS (Fig. 2) show that peroxidation apparently increases the hydrophobicity of the synaptosomal membrane, as assessed both by an increase in the fluorescence quantum yield of the probe and by a blue shift of the emission maximum (Stryer, 1968; Yoshimura et al., 1987). This increase in hydrophobicity could be related to the greater fusion activity observed with peroxidized synaptosomes.

Considering that hydrophobic interactions play an important role in the process of membrane aggregation (Wilschut, 1990), it is reasonable to suggest that the higher fusion ability of peroxidized synaptosomes is due to an enhancement of aggregation between the synaptosomal and liposomal membranes. The results obtained in binding studies are in agreement with this hypothesis, since an increase in the percentage of binding was observed for peroxidized synaptosomes, independently of medium pH (Table 1).

pH Dependence of the Fusion Reaction

Our results show that fusion is enhanced by low pH, both for peroxidized and nonperoxidized synaptosomes (Fig. 3). This indicates that in our model system nonspecific interactions may play a role in the fusion process.

Table 1. Effect of peroxidation and pH on the aggregation between synaptosomes and liposomes

	Binding (%)	
pH	Peroxidized	Nonperoxidized
7.4	60.57 ± 0.45	44.43 ± 6.88
5.5	81.51 ± 0.45	76.18 ± 1.49

Fluorescence was measured at 37°C in a final volume of 2 ml. Binding percentages were calculated from fluorescence values after addition of $C_{24}H_{50}O_7$, at a final concentration of 1 mM, both for the supernatant and the pellet (*see* Materials and Methods). Lipid peroxidation produced 8.1 ± 3.6 nmol MDA/mg protein. Values represent means of three experiments ± SD.

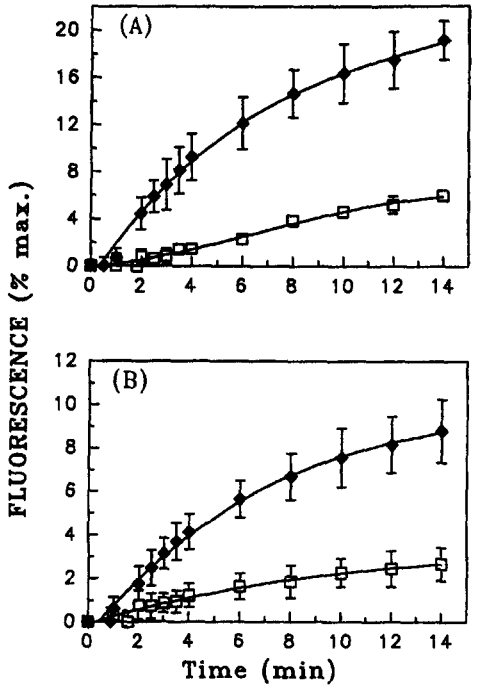

Fig. 3. pH dependence of fusion between liposomes and peroxidized (*A*) and nonperoxidized (*B*) synaptosomes. (◆) pH 5.5; (□) pH 7.4. Fluorescence was measured at 37°C in a final volume of 2 ml. Final liposome and synaptosome concentrations were 16.5 μM and 275 μg/ml, respectively. Lipid peroxidation produced 8.1 ± 3.6 nmol MDA/mg protein. Values represent means of four experiments ± SD.

It should also be noted that at pH 5.5 the hydrophobicity of both types of synaptosomes, measured by using ANS, greatly increases (*not shown*). This increase could explain that a much more extensive fusion activity in this system is observed under mildly acidic conditions.

The results obtained in binding experiments indicate that, independently of lipid peroxidation, mild acidic pH facilitates the binding step, (Table 1) allowing us to consider the possibility of a reduction in the repulsive electrostatic forces between the synaptosomal and liposomal membranes under these experimental conditions.

Table 2. Influence of varying the temperature on the extent of fusion between synaptosomes and liposomes

	Fusion (% max. fluorescence)	
Temperature	Peroxidized	Nonperoxidized
37°C	19.11 ± 1.62	8.78 ± 1.46
25°C	10.38 ± 0.93	3.72 ± 1.25

Values represent fusion extent after 14 min of interaction at pH 5.5. Fluorescence was measured at 37°C in a final volume of 2 ml. Final liposome and synaptosome concentrations were 16.5 μM and 275 μg/ml, respectively. Lipid peroxidation produced 8.1 ± 3.6 nmol MDA/mg protein. Values represent means of two experiments ± SD.

It should be stressed that the RET assay is not affected by pH changes (Eidelman et al., 1984).

The Influence of Temperature

The efficiency of the fusion reaction in this model system is higher at 37 than at 25°C. This effect can be seen both for peroxidized and nonperoxidized synaptosomes (Table 2).

