(7) W. M. Sams, *J. Amer. Med. Ass.*, **174,** 2043(1960).
(8) L. Ahtee and M. K. Paasonen, *Ann. Med. Exp. Fenn.*, **43,** 101(1965).
(9) A. Felmeister and R. Schaubman, *J. Pharm. Sci.*, **58,** 64 (1969).
(10) P. S. Guth and M. A. Sprites, *Int. Rev. Neurobiol.*, **7,** 231 (1964).
(11) C. L. Huang and F. L. Sands, *J. Pharm. Sci.*, **56,** 259(1967).
(12) C. L. Huang and F. L. Sands, *J. Chromatogr.*, **13,** 246(1964).
(13) A. Felmeister and R. Schaubman, *J. Pharm. Sci.*, **58,** 1232 (1969).
(14) G. Weissman, G. Sessa, and S. Weissman, *Biochem. Pharmacol.*, **15,** 1537(1966).

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Dermatitic Effect of Nonionic Surfactants IV: Phospholipid Composition of Normal and Surfactant-Treated Rabbit Skin

MICHAEL MEZEI and AMBROSE K. Y. LEE

Abstract ☐ Results of the authors' previous experiment indicated that the primary site of action of topically applied surfactants (polysorbate 85 and polyoxyethylene ether 96) is in the epidermal membranes. To elucidate the interaction of surfactants with biological membranes, the composition of epidermal phospholipids and the rate of biosynthesis of major phospholipid components were determined by utilizing thin-layer chromatographic, spectrophotometric, and radiotracer techniques. Results indicated that the major lipid components are cholesterol, lecithin, lysolecithin, phosphatidyl ethanolamine, and sphingomyelin. The treatment with surfactant did not induce any significant change in the phospholipid composition. The biosynthetic and turnover rates of all identified phospholipids, however, were greatly increased (two to four times) in the surfactant-treated skin. Available data suggest that the tested surfactants damaged the epidermal membranes. A role of surfactants in increasing the absorption of medicinal substances was also proposed in view of these results and other reports regarding the effect of surfactants on biological membranes.

Keyphrases ☐ Phospholipid composition, rabbits—normal, surfactant-treated skin ☐ Nonionic surfactants—dermatitic effect, rabbits ☐ TLC—analysis ☐ Scintillometry—analysis, ^{32}P-incorporation ☐ Spectrophotometry—analysis

In a multiphase system the molecules of a surface-active agent align and orient themselves at the interface. In a biological system the membranes provide the interface. The concentration of a surfactant in a tissue because of its hydrophilic–lipophilic character is the highest at, or in, the biological membranes; therefore, the site of action of a topically applied surfactant is very likely in the epidermal membranes.

Unfortunately, it is difficult to design experiments to test the action of surfactants on biological membranes. At present, the exact structural configuration of membranes is not defined because of the lack of reliable techniques to study membranes at cellular or molecular level. Recent reviews describe most of the presently accepted theories relating to the structure and function of biological membranes (1–5). Changes in membranes induced by surfactants or any other agents can be studied only by indirect methods. On the basis of the presently accepted concepts of biological membranes, a qualitative and/or quantitative change in lipid composition of a tissue may indicate structural changes and, consequently, functional changes in the membranes. Results of previous investigations (6–8) indicated that the treatment with surfactant preparations induced an increase both in the content and in the biosynthetic rate of epidermal phospholipids, nucleic acids, and acid-soluble material. This increase was explained by the assumption that the surfactants damaged the biological membranes by either rupturing the membranes or replacing certain phospholipid molecules in the continuous phospholipid micelles present in the membranes. Phospholipid molecules present in ruptured membranes and those that are possibly replaced by surfactants were measured along with newly formed molecules during the analysis of skin tissue.

