Influence of proteins on the reorganization of phospholipid bilayers into large domains

Doris M. Haverstick and Michael Glaser
Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

ABSTRACT Using large (5–10 μm) vesicles formed in the presence of phospholipids fluorescently labeled on the acyl chain and visualized using a fluorescence microscope, charge-coupled-device camera, and digital image processor, we examined the effects of membrane proteins on phospholipid domain formation. In vesicles composed of phosphatidic acid and phosphatidylcholine, incubation with cytochrome *c* induced the reorganization of phospholipids into large phosphatidic acid–enriched domains with the exclusion of phosphatidylcholine. Cytochrome *c* binding was demonstrated to be highest in the phosphatidic acid–enriched domain of the vesicle using the absorbance of the heme moiety for visualization. Both binding of cytochrome *c* and phospholipid reorganization were blocked by pretreatment of the vesicles with 0.1 M NaCl. The pore forming peptide gramicidin was examined for the effects of an integral protein on domain formation. Initially, gramicidin distributed randomly within the vesicle and showed no phospholipid specificity. Phosphatidic acid domain formation in the presence of 2.0 mM $CaCl_2$ or 100 μM cytochrome *c* was not affected by the presence of 5 mol % gramicidin within the vesicles. In both cases, gramicidin was preferentially excluded from the phosphatidic acid–enriched domain and became associated with phosphatidylcholine-enriched areas of the vesicle. Thus, cytochrome *c* caused a major reorganization of both the phospholipids and the proteins in the bilayer.

INTRODUCTION

The fluid mosaic model of membrane structure proposes that biological membranes are dynamic entities with proteins and lipids capable of lateral motion within the confines of the membrane bilayer (Singer, 1974). Free diffusion implies a random distribution of components in the membrane. Biophysical studies into protein-lipid interactions have been widespread in recent years and spectroscopic techniques have been particularly useful in examining such interactions (Devaux and Seigneuret, 1986). Most studies have examined only close interactions between proteins and lipids or the occurrence of phospholipid phase separations. In general, the lipids adjacent to membrane proteins, i.e., the boundary lipid or lipid annulus, are not immobile and exchange with the bulk lipids in the bilayer at an appreciable rate ($\sim 10^6$–10^7 s^{-1}) (Oldfield et al., 1978; Seelig and Seelig, 1978; Jost and Griffith, 1980; Smith and Oldfield, 1984). In view of this it might seem that proteins could not cause large-scale nonrandom distributions of phospholipids within membranes. Regardless of how they are formed, nonrandom distributions exist and may take the form of differences in lipid composition within various membranes of a single cell (Gilmore et al., 1979), asymmetric distribution of phospholipids across a given bilayer (Op den Kamp, 1979) or the organization of membranes into discrete lipid domains (Pessin and Glaser, 1980; Bearer and Friend, 1982; Shukla and Hanahan, 1982; Jain, 1983; Karnovsky et al., 1983; Thompson and Tillack, 1985).

The direct visualization of the interaction of acidic phospholipids and divalent cations has been described using fluorescence microscopy (Haverstick and Glaser, 1987). In phospholipid vesicles containing acidic phospholipids such as phosphatidic acid (PA)[1] or phosphatidylserine, incubation with 2.0 mM $CaCl_2$ induced the formation of acidic phospholipid–enriched domains within vesicle bilayers, while not disrupting the integrity of the vesicles. The interaction of calcium with acidic phospholipids in erythrocytes also can result in the formation of large domains. There has been a significant body of information generated suggesting that such cation-acidic phospholipid interactions may play a role in the early steps of membrane or bilayer fusion in such biological processes as exocytosis (Papahadjopoulos et al., 1977; Newton et al., 1978; Portis et al., 1979).

In the current study, the role that proteins might play in phospholipid domain formation was directly examined

Dr. Haverstick's current address is Sinai Hospital, Center for Cell Biology, 6767 West Outer Drive, Detroit, MI 48230.

[1] *Abbreviations used in this paper:* dansyl, *N*-(5-dimethylaminonaphthalene-1-sulfonyl)amino; DOPA, dioleoylphosphatidic acid; DOPC, dioleoylphosphatidylcholine; NBD, 1-acyl-2-[*N*-4-(nitrobenzo-2-oxa-1,3-diazole)amino-caproyl]; PA, phosphatidic acid; PC, phosphatidylcholine.

