Biochimica et Biophysica Acta, 551 (1979) 122—128

BBA 78273

A MONOLAYER STUDY OF THE REACTION OF TRINITROBENZENE SULPHONIC ACID WITH AMINO PHOSPHOLIPIDS

D.G. BISHOP *, E.M. BEVERS **, G. VAN MEER, J.A.F. OP DEN KAMP *** and L.L.M. VAN DEENEN

Department of Biochemistry, State University of Utrecht, Transitorium 3, Padualaan 8, De Uithof, Utrecht (The Netherlands)

(Received June 30th, 1978)

Key words: Trinitrobenzene sulfonic acid; Amino phospholipid; Phosphatidylethanolamine; (Monolayer)

Summary

The reaction of trinitrobenzene sulphonic acid with amino phospholipids, and in particular phosphatidylethanolamine has been studied by the monolayer technique. Injection of trinitrobenzene sulphonic acid under a monolayer of amino phospholipid results in an increase in surface pressure. The rate and extent of the pressure change is greatly affected by the initial surface pressure, the fatty acid composition of the lipid, and the presence of other non-reactive lipids, especially negatively charged phospholipids.

The extent of the reaction was measured with ^{32}P-labelled phospholipids isolated from *Bacillus subtilis*. Only about 80% of the phosphatidylethanolamine in the monolayer could be converted to its trinitrophenyl derivative. In the presence of negatively charged phospholipids such as cardiolipin or phosphatidylglycerol, a further 20% decrease in the trinitrophenylation of phosphatidylethanolamine was found. The pressure increase occurring during trinitrophenylation could also be correlated with the extent of the reaction by comparison of the force-area curves of pure phosphatidylethanolamine, its trinitrophenyl derivative and mixtures of both compounds.

The data may offer an explanation for the observation that incomplete labelling of amino phospholipids frequently occurs in natural membranes and furthermore indicate that the use of chemical labelling techniques in the study of lipid asymmetry in biological membranes must be approached with great caution.

* Permanent address: Plant Physiology Unit, CSIRO Division of Food Research and School of Biological Sciences, Macquarie University, North Ryde, 2113, Sydney, Australia.

** Present address: Medical Faculty Maastricht, Department of Biochemistry, Biochemical Center, Beeldsnijdersdreef 101, Maastricht, The Netherlands.

*** To whom correspondence should be sent.

Introduction

The concept of an asymmetric distribution of lipids in biological membranes is now well established, and methods employed to assess asymmetry have included the use of specific lipid-hydrolyzing enzymes and chemical reaction with specific group reagents [1,2]. Trinitrobenzene sulphonic acid is a commonly employed reagent for localization of amino groups and has been used to localize amino phospholipids in membranes of erythrocytes [3—7], sarcoplasmic reticulum [8], bacteria [4,7,9—12] and liposomes [13,14]. When such chemical labelling reagents are employed to measure lipid asymmetry, it is necessary to establish that complete reaction of the lipid with the reagent occurs under conditions where both sides of the membrane are accessible to the reagent. Such data have not always been provided, and we have recently reported that under conditions where both sides of the cytoplasmic membrane of *Bacillus subtilis* were exposed to trinitrobenzene sulphonic acid, only 60—70% of the amino phospholipids present in the membrane reacted with the reagent [11]. Under the same conditions, complete hydrolysis of the amino phospholipids by phospholipase C could be rapidly obtained. It was concluded that the introduction of the bulky trinitrophenyl group into both the proteins and lipids of the membrane during labelling with trinitrobenzene sulphonic acid, and the concomitant change in charge of the membrane components, precluded the reaction proceeding to completion.

This communication describes the reaction of trinitrobenzene sulphonic acid with phospholipid monolayers. The results demonstrate that the reaction can give rise to a significant increase in the surface pressure of the monolayer, and that it does not proceed to completion.

Materials and Methods

Monolayer measurements were performed at 24°C in a teflon trough with 50 mM potassium phosphate buffer, pH 8.4. Monomolecular films were spread from a 1% solution of the lipid in chloroform, and a 0.25 M solution of trinitrobenzene sulphonic acid was injected under the monolayer to give a final concentration in the subphase of approx. 2 mM. Surface pressure increases were measured with a Wilhelmy type surface balance. Isotherms of pure phosphatidylethanolamine, its trinitrophenyl derivative and mixtures of both compounds (cf. Fig. 3) were recorded during continuous compression of the monolayer which was formed on an initial surface of 553.8 cm^2. The compression rate was 79.3 cm^2/min. For an extensive description of the techniques involved see ref. 15. Phosphatidylethanolamine, lysyl phosphatidylglycerol, cardiolipin and phosphatidylglycerol were isolated by CM-cellulose chromatography [16] and preparative thin-layer chromatography from the lipids of *B. subtilis* grown in glucose-containing medium (if necessary in the presence of [^{32}P]phosphate [11]). Trinitrophenyl phosphatidylethanolamine was prepared by mixing the phospholipid with a large excess of trinitrobenzene sulphonic acid in chloroform and allowing the reaction to proceed overnight at room temperature. Other, highly purified lipids were provided by Drs. van Dijck and van Zoelen.

In order to measure the extent of labelling, monolayers were collected after

the observed pressure change had ceased and the reaction had been stopped by the addition of 500 μl 2 M HCl to the subphase. The lipids were extracted and separated by thin-layer chromatography [11]. Spots were visualized by iodine vapour and autoradiography, and the radioactivity was measured by scintillation counting.

Results and Discussion

The pressure changes observed when monolayers of dioleoyl phosphatidylethanolamine react with trinitrobenzene sulphonic acid are shown in Fig. 1. Over the range of initial surface pressures tested, from 8 to 36 dynes/cm, a substantial rise in pressure was observed immediately upon addition of trinitrobenzene sulphonic acid to the subphase. The greatest pressure increase occurs when the initial pressure of the monolayer was in the range 15—25 dynes/cm, an increase of 8—10 dynes being obtained within 15 min after the addition of the reagent, and subsequent experiments were therefore carried out with monolayers spread at an initial pressure of 25 dynes/cm.

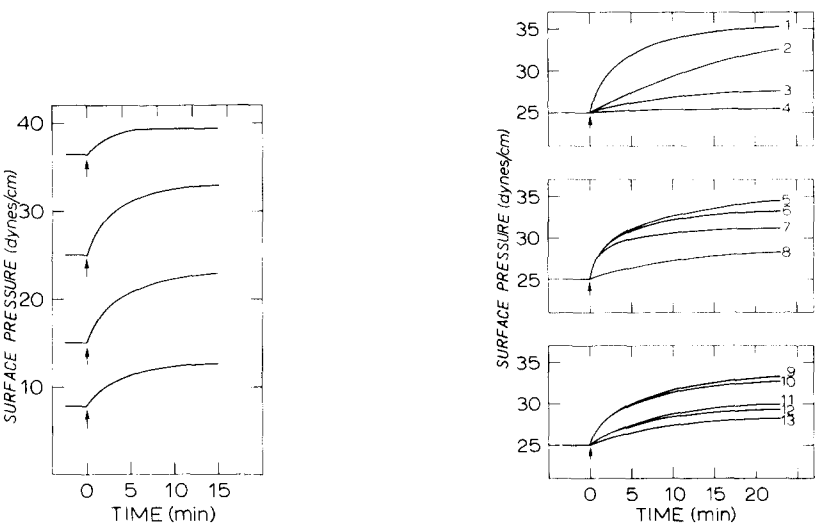

Fig. 1. Increase in surface pressure of a dioleoyl phosphatidylethanolamine monolayer by reaction with trinitrobenzene sulphonic acid. Monolayers of dioleoyl phosphatidylethanolamine were spread at different initial surface pressures and trinitrobenzene sulphonic acid solution was injected under the monolayer at zero time to give a final concentration of 2 mM.

Fig. 2. Effect of trinitrobenzene sulphonic acid on the surface pressure of phospholipid monolayers. All monolayers were spread at an initial surface pressure of 25 dynes/cm. The curves show pressure increases which are obtained by injection of trinitrobenzene sulphonic acid into the subphase. The components are: 1, dioleoyl phosphatidylethanolamine; 2, dipalmitoyl phosphatidylethanolamine (a similar curve was obtained with phosphatidylserine from beef brain); 3, dimyristoyl phosphatidylserine; 4, *B. subtilis* phosphatidylglycerol; 5, dioleoyl phosphatidylethanolamine; 6, dioleoyl phosphatidylethanolamine + dioleoyl phosphatidylcholine (4 : 1); 7, as in 6 but at a ratio 1 : 1; 8, dioleoyl phosphatidylethanolamine + cardiolipid from beef heart (4 : 1); 9, *B. subtilis* phosphatidylethanolamine; 10, *B. subtilis* lysylphosphatidylglycerol; 11, phosphatidylethanolamine + cardiolipin both from *B. subtilis*; 12, phosphatidylethanolamine + phosphatidylglycerol both from *B. subtilis* (4 : 1); 13, total lipid extract from *B. subtilis* (for composition see Table I).

The effect of trinitrobenzene sulphonic acid on monolayers of various phospholipids and phospholipid mixtures is shown in Fig. 2. Monolayers of phosphatidylethanolamine, phosphatidylserine, and lysyl phosphatidylglycerol show a pressure increase when trinitrobenzene sulphonic acid is added to the subphase. The maximum pressure increase as well as the rate at which this value is obtained differ greatly. The influence of the fatty acid composition of the phospholipids is shown by the fact that dioleoyl phosphatidylethanolamine shows a much higher increase in surface pressure than dipalmitoyl phosphatidylethanolamine or phosphatidylethanolamine isolated from *B. subtilis* which contains mainly branched chain fatty acids [11] (Fig. 2A). This difference is no doubt due to the physical state of the molecules in the monolayer, dioleoyl phosphatidylethanolamine being in the liquid crystalline state (transition temperature -10°C) whereas the dipalmitoyl derivative is in the gel state (transition temperature 60°C) [17]. A similar result was obtained with the two phosphatidylserines, the reaction occurring with the saturated compound showing a smaller increase in surface pressure than that with the unsaturated compound. No pressure increase was observed in monolayers of phospholipid which lack amino groups, such as phosphatidylglycerol (Fig. 2A), cardiolipin and phosphatidylcholine.

The data in Fig. 2 also indicate that phosphatidylserine reacts at a slower rate than phosphatidylethanolamine, whereas lysyl phosphatidylglycerol reacts with a similar rate. This might indicate that the charge of the lipids in the monolayer is another rate-determining factor in the reactivity with trinitrobenzene sulphonic acid. The effect of the charge of the polar headgroup was clearly demonstrated by mixing phosphatidylethanolamine with various neutral and negatively charged phospholipids (Fig. 2). The presence of 20 mol% of phosphatidylcholine in the phosphatidylethanolamine monolayer had little effect on the rate and extent of pressure increase (Fig. 2B) whereas cardiolipin or phosphatidylglycerol decreased the rate and resulted in a very limited pressure increase (Figs. 2B and 2C). The isolated phospholipid fraction of *B. subtilis*, which contains substantial amounts of cardiolipin and phosphatidylglycerol [11], also showed only a small pressure increase.

It is apparent therefore that the rate and extent of the observed pressure increase are influenced by several factors such as: the initial surface pressure; the fatty acid composition of the lipids and the surface charge of the monolayer. We have used two different approaches to determine whether these factors also affect the extent of the labelling reaction.

In the first approach we have measured the force-area curves of egg-yolk phosphatidylethanolamine, its trinitrophenyl derivative and a number of mixtures of these two compounds (Fig. 3). From this data we have obtained a relation between the surface pressure increase and extent of labelling. As the initial trinitrophenylation experiments shown in Figs. 1 and 2 were carried out at a fixed surface area, the average area occupied by each molecule did not change during the course of the reaction. In the experiment shown in Fig. 3, however, each monolayer contains the same total number of molecules, but in different proportions, and the average area per molecule has been varied. The surface pressure corresponding to each area can be therefore measured. For example at 15 dynes/cm, pure phosphatidylethanolamine occupies an area of

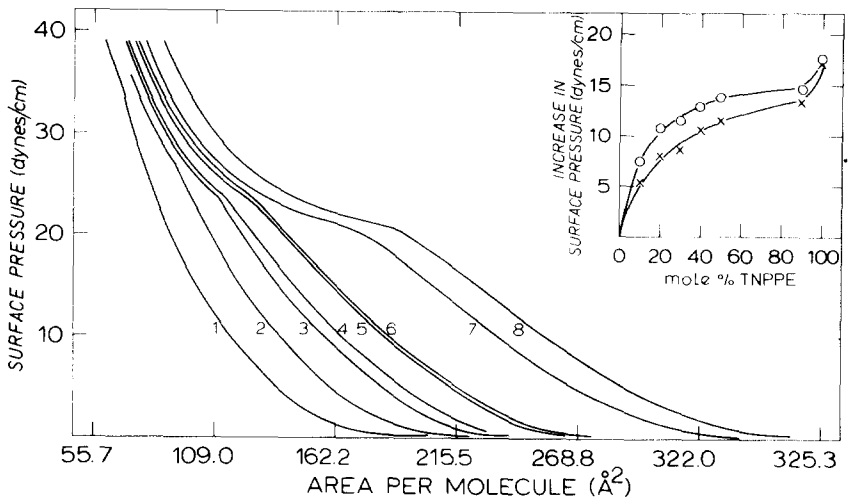

Fig. 3. Force area curves of phosphatidylethanolamine, its trinitrophenyl derivative and mixtures of both compounds. Force area curves are shown of the pure phosphatidylethanolamine from egg yolk (1), pure trinitrophenyl phosphatidylethanolamine (8) and mixtures containing 10 (2); 20 (3); 30 (4); 40 (5); 50 (6) and 90 (7) mol% of the trinitrophenyl derivative. The insert shows the correlation between the amount of trinitrophenyl phosphatidylethanolamine (TNPPE) present and the increase in surface pressure when compared with pure phosphatidylethanolamine, at a surface pressure of 15 dynes/cm (○———○) and 25 dynes/cm (X———X).

100 Å²/mol, and the surface pressure created by the same number of molecules in varying mixtures of phosphatidylethanolamine and trinitrophenyl phosphatidylethanolamine occupying the same surface area can be measured from the other curves in Fig. 3. The increases in pressure due to the presence of trinitrophenyl phosphatidylethanolamine molecules are plotted in the insert to Fig. 3, for the two cases where the surface pressure of pure phosphatidylethanolamine monolayers was 15 and 25 dynes/cm. It is clear that the correlation between pressure increase and extent of labelling is not linear and is dependent on the initial surface pressure. This result also implies that the correlation is valid only for the specific type of phosphatidylethanolamine used in this experiment and that any extrapolation of this data to other types of phosphatidylethanolamines (or mixtures) is not possible.

An interesting phenomenon is observed in the force-area curve of pure trinitrophenyl phosphatidylethanolamine (Fig. 3) which shows a plateau, thus suggesting that the lipid is undergoing a lipid-phase transition. The occurrence of a gel to liquid crystalline phase transition is excluded, however, by the observations that the force-area curve was nearly identical at 24 and 37°C, and that differential scanning calorimetry of the trinitrophenyl phosphatidylethanolamine showed a lipid-phase transition below 0°C. An alternative explanation could be that the plateau in the force-area curve is due to another type of lipid phase transition caused by an alteration in the orientation of the bulky polar headgroup. At low pressures the trinitrophenyl group might be oriented parallel to the water surface whereas a compression of the film could force the group to become oriented perpendicular to the surface.

The second approach to determine the extent of the trinitrophenylation

reaction utilized the more direct procedure of chemical analysis by thin-layer chromatography. Monolayers of ^{32}P-labelled phospholipids isolated from *B. subtilis* were prepared at a pressure of 25 dynes/cm. After the completion of the pressure increase due to trinitrophenylation, the reaction was terminated by addition of HCl to the subphase and the monolayer was collected. The lipids were extracted, separated by thin-layer chromatography and radioactivity determined. The data obtained by this procedure showed that a maximum of 80% of pure phosphatidylethanolamine in a monolayer could be trinitrophenylated. The incorporation of 20 mol% phosphatidylglycerol or cardiolipin to the monolayer resulted in a further decrease of about 20% in the labelling of phosphatidylethanolamine. In a monolayer containing a total lipid extract of *B. subtilis* it was found that both phosphatidylethanolamine and lysyl phosphatidylglycerol were incompletely trinitrophenylated (Table I).

The above data obtained with the monolayer technique may offer an explanation for the incomplete labelling of phosphatidylethanolamine [8,11] and phosphatidylserine [3,5] in some natural membranes. A high surface pressure as well as a negative surface charge clearly inhibit the reaction and it is obvious that both parameters become more effective as more amino phospholipids are converted into the trinitrophenyl derivatives. The data presented here may also have implications for the interpretation of data obtained when biological membranes are labelled with trinitrobenzene sulphonic acid. Different rates of trinitrobenzene sulphonic acid labelling can be the consequence of local differences in packing of the amino phospholipids in the membrane. It has been recently shown in this laboratory (Demant, E.J.F., unpublished) that whereas phosphatidylethanolamine in isolated membranes of *Bacillus megaterium* is completely accessible to trinitrobenzene sulphonic acid at 37°C, the accessibility is reduced at temperatures below the onset of the liquid crystalline to gel phase transition in these membranes. Trinitrobenzene sulphonic acid also reacts readily with protein amino groups and such a reaction with membrane proteins could conceivably affect the extent of the reaction with membrane lipids, due to the introduction of further bulky trinitrophenyl groups and consequent change in charge near the membrane surface.

TABLE I

REACTION OF TRINITROBENZENE SULPHONIC ACID WITH A TOTAL LIPID EXTRACT OF *B. SUBTILIS* SPREAD ON A MONOLAYER

Data are given as percentage of total ^{32}P-labelled phospholipid.

	Lipid composition	
	Initial	Final
Cardiolipin	27	30
Trinitrophenyl phospholipid	—	30
Phosphatidylethanolamine	41	16
Phosphatidylglycerol	15	14
Lysyl phosphatidylglycerol	15	6
Unknown	2	4

Acknowledgements

We thank Drs. van Dijck and van Zoelen for their gift of purified phospholipids and carrying out the differential scanning calorimetry measurements and Dr. Demel for his assistance with the monolayer technique. The present investigation was carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial support from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). D.G.B. was on leave from the Division of Food Research, C.S.I.R.O., Sydney, Australia.

References

1 Rothman, J.E. and Lenard, J. (1977) Science 195, 743—753
2 Bergelson, L.D. and Barsukov, L.I. (1977) Science 197, 224—230
3 Gordesky, S.E. and Marinetti, G.V. (1973) Biochem. Biophys. Res. Commun. 50, 1027—1031
4 Haest, C.W.M. and Deuticke, B. (1975) Biochim. Biophys. Acta 401, 468—480
5 Marinetti, G.V. and Love, R. (1976) Chem. Phys. Lipids 16, 239—254
6 Gordesky, S.E., Marinetti, G.V. and Love, R. (1975) J. Membrane Biol. 20, 111—132
7 Marinetti, G.V. and Love, R. (1977) Chem. Phys. Lipids 18, 170—180
8 Vale, M.G.P. (1977) Biochim. Biophys. Acta 471, 39—48
9 Shimada, K. and Murata, N. (1976) Biochim. Biophys. Acta 455, 605—620
10 Rothman, J.E. and Kennedy, E.P. (1977) J. Mol. Biol. 110, 603—618
11 Bishop, D.G., Op den Kamp, J.A.F. and van Deenen, L.L.M. (1977) Eur. J. Biochem. 80, 381—391
12 Litman, B.J. (1974) Biochemistry 13, 2844—2848
13 Roseman, M., Litman, B.J. and Thompson, T.E. (1975) Biochemistry 14, 4826—4830
14 Paton, J.C., May, B.K. and Elliot, W.H. (1978) J. Bacteriol. 135, 393—401
15 Demel, R.A. (1974) in Methods in Enzymology, Vol. 32, pp. 539—545, Academic Press, New York
16 Comfurius, P. and Zwaal, R.F.A. (1977) Biochim. Biophys. Acta 488, 36—42
17 van Dijck, P.W.M., de Kruijff, B., van Deenen, L.L.M., de Gier, J. and Demel, R.A. (1976) Biochim. Biophys. Acta 455, 576—587