The higher rate of biosynthesis of epidermal phospholipids was explained by the reasoning that it was expected in order to repair the surfactant-damaged membranes or to regenerate membranes. A further step in this project was to find out whether the surface-active agents interact with the membrane as a whole or with only certain components of the membrane. If the surfactants disrupt or damage the membrane as a result of hydrophobic or micellar interactions or by hydrogen bonding and a completely new membrane is regenerated, then the content and the rate of biosynthesis of all phospholipid membrane components will be increased. On the other hand, if the surfactant interacts with certain phospholipid molecules participating in the membrane, *i.e.*, a molecule can replace one phospholipid molecule present in the lipid micelles, one should find that the content and the rate of biosynthesis of those particular phospholipids would be increased more than that of other phospholipid components. The determination of phospholipid composition and the rate of biosynthesis of each phospholipid component in the control and sur-

Table I—Relative Percent Composition of Epidermal Phospholipids

Phospholipids	Untreated, mean ± *SD*	Relative Percent[a]: Petrolatum, mean ± *SD*	Polysorbate 85, mean ± *SD*	Polyoxyethylene Ether 96, mean ± *SD*
Lecithin	41.6 ± 5.0	41.6 ± 5.2	43.3 ± 4.8	40.2 ± 4.6
Lysolecithin	10.9 ± 4.3	10.4 ± 3.9	10.3 ± 4.6	10.4 ± 4.8
Phosphatidyl ethanolamine	27.1 ± 4.0	27.7 ± 4.6	27.5 ± 3.4	29.2 ± 3.6
Sphingomyelin	20.3 ± 5.1	20.4 ± 2.0	18.8 ± 5.5	20.1 ± 5.4

[a] Results indicated represent the mean value of percentage composition, on the basis of lipid phosphorus, with standard deviation (±*SD*, N = 31). The percentage composition was calculated, in case of each rabbit, from the sum of phosphorus recovered from spots on the thin-layer plate corresponding to the four phospholipids which was accepted as 100%. Epidermal samples were obtained from three groups of rabbits: 21 rabbits were treated for 4 days, five for 2 days, and five for 7 days.

factant-treated skin, as described in this report, was another attempt to test, although indirectly, the site of action of the selected surfactant at the molecular level.

MATERIALS AND METHODS

The selection and the treatment of the experimental animals and the isolation of epidermis and epidermal lipids have been described in the authors' previous reports (6–8). Aliquots of the lipid extract were used for the separation of phospholipids by TLC.

Thin-Layer Chromatography—Thirty grams of silica gel G (E. Merck, A. G., Darmstadt, Germany) was mixed with 63 ml. of 0.01 *M* Na_2CO_3 solution and spread on 20 × 20-cm. glass plates in 0.25-mm. thickness. The plates were dried at room temperature and were activated for 1 hr. at 105° just before use. Aliquots of lipid extracts were applied along with standard solutions of known phospholipids with a microliter syringe as narrow streaks on 2-cm. wide lanes, 1.5 cm. from the bottom of the plate. The plates were subjected to ascending chromatography in a closed glass developing tank, which contained 100 ml. of chloroform–methanol–distilled water, 65:30:5. This solvent was allowed to rise to 15 cm. from the starting line.

The developed chromatograms were examined by various detection methods, such as exposure to iodine vapor, spraying with ammonium molybdate–perchloric acid, rhodamine B, and Dragendorff reagents (9). Individual lipids were identified by comparison with standard lipid applied on the same thin-layer plate. Additional evidence of identity of the various phospholipids was obtained by recent experiment using the Zeiss chromatogram spectrophotometer (10). The identified components, as well as standard known lipids and blank areas, were scraped into glass-stoppered test tubes, and the phospholipids were digested with sulfuric acid and hydrogen peroxide and the phosphorus was determined as described by Keenan *et al.* (11).