The differences in rate and extent of fusion at the two temperatures studied (37 and 25°C) could be due to differences in the degree of membrane aggregation. However, it is unlikely that an enhancement in aggregation is responsible for the behavior of the system observed upon temperature increase, since we recorded fairly high percentages of binding when synaptosomes and liposomes were incubated on ice (0–4°C), indicating that aggregation cannot be substantially increased by an increase in temperature (*see* Table 1). Therefore, the inhibition of the fusion reaction by a decrease in temperature may correspond to events such as the reduction in lateral and rotational mobility of membrane lipids as well as that of proteins embedded in the membrane.

Effect of Trypsin Pretreatment of Synaptosomes

Our results show a significant inhibition of the interaction by trypsin pretreatment of synaptosomes (Fig. 4), indicating that the fusion reaction in this model system is a protein-mediated process. At neutral pH, there is almost a complete inhibition of the interaction between synaptosomes and liposomes following trypsin pretreatment. This finding shows that under these conditions fusion is strongly dependent on the presence of proteins in synaptosomal membranes. The inhibitory effect of trypsin at low pH (5.5) is not as strong as that at physiological pH. This suggests that, in addition to the role of proteins, nonspecific interactions between the synaptosomal and liposomal membranes, under acidic conditions, may also be involved in the fusion process (*as mentioned above*). Control experiments demonstrate that under trypsinization conditions (presence of a trypsin/trypsin-inhibitor complex and preincubation at 37°C for 30 min) the fusion reaction is not affected.

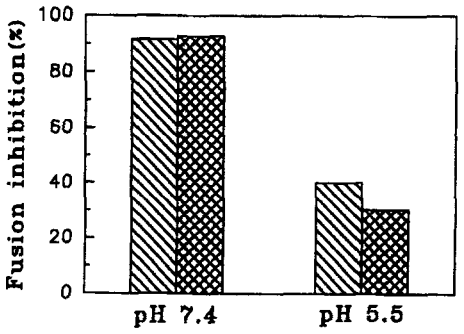

Fig. 4. Trypsin-induced inhibition of the fusion reaction. Percentages of inhibition were calculated from the extents of fusion, after 14 min of membrane interaction, for control and trypsin-treated synaptosomes (n = 3). Fluorescence was measured at 37°C in a final volume of 2 ml. Final liposome and synaptosome concentrations were 16.5 μM and 275 μg/ml, respectively. Lipid peroxidation produced 8.1 ± 3.6 nmol MDA/mg protein. (▨) Peroxidized; (▩) non-peroxidized.

Role of Proteins in the Aggregation Process

Viral fusion studies suggest that, in addition to its role in intermembrane specific recognition, proteins are also required for the subsequent steps of fusion, namely in the establishment of molecular contact between the membranes and in the removal of the hydration forces barrier (Stegmann, Doms & Helenius, 1989).

Our work provides conclusive evidence of an involvement of proteins in the aggregation process, since a significant reduction of binding percentages following trypsin pretreatment of synaptosomes is observed (Fig. 5). It should be emphasized, however, that our results do not rule out the possibility of the participation of proteins in subsequent steps of the fusion process.

At neutral pH, trypsin-induced inhibition of the aggregation process is considerably higher than at moderately acidic pH (Fig. 5), suggesting that, similarly to what occurs in fusion, nonspecific interactions may participate in the aggregation process under acidic conditions.

Another aspect worth mentioning is that trypsin pretreatment causes a higher binding inhibition in peroxidized synaptosomes than in nonperoxidized ones, independently of the medium pH (Fig. 5). This reveals that proteins play a more important role in the aggregation process involving peroxidized membranes than in that involving nonperoxidized membranes. It is interesting to note that these results contrast with those obtained in the fusion experiments (Fig. 4) where proteins seem to ex-

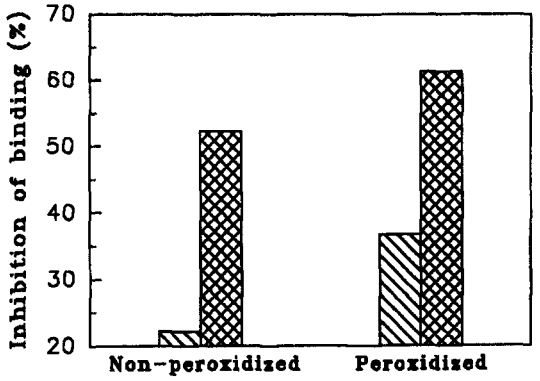

Fig. 5. Percentages of trypsin-induced inhibition of the aggregation between synaptosomes and liposomes. Binding percentages were calculated using the fluorescence values of the pellet after addition of detergent (*see* Materials and Methods), both for control and trypsin-treated synaptosomes ($n = 2$). Fusion was measured at 37°C in a final volume of 2 ml. Lipid peroxidation produced 8.1 ± 3.6 nmol MDA/mg protein. (▨) pH 5.5; (▩) pH 7.4.

hibit a similar role for both peroxidized and nonperoxidized synaptosomes.

In conclusion, although electrostatic and hydrophobic interactions are involved in fusion in this model system, proteins play an important role in both stages of the fusion process, even in peroxidized synaptosomes.

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References

Barttlet, G.R. 1959. Phosphorous assay in column chromatography. *J. Biol. Chem.* **234**:466–468

Ben-Shachar, D., Riederer, P., Youdim, M.B.H. 1991. Iron-melanin interaction and lipid peroxidation: implications for Parkinson's disease. *J. Neurochem.* **57**:1609–1614

Buege, J.A., Aust, S.D. 1978. Microsomal lipid peroxidation. *In:* Methods in Enzymology. Vol. **52.** S.P. Colowick and N.O. Kaplan, editors. pp. 302–310. Academic, New York

Dean, R.T., Hunt, J.V., Grant, A.J., Yamamoto, Y., Niki, E. 1991. Free radical damage to proteins: the influence of the relative localization of radical generation, antioxidants, and target proteins. *Free Rad. Biol. Med.* **11**:161–168

Düzgünes, N. 1985. Membrane fusion. *In:* Subcellular Biochemistry. Vol. 11. D.B. Roodyn, editor. pp. 195–286. Plenum, New York

Eidelman, O., Schlegel, R., Tralka, T.S. Blumenthal, R. 1984. pH-dependent fusion induced by Vesicular Stomatitis virus glycoprotein reconstituted into phospholipid vesicles. *J. Biol. Chem.* **259**:4622–4628

Gonçalves, T., Carvalho, A.P., Oliveira, C.R. 1991. Antioxidant effect of calcium antagonists on microsomal membranes isolated from different brain areas. *Eur. J. Pharmacol.* **204**:315–322

Hajós, F. 1975. An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res.* **93**:485–489

Halliwell, B., Gutteridge, J.M.C. 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **219**:1–14

Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. *In:* Methods in Enzymology. Vol. 3. S.P. Colowick and N.O. Kaplan, editors. pp. 447–454. Academic, New York.

Palmeira, C., Oliveira, C.R. 1992. Partitioning and membrane disordering effects of dopamine antagonists: Influence of lipid peroxidation, temperature, and drug concentration. *Arch. Biochem. Biophys.* **295**:161–171

Pedroso de Lima, M.C., Hoekstra, D. 1994. Liposomes, viruses, and membrane fusion. *In:* Liposomes as Tools in Basic Research and Industry. J.R. Philippot and F. Schuber, editors. pp. 137–156. CRC, London (*in press*)

Richter, C. 1987. Biophysical consequences of lipid peroxidation in membranes. *Chem. Phys. Lipids* **44**:175–189

Stegmann, T., Doms, R.W., Helenius, A. 1989. Protein-mediated membrane fusion. *Annu. Rev. Biophys. Biophys. Chem.* **18**:187–211

Struck, D.K., Hoekstra, D., Pagano, R.E. 1981. Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* **20**:4093–4099

Stryer, L. 1968. Fluorescence spectroscopy of proteins. *Science* **162**:526–533

Szoka, F., Papahadjopoulos, D. 1978. Procedure for the preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. USA* **75**:4194–4198

Viani, P., Cervato, G., Fiorilli, A., Cestaro, B. 1991. Age-related differences in synaptosomal peroxidative damage and membrane properties. *J. Neurochem.* **56**:253–258

Yoshimura, T., Maezawa, S., Hong, K. 1987. Exposure of hydrophobic domains of clathrin in its membrane fusion-inducible pH region. *J. Biochem.* **101**:1265–1272

Wills, E.D. 1969. Lipid peroxide formation in microsomes. General considerations. *Biochem. J.* **113**:315–324

Wilschut, J. 1990. Membrane fusion in lipid vesicle systems. *In:* Membrane Fusion: Mechanisms, Cell Biology and Applications in Biotechnology. J. Wilschut and D. Hoekstra, editors. pp. 89–125. Marcel Dekker, New York.

Zaleska, M.M., Floyd, R.A. 1985. Regional lipid peroxidation in rat brain in vitro: possible role of endogenous iron. *Neurochem. Res.* **10**:397–410