0006-3495/89/04/677/06 $2.00

using fluorescence microscopy. The role of a protein known to bind to acidic phospholipids was examined using the basic protein cytochrome *c*. The heme moiety allowed for the direct visualization of protein binding and the visualization of both protein and fluorescent phospholipids within a single vesicle. Thus it was possible to determine whether specific phospholipids were associated with cytochrome *c* or excluded from the immediate environment of the protein. For studies examining the effects of an integral membrane protein, the polypeptide ionophore gramicidin was used. This polypeptide shows no lipid specificity, inserts completely into phospholipid vesicles, and forms a dimer spanning the bilayer. With gramicidin incorporated into the vesicles, the effects of an integral membrane protein on domain formation induced by divalent cations and cytochrome *c* could be studied.

MATERIALS AND METHODS

Sources and preparation of phospholipids and fluorescent phospholipids were previously described (Haverstick and Glaser, 1987). Cytochrome *c* (horse heart) and gramicidin (*Bacillus brevis,* Dubos) were purchased from Sigma Chemical Co., St. Louis, MO.

Gramicidin was dansylated by the procedure of Veatch and Blout (1976). The protein (25 mg) was dissolved in 2 ml of acetone and mixed with 50 mg of dansyl chloride (Molecular Probes, Inc., Eugene, OR). After the addition of sodium bicarbonate to adjust the pH to 8.5, the mixture was stirred in the dark for 3 h. Gramicidin and dansyl-gramicidin were separated from free dansyl chloride by passing the reaction mixture over Sephadex LH-20 equilibrated in methanol. Product was characterized by tryptophan and dansyl absorbance and fluorescence spectra.

Vesicles for viewing with the fluorescence microscope and imaging system were prepared according to the methods of Darzon et al. (1980). Specifically, 250 nmol lipid were mixed in chloroform at the ratios indicated for the specific experiments listed below and taken to dryness under a stream of N_2 in a flat vessel. Deoxygenated water (500 μl) was carefully layered over the phospholipid film and the vessel was left undisturbed for 48 h. Vesicles for study were chosen randomly with only size and freedom from surrounding debris as criteria. Although there was some heterogeneity among the vesicles, the phenomena illustrated in the figures were representative of at least 90% of the randomly selected vesicles that were studied. Preparation of slides for viewing, image capture, and the pseudocolor program were as outlined previously (Haverstick and Glaser, 1987). All viewing images have had a pseudocolor program applied to them. In essence, the 256 gray tones viewed by the charge-coupled-device have been assigned colors by the computer ranging from blue (0) to green to yellow to red (256). For those images taken with transmitted light (Fig. 1), the pseudocolor program was not applied and the images are shown in gray tones.

RESULTS

Because cytochrome *c* is known to bind to acidic phospholipids, initial experiments were directed at determining if phospholipid domains would be formed in vesicles containing PA and if they would be of sufficient size to be visualized. Fig. 1 *A* shows a vesicle containing 1 mol % NBD-PA, 4 mol % DOPA, and 95 mol % DOPC under lighting conditions for viewing fluorescence (*left*) and transmitted light (*right*). Initially there was a uniform distribution of the fluorescently labeled phospholipid, whereas under transmitted light conditions the vesicle was invisible. After incubation with 100 μM cytochrome *c* for 30 min (Fig. 1 *B*), the fluorescent PA had migrated into two large patches within the vesicle (*left*). The domains were enriched two- to threefold in the concentration of PA. Precise quantitation of the PA concentration cannot be done due to the self-quenching of the fluorophore at high concentrations (Nichols and Pagano, 1981; Hoekstra, 1982) and other technical problems.[2] At the same time it was now possible to see the vesicle under conditions of transmitted light (Fig. 1 *B*, *right*) due to the binding of cytochrome *c* to the vesicle. It was evident that the areas of the vesicle that were enriched in PA were the same areas of the vesicle that had the highest amount of cytochrome *c* bound to them. These results show that cytochrome *c* was, in fact, responsible for the redistribution of the fluorescent PA into a large domain within the vesicle.