Radiotracer Technique—Recent experiments were extended to measure the rate of *in vivo* incorporation of ^{32}P into the epidermal phospholipid components. In this part of the project, rabbits (five in a group) were treated for 2, 4, and 7 days. Twenty-four hours before the end of treatment, approximately 5 mc. inorganic ^{32}P (Na_2HPO_4) was injected intravenously through the ear veins. The experimental procedure for extracting the epidermal lipids was the same as with other rabbits treated for 4 days without the use of an isotope (7). To minimize the error introduced by the TLC technique, the same sample that was obtained from the identified and marked spots was used for both spectrophotometric determination of phosphorus (11) and for the measurement of ^{32}P incorporation. In this way a more reliable specific radioactivity (c.p.m./mcg. lipid-P) could be calculated. Aliquots (3.0 ml.) of the isobutanol layer, which was used for the spectrophotometric determination of phosphorus, were mixed with scintillator solvent (7.0 ml. Bray's solution) composed of 60 g. naphthalene, 20 ml. ethylene glycol, 100 ml. methanol, 4 g. PPO, and 0.2 g. POPOP, and dioxane made up the volume to 1.0 l. The radioactivity was measured by Unilux I liquid scintillation counter (Nuclear Chicago).

RESULTS AND DISCUSSION

Thin-layer chromatographic analyses of epidermal lipid extracts indicated that there are four phospholipids—lecithin, lysolecithin, phosphatidyl ethanolamine, and sphingomyelin—present in rabbit skin in amounts that could be measured and identified. Besides these four components, three other phospholipids were detected but not identified, mainly because of the minute amount present. Table I demonstrates the relative percent composition of the four major epidermal phospholipids of untreated and surfactant-treated rabbit skin. The relative percent composition was calculated from the sum of phosphorus recovered from spots containing these four phospholipids. The unidentified phospholipids, which probably compose 10–20% of the total phospholipid content, were disregarded.

According to the data shown by Table I, there is no statistically significant change in the percent composition of these phospholipids after the treatment with the tested surfactant preparations (10% in white petrolatum). There is, however, a slight increase in lecithin content as well as a slight decrease in sphingomyelin content of samples treated with polysorbate 85 compared with that of untreated skin samples. The distribution of incorporated radioactivity in epidermal phospholipids is shown by Table II. Due to the treatment with polysorbate 85, the content of lecithin seemed to be increased (Table I) but the reverse is true regarding the radioactivity (Table II). The same treatment resulted in a decrease in the content (Table I) and an increase in the radioactivity (Table II) of sphingomyelin. That the treatment with surfactants has an effect, however slight, on the content but more on the rate of biosynthesis of these four phospholipids becomes evident if the results shown in Table III are considered. Table III demonstrates the effect

Table II—Distribution of Incorporated Radioactivity in Epidermal Phospholipids

Phospholipids	Untreated, mean ± *SD*	Relative Percent[a]: Petrolatum, mean ± *SD*	Polysorbate 85, mean ± *SD*	Polyoxyethylene Ether 96, mean ± *SD*
Lecithin	50.0 ± 4.7	48.5 ± 5.2	46.9 ± 5.8	43.2 ± 5.8
Lysolecithin	7.5 ± 2.9	7.6 ± 2.1	6.6 ± 1.7	8.6 ± 2.8
Phosphatidyl ethanolamine	28.9 ± 3.2	31.2 ± 3.7	27.6 ± 5.7	29.6 ± 2.8
Sphingomyelin	13.5 ± 4.4	12.8 ± 5.3	18.8 ± 7.2	18.7 ± 5.3

[a] Values represent the mean percentage of counts measured in the four phospholipids recovered from the thin-layer plates. The percentage of radioactivity was calculated, in case of each rabbit, from the sum of counts in the four phospholipids recovered which was accepted as 100%. The mean of these percentages were calculated, in case of each phospholipid component, with standard deviations (±*SD*, N = 15). Epidermal samples were obtained from three groups of rabbits (five in each group) treated for 2, 4, and 7 days, respectively.

Table III—Incorporation of ^{32}P into Epidermal Phospholipids

Phospholipids	Length of Treatment, days	Specific Activity as Percent of Control[a]: Petrolatum, mean ± *SD*	Polysorbate 85, mean ± *SD*	Polyoxyethylene Ether 96, mean ± *SD*
Lecithin	2	128 ± 35	194 ± 52	179 ± 52
	4	118 ± 15	202 ± 12	151 ± 6
	7	113 ± 14	196 ± 85	194 ± 60
Lysolecithin	2	142 ± 33	159 ± 29	117 ± 19
	4	104 ± 6	466 ± 69	221 ± 40
	7	103 ± 37	145 ± 22	209 ± 78
Phosphatidyl ethanolamine	2	120 ± 32	155 ± 19	129 ± 23
	4	120 ± 25	211 ± 45	197 ± 27
	7	142 ± 33	222 ± 84	216 ± 27
Sphingomyelin	2	112 ± 8	290 ± 148	226 ± 20
	4	133 ± 30	410 ± 55	341 ± 50
	7	103 ± 16	283 ± 132	269 ± 87

[a] The results are expressed as percent differences of specific activity (c.p.m./mcg. lipid-P) of skin samples treated with the ointment base: white petrolatum, and nonionic surfactants: polysorbate 85 and polyoxyethylene ether 96, as compared with that of the untreated skin, which is accepted as 100%. Values show the mean ± standard deviation (± *SD*, N = 5).

of treatment with surfactant preparations on the rate of incorporation of ^{32}P into epidermal lipid components.

To assess statistical significance of the data, the percentage stimulations of ^{32}P incorporation into the epidermal phospholipid component of each individual rabbit were calculated. These percentage increases were averaged, and then the mean values were calculated with standard deviations. This was necessitated by the variation in the absolute incorporation of ^{32}P into the various epidermal phospholipids of different rabbits. This variation was due not only to individual biological variation but, to a greater extent, to the fact that it was almost impossible to inject intravenously exactly 5.0 mc. ^{32}P. Since the amount of ^{32}P in the circulatory blood and, consequently, at the site of the designated epidermal tissue varied with each rabbit, the variation in the rate of incorporation of ^{32}P into phospholipid components was very large, even within one type of skin. This variation would tend to decrease the statistical significance of any differences that might be observed between the specific activity of a particular phospholipid extracted from the untreated and surfactant-treated skin of one rabbit. The rate of ^{32}P incorporation was expressed as specific activity in counts per minute per phosphorus (c.p.m./mcg.-P) present in the particular phospholipid recovered from the thin-layer plate.

In all cases the incorporation of ^{32}P into phospholipids of the surfactant-treated skin was much greater than that of the untreated skin samples. The treatment with petrolatum also seems to stimulate the incorporation of ^{32}P, but this stimulation is not significant. The highest increase in the rate of ^{32}P incorporation is observed in skin samples treated with polysorbate 85 preparation for 4 days. This increase is more than fourfold in the case of lysolecithin and sphingomyelin and somewhat more than twofold with lecithin and phosphatidyl ethanolamine. Treatment with polysorbate 85 for 2 and 7 days induced a less dramatic increase in the specific activity of each of the four phospholipids. The explanation for the relationship between the length of time of treatment and the extent of increase in the specific activity of the phospholipids is rather complex. Speculations for possible mechanisms of reaction to injury caused by the surfactant at various stages of the treatment are presented elsewhere (8). The treatment with polyoxyethylene ether 96 also induced a considerable increase in the rate of incorporation of ^{32}P into each of the phospholipids. These increases, however, were almost independent of the length of time of treatment.

In most cases the standard deviation is rather large, mainly because of the thin-layer chromatographic technique and partly because of other numerous steps in the experimental procedure. The quantitative recovery of phospholipids from the corresponding spots on the thin-layer plates and the spectrophotometric and radioactive measurement of the phospholipid components in the presence of silica gel are the major factors in the spread of results. In spite of these problems, the effect of the tested surfactants on the rate of biosynthesis of the major epidermal phospholipids is apparent.

Results reported previously (6–8) and data presented herein are clear, although indirect, indications for changes in epidermal membranes as a result of treatment with surfactants. Many investigators (12–20) reported that surfactants in a variety of dosage forms increase the absorption of medicinal substances. In most of these reports (12–20) the role of surfactants in enhancing absorption was explained by the physicochemical properties of the tested surfactants; a surfactant may reduce the disintegration time of a tablet or may enhance the dissolution or diffusion of an active ingredient by various physicochemical phenomena, thereby influencing the rate of absorption of agents administered along with a surface-active agent. These are sound explanations, and no doubt a surfactant can influence absorption on these bases due to its physicochemical properties. The authors would like to propose, on the bases of previous studies (6–8), that the role of a surfactant in enhancing absorption of other agents present in the same product is also due to its physiological properties. As the authors postulated (21), the surfactants may act on the membranes by various possible mechanisms, and this is also a contributing factor for the reported increased rate of absorption. A damaged or regenerating membrane is less of a barrier to penetrating substances than are intact membranes. The question now is whether it is wise to increase the absorption of a substance by damaging the membranes, especially in the case of chronic treatment. The surfactants increase the permeability of membranes, which may lead not only to higher penetration of medicinal substances but, by disturbing the balance of a cell system, also to metabolic disorder.

REFERENCES

(1) V. P. Whittaker, *Brit. Med. Bull.*, **24**, 101(1968).
(2) J. A. Lucy, *ibid.*, **24**, 127(1968).
(3) A. Rothstein, *Ann. Rev. Physiol.*, **30**, 15(1968).
(4) E. D. Korn, *Ann. Rev. Biochem.*, **38**, 263(1969).
(5) D. E. Green and D. H. MacLennan, *BioScience*, **19**, 213 (1969).
(6) M. Mezei and R. W. Sager, *J. Pharm. Sci.*, **56**, 1604(1967).
(7) M. Mezei and G. N. White, *ibid.*, **58**, 1209(1969).
(8) M. Mezei, to be published.
(9) D. Waldi, in "Thin-Layer Chromatography," E. Stahl, Ed., Springer-Verlag, New York, N. Y., 1965.
(10) M. Mezei and A. K. Y. Lee, to be published.
(11) R. W. Keenan, G. Schmidt, and T. Tanaka, *Anal. Biochem.*, **23**, 555(1968).
(12) R. W. Wissler, W. F. Bethard, P. Baker, and H. D. Mori, *Proc. Soc. Exp. Biol. Med.*, **86**, 170(1954).
(13) G. Levy and R. H. Reuning, *J. Pharm. Sci.*, **53**, 1471(1964).
(14) G. Levy, K. E. Miller, and R. H. Reuning, *ibid.*, **55**, 394 (1966).
(15) H. Matsumoto, *Chem. Pharm. Bull.*, **14**, 398(1966).
(16) H. Yamada, T. Ichihashi, F. Kogishi, and R. Yamamoto, *ibid.*, **14**, 786(1966).
(17) K. Kakemi, H. Sezaki, S. Muranishi, and H. Matsui, *ibid.*, **15**, 172(1967).
(18) T. Matsuzawa, H. Fujisawa, K. Aoki, and H. Mima, *ibid.*, **17**, 999(1969).
(19) L. J. Ravin, E. G. Shami, A. Intoccia, E. Rattie, and G. Joseph, *J. Pharm. Sci.*, **58**, 1242(1969).

(20) C. W. Whitworth and E. R. Carter, *ibid.*, **58**, 1285(1969).
(21) M. Mezei, R. W. Sager, W. D. Stewart, and A. L. deRuyter, *ibid.*, **55**, 584(1966).

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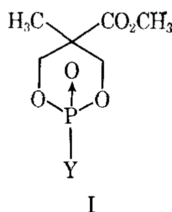
1,3,2-Dioxaphosphorinane 2-Oxides IV: Preparation of Some 2-Substituted-5-carbomethoxy-5-methyl-1,3,2-dioxaphosphorinane 2-Oxides as Potential Antitumor Agents

JOHN H. BILLMAN and GERALD R. ROEHRIG

Abstract □ Twenty-two of the title compounds, in which the substituents are chloro, alkylamino, dialkylamino, arylamino, hydroxy, and amine salts, as well as the pyrophosphate, have been synthesized and submitted for antitumor evaluation.

Keyphrases □ 1,3,2-Dioxaphosphorinane 2-oxides—synthesis □ Antitumor agents—synthesis, 1,3,2-dioxaphosphorinane 2-oxides □ IR spectrophotometry—structure, analysis

Previous work in the authors' laboratory (1) has led to consideration of the dioxaphosphorinane 2-oxides as potential antitumor agents. