BBA 75013

MONOLAYER INTERACTIONS OF PHOSPHOLIPIDS AND CHOLESTEROL

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(Received August 11th, 1966)

SUMMARY

1. The force–area characteristics of monolayers of saturated and unsaturated phospholipids have been studied.

2. Interaction in phospholipid monolayers depends on the nature of the fatty acid constituents of the phospholipid, and on the temperature.

3. The mean area per molecule in mixed films of cholesterol with (1,2-distearoyl)-3-lecithin and (1,2-didecanoyl)-3-lecithin at 22° practically followed the simple additivity rule.

4. A condensing effect of cholesterol was evident with films of (1-stearoyl-2-lauroyl)-3-lecithin; (1,2-ditetradecanoyl)-3-lecithin, (1-stearoyl-2-oleoyl)-3-lecithin and the corresponding ethanolamine analogue, as well as with (1,2-dioleoyl)-3-lecithin at 22°. At 5° the condensation effect with (1,2-ditetradecanoyl)-3-lecithin was much reduced.

5. The very expanded films of synthetic lecithins and phosphatidyl ethanolamines containing linoleic and linolenic acid showed no appreciable condensation effects with cholesterol.

6. The behaviour of the mixed cholesterol–phospholipid films is governed by a number of factors, including Van der Waals' interactions, configurational entropy effects and alterations in the structure of water adjacent to the monolayers. These factors depend on chain length and degree of unsaturation.

INTRODUCTION

It is well known that the physical properties of monomolecular layers formed by mixed lipids may be quite different from the films of the single lipid components. In this way the interactions between several classes, *e.g.* fatty acids and triglycerides, fatty acids and cholesterol, and glycerides and phospholipids have been established by DERVICHIAN AND DE BERNARD[1], ADAM AND JESSOP[2], and DESNUELLE, MOLINES AND DERVICHIAN[3], respectively. As regards the influence of cholesterol on the behaviour of lecithin in biological systems, LEATHES[4] showed that the presence of cholesterol, which is assumed to have a practically invariant molecular cross section, causes a

decrease of the apparent area occupied by lecithin molecules. The study of this so-called condensing effect of cholesterol was extended to mixtures of egg lecithin and cholesterol by DE BERNARD[5], who determined the variation of the mean molecular area at constant surface pressure as a function of the mole ratios of lecithin and cholesterol in mixed monolayers at the air–water interface. Because of the abundance of cholesterol in a number of cell membranes this phenomenon was considered to have biological relevance. A natural phospholipid such as egg lecithin is known to consist of a great number of molecular species differing with regard to the composition of their fatty acid constituents. Some preliminary experiments on synthetic substances[6] indicated that the magnitude of the reduction of cross-sectional area by the presence of cholesterol in the phospholipid film may differ significantly among different species. In the present study, these experiments were extended to a number of synthetic phosphoglycerides containing a great variety of fatty acid constituents.

EXPERIMENTAL

Materials

The phospholipids were synthesized by Dr. G. H. DE HAAS and co-workers by methods described previously[7–9]. The purity of the compounds was verified by thin-layer chromatography with diisobutyl ketone–acetic acid–water (40:25:5, v/v/v) and chloroform–methanol–water (65:35:4, v/v/v) as developers. Lipid extracts from tissue were prepared according to a minor modification of the procedure of BLIGH AND DYER[10]. Separation of these extracts into different phospholipid fractions was performed by column chromatography on silicic acid according to the procedure of HANAHAN[11]. Cholesterol was purchased from Fluka, A.G., Switzerland. The sample showed no impurities in thin-layer chromatography.

Determination of force–area curves

Force–area measurements were performed at the air–water interface with a conventional Langmuir–Adam surface balance, using a paraffin-coated glass trough. The trough was filled with salt-free unbuffered water that had been distilled from alkaline permanganate and then redistilled in a quartz still. The pH of this water in equilibrium with laboratory air was about 5.4. The aqueous surface was swept clean with a teflon bar. Most experiments were performed at room temperature (22°) but a number of experiments with phospholipids containing polyunsaturated fatty acid constituents were carried out at 7° and 37°, under anaerobic conditions. Further experiments were made at 5° with (1,2-ditetradecanoyl)-3-lecithin and mixtures with cholesterol.