The size and pattern of domain formation in the presence of cytochrome *c* could be altered by the concentration of cytochrome *c* used. In the presence of 10 μM cytochrome *c*, smaller, multiple domains were formed (Fig. 1 *C*) suggesting that the lesser amount of cytochrome *c* did bind to the acidic phospholipids and cause domain formation. Vesicles treated with 10 μM cytochrome *c* were not visible under transmitted light, however, due to the lower concentration of the cytochrome.

The interactions of cytochrome *c* and acidic phospholipids are primarily electrostatic. Previous studies indicated that in an environment of increased ionic strength such as 0.1 M NaCl, cytochrome *c* did not bind to acidic phospholipids, and previously bound protein was partially displaced (Kimmelberg et al., 1970). Because calcium-induced domain formation is readily reversible by EGTA, an experiment was carried out to determine whether displacement of cytochrome *c* from the vesicle by NaCl would also cause the dissolution of the PA domain. Fig. 1 *D* indicates that the addition of 0.1 M NaCl to vesicles such as those in Fig. 1 *B*, did not affect the PA-enriched domain within the vesicle. At the same time, however, under conditions of transmitted light, the vesicle had become invisible once again, suggesting that a large

[2]At high NBD-PA concentrations, particularly those found in calcium-induced domains, the PA concentration can be estimated by looking at the nondomain region. That is, the amount of fluorescence in the nondomain region can be measured and subtracted from the total fluorescence before calcium was added. This gives the amount of fluorescence in the domain.

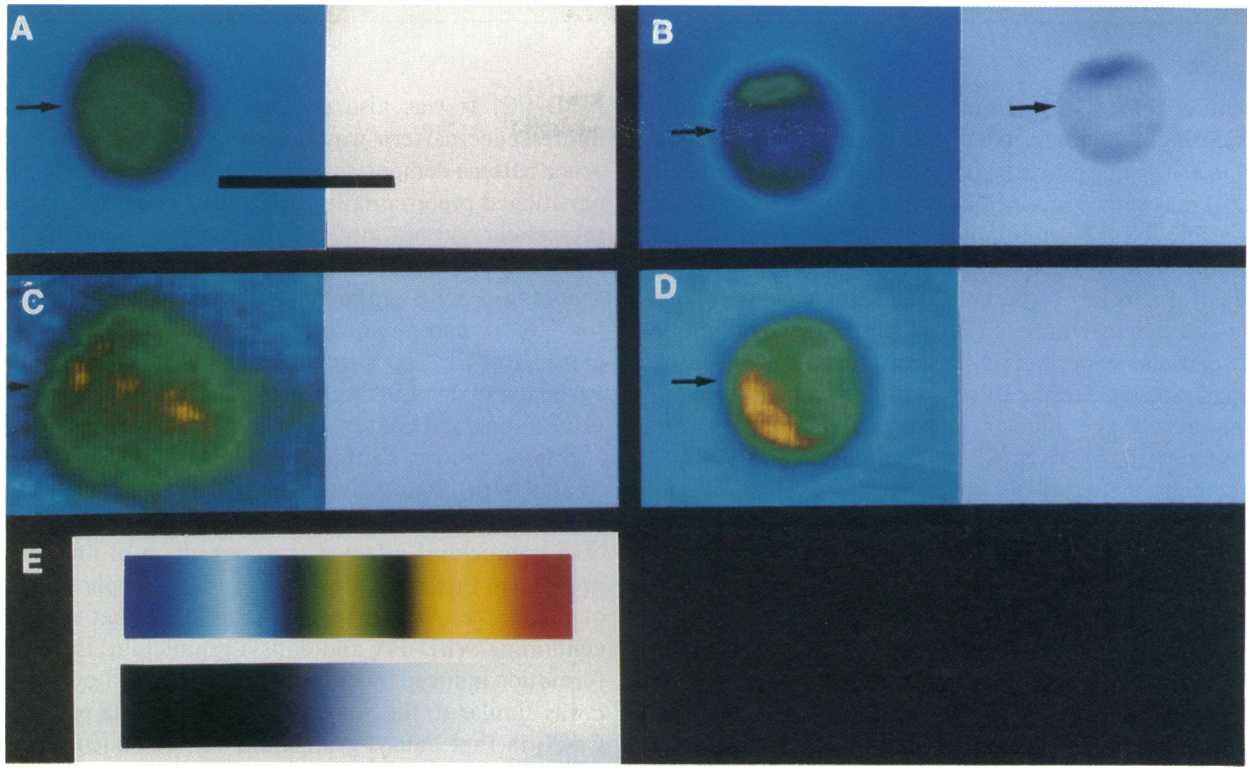

FIGURE 1 Cytochrome *c*-induced domain formation in phospholipid vesicles. Vesicles were formed containing 1 mol % NBD-PA, 4 mol % DOPA, and 95 mol % DOPC. For all panels, vesicles were viewed for NBD fluorescence (*left*) and for visibility under transmitted light conditions (*right*). Arrows indicate the edge of the vesicles. Bar indicates 10 μm. (*A*) A vesicle viewed immediately after the addition of 100 μM cytochrome *c*. (*B*) A similar vesicle after 30 min in the presence of 100 μM cytochrome *c*. (*C*) A vesicle as in *A* viewed after 30 min in the presence of 10 μM cytochrome *c*. (*D*) A vesicle as in *B* (domain formation induced by 100 μM cytochrome *c*), further incubated for 30 min with 0.1 M NaCl.