Known amounts of the pure lipids in solvent were delivered on to the surface with an Agla microsyringe, and the solvent was allowed to evaporate for 3 min before compressing the spread films. Chloroform was used as a solvent for all the lipids except (1,2-dipalmitoyl)-3-phosphatidyl ethanolamine, for which 30% ethanol in chloroform was used. Some of the results shown in the figures were reproduced satisfactorily using petroleum ether as solvent. The time required to carry out a complete force–area curve was 10–15 min, and oxidation of the lipids at the interface was reduced by enclosing the trough in a N_2-filled box. For mixed films, the surface pressure was plotted against the mean area per molecule, by which is understood the total area divided by

the total number of cholesterol and phospholipid molecules at the air–water interface. For a quantitative evaluation of the effect of cholesterol, the variation of the mean molecular area at a given constant surface pressure was plotted as a function of the mole fraction. In the figures the mean molecular areas are shown at a pressure of 12 dyne/cm. In general, the same qualitative form for the curves was found at other surface pressures at the same temperature, although, of course, some quantitative differences appear. In particular, where the condensation effect occurs, the fractional condensation for a given mixture diminishes as the surface pressure increases.

RESULTS

Phospholipid monolayers

The force–area curves of synthetic phosphoglycerides containing different combinations of saturated and unsaturated fatty acids are compiled in Figs. 1–3. According to expectation, the force–area characteristics of phosphoglycerides containing

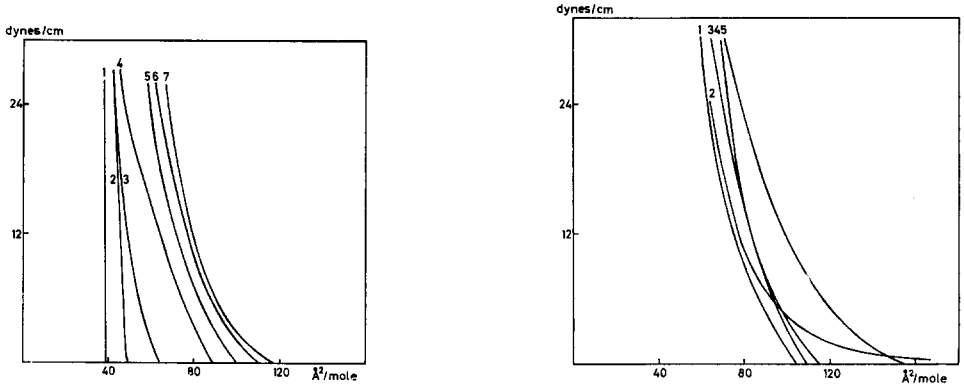


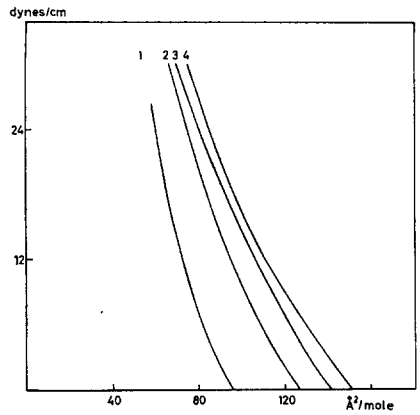
Fig. 1. Force–area characteristics of various saturated phospholipids and cholesterol at 22° (except Curve 4, which is at 5°). 1, cholesterol; 2, (1,2-dipalmitoyl)-3-phosphatidyl ethanolamine; 3, (1,2-distearoyl)-3-lecithin; 4, (1,2-ditetradecanoyl)-3-lecithin at 5°; 5, (1,2-ditetradecanoyl)-3-lecithin at 22°; 6, (1-stearoyl-2-lauroyl)-3-lecithin; 7, (1,2-didecanoyl)-3-lecithin.

Fig. 2. Force–area characteristics of various unsaturated lecithins. 1, (1-stearoyl-2-oleoyl)-3-lecithin; 2, (1-butylryl-2-oleoyl)-3-lecithin; 3, (1-linoleoyl-2-stearoyl)-3-lecithin; 4, (1,2-dioleoyl)-3-lecithin; 5, (1,2-dilinoleoyl)-3-lecithin.

two saturated fatty acids exhibit a shift from a condensed to an expanded type of film with decrease of fatty acid chain length. The same is true when the number of unsaturated bonds in the paraffinic chains increases. Some of these results were recorded in an earlier publication[6]. The monolayer packing shown is in fair agreement with the earlier work. However, some differences are to be noted, which may be due to improvements in the purity of the water utilised for the sub-phase.

Mixed films of cholesterol and phospholipids

In order to facilitate a comparison between our results on the monolayers of individual phosphoglycerides with the observations obtained by DE BERNARD, the results of this investigator on egg lecithin are reproduced in Fig. 4. The curve shows



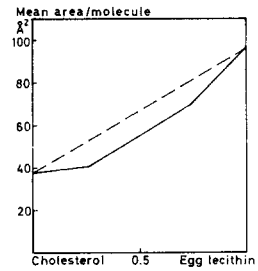
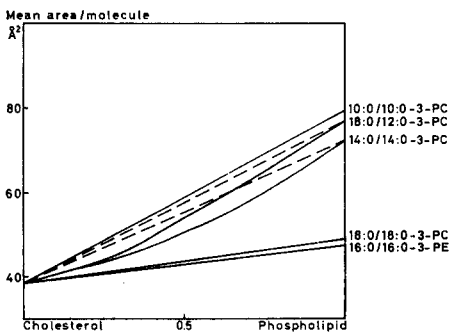


Fig. 3. Force–area characteristics of various unsaturated phosphatidyl ethanolamines. 1, (1-stearoyl-2-oleoyl)-3-phosphatidyl ethanolamine; 2, (1-palmitoyl-2-linoleoyl)-3-phosphatidyl ethanolamine; 3, (1-palmitoyl-2-linolenoyl)-3-phosphatidyl ethanolamine; 4, (1-linolenoyl-2-palmitoyl)-3-phosphatidyl ethanolamine.

Fig. 4. Variations of the mean molecular area, as a function of the composition, for mixed monolayers of cholesterol and egg lecithin as reproduced from the paper of DE BERNARD[5]. The straight dotted line represents the simple additivity rule of the molecular areas of cholesterol and egg lecithin.

that the mean molecular area of the lipid molecules in the mixed films falls below the proportionate average of the areas in the pure films (condensation). The curve shows two breaks at mixtures corresponding to cholesterol:egg lecithin ratios of 3:1 and 1:3, respectively. The area of cholesterol undergoes very little variation either on compression or on change in temperature[2,12], and DE BERNARD assumed that the reduction of the total area can be attributed to a decrease of the molecular area of egg lecithin.

Our results on the various synthetic compounds in principle confirmed this



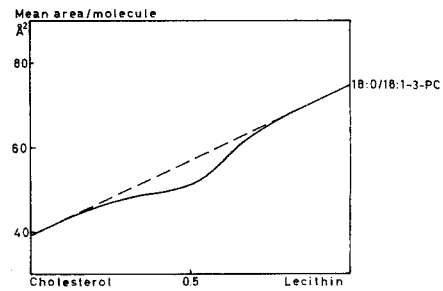


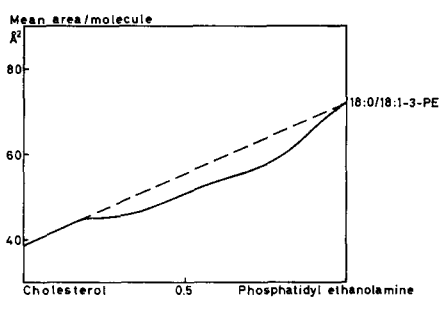
Fig. 5. Variation of the mean molecular area, as a function of the composition, for mixed monolayers of cholesterol and, respectively, (1,2-didecanoyl)-3-lecithin, (1-stearoyl-2-lauroyl)-3-lecithin, (1,2-ditetradecanoyl)-3-lecithin, (1,2-distearoyl)-3-lecithin, (1,2-dipalmitoyl)-3-phosphatidyl ethanolamine. PC, phosphatidyl cholines; PE, phosphatidyl ethanolamines.

Fig. 6. Variation of the mean molecular area, as a function of the composition, for mixed monolayers of cholesterol and (1-stearoyl-2-oleoyl)-3-lecithin (18:0/18:1-3-PC).

condensing effect of cholesterol, but considerable differences were found to exist between phosphoglycerides containing different fatty acid chains. As is demonstrated in Fig. 5, a number of long-chain saturated synthetic phospholipids, when mixed with cholesterol in various proportions, did not reveal any measurable reduction of the cross-sectional area. The mean area per molecule in mixed films of cholesterol with (1,2-distearoyl)-3-lecithin and (1,2-dipalmitoyl)-3-phosphatidyl ethanolamine (as measured by STANDISH[13]) practically follow the simple additivity rule as indicated by the straight lines obtained. These results are not surprising inasmuch as these saturated phospholipids form rather condensed films (Fig. 1). The interactions between the molecules is strong in the pure phospholipid films, and the addition of cholesterol gives only small, barely measurable, changes in packing.

When mixed films of cholesterol with saturated lecithins which themselves give expanded films are considered the picture is more confused. (1,2-Didecanoyl)-3-lecithin, which itself gives an expanded film (Fig. 1), shows no condensation effect with cholesterol. On the other hand, (1,2-ditetradecanoyl)-3-lecithin and (1-stearyol-2-lauroyl)-3-lecithin, which also give expanded films (Fig. 1), exhibit marked condensation effects.

Turning to the very expanded films of unsaturated phosphoglycerides, it will be seen that cholesterol causes condensation with phospholipids containing oleic acid (Figs. 6–8), except for the case of (1-butyryl-2-oleoyl)-3-lecithin (Fig. 9). In contrast,



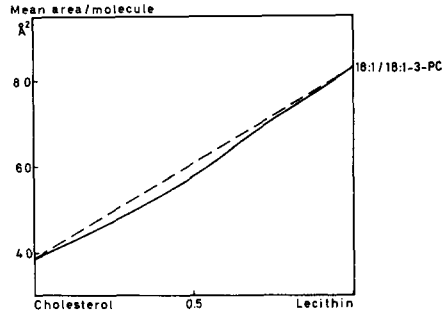
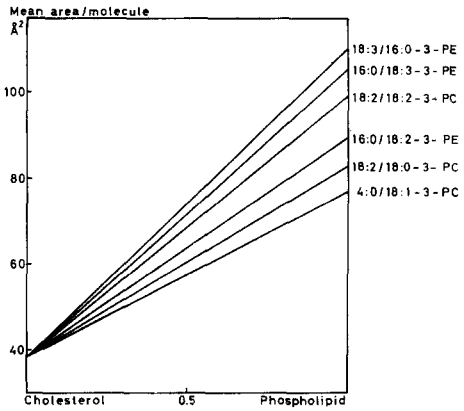


Fig. 7. Variation of the mean molecular area, as a function of the composition, for mixed monolayers of cholesterol and (1-stearoyl-2-oleoyl)-3-phosphatidyl ethanolamine (18:0/18:1-3-PE).

Fig. 8. Variation of the mean molecular area, as a function of the composition, for mixed monolayers of cholesterol and (1,2-dioleoyl)-3-lecithin (18:1/18:1-3-PC).

the phosphoglycerides containing the polyunsaturated linoleic or linolenic acids showed no condensation effects (Fig. 9) at 7°, 22° or 37°.

The effects observed for the oleoyl compounds show that two oleoyl chains in the phospholipid cause less condensation with cholesterol than one oleoyl chain with another saturated chain of similar length. The film of (1-stearoyl-2-oleoyl)-3-lecithin shows a particularly striking condensation effect at a mole ratio of about 1:1 (Fig. 6), with much less interaction at low and high mole ratios. The corresponding ethanol-



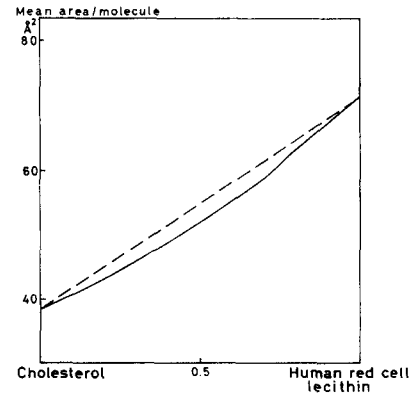


Fig. 9. Variation of the mean molecular area, as a function of the composition, for mixed monolayers of cholesterol and, respectively, (1-linolenoyl-2-palmitoyl)-3-phosphatidyl ethanolamine, (1-palmitoyl-2-linolenoyl)-3-phosphatidyl ethanolamine, (1,2-dilinoleoyl)-3-lecithin, (1-palmitoyl-2-linoleoyl)-3-lecithin, (1-butyryl-2-oleoyl)-3-lecithin. PC, phosphatidyl cholines; PE, phosphatidyl ethanolamines.

Fig. 10. Variation of the mean molecular area, as a function of the composition, for mixed monolayers of cholesterol and lecithin extracted from human red cells.

amine compound also showed a significant condensation with cholesterol, but over a more extended range of mole ratios (Fig. 7).

In agreement with DE BERNARD, purified lipids from natural sources were found to be reduced in molecular area by cholesterol (Fig. 10). In contrast to the results of DE BERNARD on egg lecithin, there is no evidence in our experiments of phospholipid–cholesterol complexes at 1:3 and 3:1 mole ratios for human red-cell lecithin (Fig. 10), chromatographically pure egg lecithin, human plasma sphingomyelin or any of the pure synthetic phospholipids studied.

DISCUSSION

The data on the monolayer interactions of phospholipids and cholesterol show that these interactions are far from simple. There is no direct correlation, for example, between the state of expansion of the phospholipid and its interaction with cholesterol, showing that simple packing of molecules interacting by Van der Waals' forces will not explain the results. Nor is there a specific interaction between cholesterol and the oleoyl chain of the phospholipids, in view of the results for (1-butyryl-2-oleoyl)-3-lecithin (Fig. 9) and in view of the lack of interaction between cholesterol and lysooleoyl lecithin[25]. Indeed, the condensation effect is not specific for cholesterol itself, since a condensation of an oleoyl phospholipid can be obtained by adding (1,2-distearoyl)-3-lecithin to the monolayer[25]. Van der Waals' interactions will play a part in the condensation effect, and may partially account for the fact that several saturated and oleoyl phosphoglycerides condense with cholesterol, whereas the polyunsaturated molecules do not. The polyunsaturated molecules, at the temperatures studied, will be unable to approach closely to the cholesterol molecule because of the double-bond-induced distortion of the chain, and the CH_2 interactions with cholesterol

will be accordingly reduced[20]. Similarly, short-chain saturated phosphoglycerides will also undergo smaller Van der Waals' interactions and therefore be less able to give condensation effects. It should be noted that the Van der Waals' interaction between cholesterol and phospholipids as evaluated by VANDENHEUVEL[23] may be less than the mutual interaction of the long-chain saturated phospholipids themselves[24], and that the Van der Waals' contribution to the mixed-film interaction could in principle tend to give an expansion effect.

Further information on the nature of the interactions can be obtained from the effect of temperature on the force–area curves, and on the condensation effect itself. The force–area curves for (1,2-ditetradecanoyl)-3-lecithin at 5° and 22° are shown in Fig. 1. Using the thermodynamic approach of GOODRICH[21], it can be calculated that the compression of the monolayer is accompanied by a positive change of the heat content. This result shows that the interaction in the pure monolayer is not simply one of Van der Waals' attraction alone, but that other contributions to the interaction energy must be present. The excess free energy of mixing an equimolar amount of cholesterol with (1,2-ditetradecanoyl)-3-lecithin at 12 dyne/cm may be calculated by the GOODRICH method from the force–area curves of the pure and mixed films, and the excess entropy and heat of mixing thereby obtained from the temperature effect. The calculation shows that over the temperature range studied the excess entropy of mixing is positive ($T\Delta S$ = approx. 725 cal/mole), with an associated positive excess enthalpy of mixing (approx. 650 cal/mole). Once again, simple Van der Waals' attraction cannot explain these results, although Van der Waals' interaction undoubtedly plays a part. Mixing phospholipid and cholesterol would be expected to lead to a reduction in the configurational entropy of the hydrocarbon chains. The positive excess entropy of mixing suggests that the expected negative contribution arising from chain-configurational factors is swamped by a substantial positive entropy contribution from another source. One such possible source of positive entropy would be net structure-breaking effects in the water adjacent to the monolayer. On this view, a positive excess entropy of mixing will arise from a decrease of water structure in the mixed films compared with that found in the pure monolayers. The positive excess heat of mixing (for the one case considered) also points in the same direction. The condensation phenomenon, where it occurs, is thus seen as the result of a balance of effects, notably chain-configurational terms, Van der Waals' interaction and water structure changes. All three will depend on the temperature, the length and shape of the chains, and their consequent ability to interact with each other, with cholesterol and with the substrate water. Further thermodynamic measurements will be particularly useful for further understanding of the monolayer interactions, and the present calculations are to be regarded as preliminary.

CHAPMAN, OWENS AND WALKER[26] have recently studied cholesterol monolayer interactions with phosphatidyl ethanolamines containing elaidoyl chains, and have shown that interaction occurs at lower surface pressures at temperatures at which the elaidoyl compounds show a phase transition in the pure film. At lower temperatures and higher pressures, where the elaidoyl phospholipids are more condensed, the cholesterol interaction is absent or much reduced. This interesting association of a phase transition with interaction with cholesterol may not be general for elaidoyl phospholipids and further measurements on the dielaidoyl phosphatidyl choline are desirable. This choline compound does not exhibit a phase transition at room temper-

ature, but is rather expanded[26] and may well exhibit interaction with cholesterol. Similarly the (2-oleoyl-3-elaidoyl)-1-phosphatidyl ethanolamine shows no phase transition at room temperature and may likewise give a strong interaction with cholesterol. It is of significance that further measurements by these authors indicate that with (2-stearoyl-3-elaidoyl)-1-phosphatidyl ethanolamine at 26°, the film is somewhat expanded, does not show a phase transition, but does show an interaction with cholesterol*.

In considering the biological relevance of the results, it is interesting to note that environmentally induced alterations in fatty acid composition may be compensated for by living organisms so that the physical character of the membrane lipids is preserved to a great extent. MEYER AND BLOCH[14] found that in lecithin from yeast cells which were grown anaerobically, the decrease of unsaturated fatty acids was compensated for by an appearance of saturated fatty acids with shorter chain length such as capric, lauric, and myristic acids. Remarkably, these constituents replace the unsaturated fatty acids by taking preferentially the 2 ester position. A comparison of the film characteristics of (1-stearoyl-2-oleoyl)-3-lecithin and (1-stearoyl-2-lauroyl)-3-lecithin demonstrates that their pressure–area curves are rather similar. Another example was provided by lecithins isolated from the livers of rats kept on coconut, corn oil, and fat-free diets. These phospholipid preparations revealed only small differences in force–area plots at the air–water interface. Detailed chemical analysis showed that this effect may be attributed to (a) a replacement of fatty acids of related unsaturation and (b) shifts in the relative proportions of several molecular lecithin species[15,16].

The presence of cholesterol may cause a significant limitation of the liquid-expanded nature of lipid films. In this respect it is interesting to note that the sterol content of the lipid fraction from different membranes varies considerably. A high proportion of cholesterol has been found in erythrocyte membranes (a phospholipid: cholesterol ratio close to 1)[17] and myelin sheath[18], whereas the sterol content of subcellular membranes such as those of mitochondrial is known to be rather low. Recent work by COLEMAN AND FINEAN[22] shows that in intestinal epithelium cells and in liver cells, the isolated protoplasmic membrane contains a high proportion of cholesterol. Indirect evidence suggests that in *Neurospora crassa* the protoplasmic membrane may be more rich in sterol than the membrane structures of mitochondria and nuclei[19]. Thus it is tempting to speculate that sterols are prominent lipid constituents of most cell envelopes, although the bacterial cell membrane lacks these compounds.

The consequences of a high sterol content for the molecular arrangement of the lipids in the membrane require further investigation, the studies on monomolecular films suggesting that cholesterol may contribute to a high degree of molecular organisation apart from the decrease of the mean molecular area in the mixed films. Although the validity of extrapolation from monolayer experiments at the air–water interface to complex biological structures is debatable, it is worth noting that several phospholipid species (*e.g.* those containing oleic acid) giving pronounced effects with sterol in monolayers are indeed abundant in a number of membranes such as myelin sheath and erythrocyte membranes.

* D. CHAPMAN, private communication.

ACKNOWLEDGEMENT

We wish to thank Dr. M. M. STANDISH for confirmatory experiments on the condensation in mixed monolayers of cholesterol and (1,2-ditetradecanoyl)-3-lecithin.

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