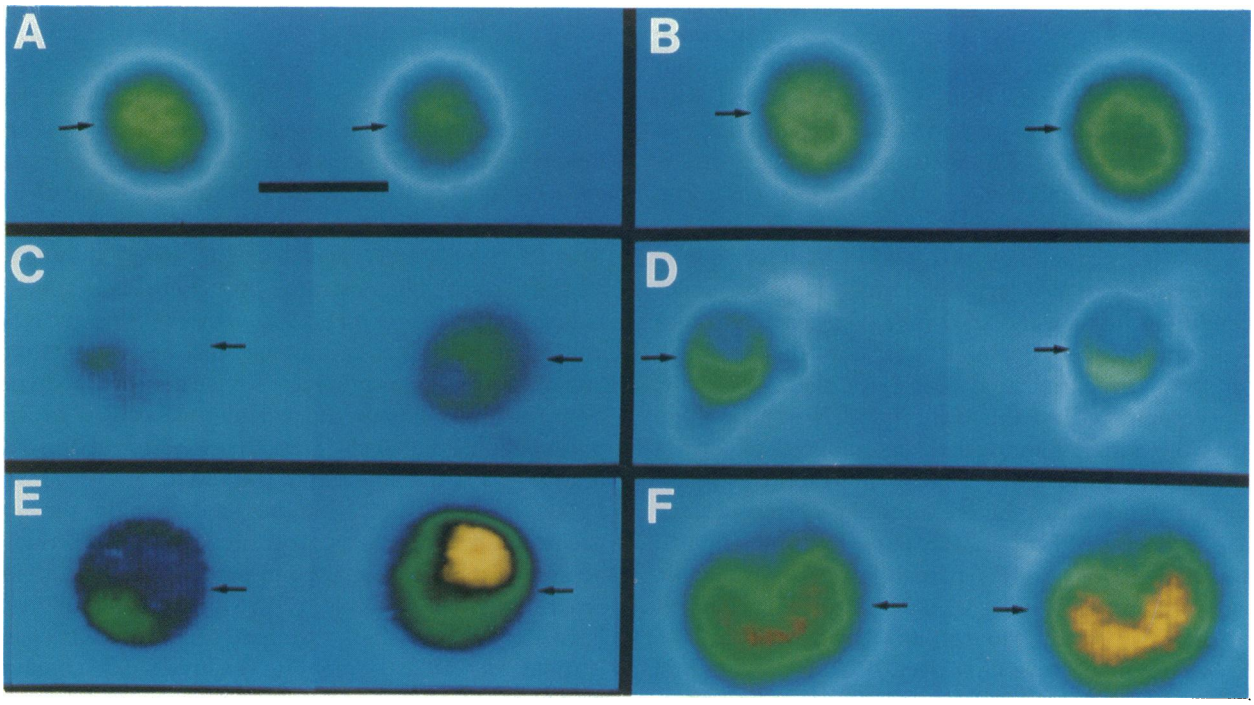

FIGURE 2 Effect of gramicidin on PA domain formation. For all panels, the vesicle was viewed for NBD fluorescence (*left*) and dansyl fluorescence (*right*). Arrows indicate the edge of each vesicle. Bar indicates 10 μm. (*A*) Vesicles containing 1 mol % NBD-PA, 4 mol % DOPA, 5 mol % dansyl-gramicidin, and 90 mol % DOPC were formed and viewed. (*B*) Vesicles containing 1 mol % NBD-PC, 89 mol % DOPC, 5 mol % dansyl-gramicidin, and 5 mol % DOPA were formed and viewed. (*C*) Vesicles were formed as in *A* and viewed after a 30-min incubation with 2.0 mM $CaCl_2$. (*D*) Vesicles were formed as in *B* and viewed after a 30-min incubation with 2.0 mM $CaCl_2$. (*E*) Vesicles were formed as in *A* and viewed after a 30-min incubation with 100 μM cytochrome *c*. (*F*) Vesicles were formed as in *B* and viewed after a 30-min incubation with 100 μM cytochrome *c*.

percentage of the cytochrome *c* had been displaced. Considering the results cited above using 10 μM cytochrome *c*, it seems probable that some of the cytochrome *c* (~10%) may have remained bound to the vesicle and been responsible for the retention of the domain even though the heme moiety of cytochrome *c* was not present in sufficient quantities to be visible. Higher concentrations of NaCl to remove more of the cytochrome *c* could not be used for these experiments because above 0.1 M NaCl vesicles implode, presumably due to unequal salt concentration across the bilayer. However, if vesicles were incubated with 0.1 M NaCl before addition of 10 or 100 μM cytochrome *c*, no phospholipid reorganization was seen, supporting the concept that the interaction of cytochrome *c* and acidic phospholipids is primarily electrostatic.

To investigate the role of an integral membrane protein on phospholipid domain formation, vesicles were made containing gramicidin, a polypeptide ionophore that completely inserts into a bilayer and forms a dimer (Veatch and Stryer, 1977), although it shows no phospholipid specificity. For these experiments, advantage was taken of vesicles formed using two different fluorescent compounds and the capacity to view the two fluorophores separately. Vesicles composed of 90 mol % DOPC, 1 mol % NBD-PA, 4 mol % DOPA, and 5 mol % dansyl-gramicidin were viewed at zero time and both fluorophores were uniformly and randomly distributed (Fig. 2 *A*). The concentrations of NBD-PA and dansyl-gramicidin were chosen so that they produced comparable fluorescence intensities. Random and uniform distribution of protein was also seen in vesicles formed with fluorescently labeled PC instead of fluorescently labeled PA, i.e., 1 mol % NBD-PC, 89 mol % DOPC, 5 mol % dansyl-gramicidin, and 5 mol % DOPA (Fig. 2 *B*). Fig. 2 *C* shows a vesicle containing fluorescent NBD-PA (as in Fig. 2 *A*) after incubation with 2.0 mM $CaCl_2$ for 30 min. The formation of a PA-enriched domain is visible under the conditions for viewing NBD (*left*). The enrichment in the calcium-induced domains was consistently much higher than the enrichment seen with the cytochrome *c*–induced domains. At the same time it was possible to see that the dansyl fluorescence was induced to form a complimentary pattern to the NBD fluorescence (*right*), suggesting that the two fluorophores had been separated from one another. That is, the dansyl-gramicidin had been excluded from the PA patch. That the gramicidin was now preferentially associated with PC rather than PA was demonstrated by the results shown in Fig. 2 *D*. Vesicles were made with fluorescent PC (as in Fig. 2 *B*) and treated with 2.0 mM $CaCl_2$ for 30 min. The formation of a PA-enriched domain was demonstrated by the lack of fluorescence in a small area of the vesicle, with the rest of the vesicle still visible due to the presence of NBD-PC. It was also possible to see that the dansyl fluorescence pattern was the same as the NBD fluorescence pattern demonstrating that the gramicidin has been partitioned preferentially within the PC-enriched area of the vesicle and not into the PA-enriched domain. These results also showed that the presence of the membrane spanning protein gramicidin did not affect the capacity for lateral phospholipid motion by PA or PC in the presence of Ca^{2+} because the size and intensity of both the PA and PC domains were consistent with earlier results (Haverstick and Glaser, 1987).

Similar vesicles to those used in Fig. 2, *C* and *D*, were treated with 100 μM cytochrome *c* to determine whether or not the presence of a protein within the bilayer (in this case gramicidin) affected the interaction of an extrinsic protein (in this case cytochrome *c*) and the phospholipids of the bilayer. It can be seen (Fig. 2 *E*) that in vesicles containing NBD-PA and dansyl-gramicidin, the domain formation induced by the presence of 100 μM cytochrome *c* was similar to the domain formation in the presence of $CaCl_2$ in that dansyl-gramicidin was excluded from the NBD-PA patch. When NBD-PC was used to form the vesicles, it was possible to see that PC and gramicidin were again sequestered into the same area of the vesicle (Fig. 2 *F*). Gramicidin was specifically excluded from the PA-enriched domain and became preferentially associated with the more fluid PC-enriched domain.

DISCUSSION

The ability of cytochrome *c* to bind to acidic phospholipids and to induce phase separations in acidic phospholipid containing vesicles is well documented (Gorrissen et al., 1986; de Kruijff and Cullis, 1980; Birrell and Griffith, 1976; Killian and de Kruijff, 1986; Mannella et al., 1987; Mustonen et al., 1987; Rietveld et al., 1986) although not all studies have demonstrated that phase separations are induced (Devaux et al., 1986). In the present report, advantage was taken of methods for the visualization of domain formation using fluorescence microscopy to demonstrate that not only did cytochrome *c* bind to acidic phospholipids, but such interactions led to the formation of a large PA domain within the vesicle bilayer (Fig. 1). In the present study, binding and domain formation occurred in the presence of similar concentrations of acidic phospholipids as used in previous studies on phase separations. For example, Caffrey and Feigenson (1984) have shown cation-induced phase separations at concentrations as low as 5 mol %. When vesicles containing phosphatidylserine as the acidic phospholipid were pretreated with 0.1 M NaCl before the addition of 100 μM cytochrome *c*, small domains enriched in phosphatidylserine were transiently formed before vesicle fusion (data not

shown). Consequently, the size and extent of domain formation depends on the cytochrome *c* concentration, ionic strength, and phospholipid composition. Gupte et al. (1984) concluded that mitochondrial electron transport, of which cytochrome *c* is a component, proceeds by a diffusion coupled, random-collision model. They suggest that under the conditions in mitochondria (~150 mM salt concentration and 100 μM cytochrome *c*), cytochrome *c* can diffuse laterally on the membrane surface and exchange with the surrounding aqueous phase. Mitochondria are enriched in acidic phospholipids, particularly cardiolipin and some phosphatidylserine, and, if the present results can be extrapolated, cytochrome *c* would induce the formation of large phospholipid domains under physiological conditions.

When vesicles were formed containing dansyl-gramicidin and either fluorescent PA or PC, both fluorophores were initially uniformly distributed. Domain formation was induced with either calcium or cytochrome *c* and it was seen (Fig. 2) that gramicidin was specifically excluded from the PA-enriched domain and became preferentially associated with the more fluid PC-enriched domain as had been suggested by earlier studies (Feigenson, 1983; London and Feigenson, 1981; Wang et al., 1987). In this case, the presence of protein within the vesicle had no effect on the calcium- or cytochrome *c*–induced domain formation. The net result of the formation of the PA-enriched domain, however, was the formation of an area of the vesicle that was enriched in both a specific phospholipid (PC) and the protein gramicidin.

Although cytochrome *c* is known to bind to acidic phospholipids and cause phase separations, there was no reason to expect that large domains enriched in acidic phospholipids would be formed. While this represents only one example, other proteins bind to acidic phospholipids and cause phase separations or show preferential lipid specificity (Devaux and Seigneuret, 1986). These proteins might also cause the formation of large domains of different phospholipid compositions as shown here. Such a sequestering of lipids by different proteins could explain the differences in lipid composition within various membranes of a single cell, the asymmetric distribution of phospholipids across a bilayer or the organization of lipids into domains within a membrane. In the case of budding of enveloped viruses through the plasma membrane of a cell, for example, if the viral proteins had different binding affinities for different phospholipids, it would account for the differences in phospholipid compositions found between the viruses and the plasma membrane (Pessin and Glaser, 1980).

The authors wish to thank Cynthia Sanders for the synthesis of dansyl-gramicidin and Jean Lewis for preparation of the manuscript.

This work was supported by National Institutes of Health grant GM 21953.

Received for publication 7 March 1988 and in final form 14 November 1988

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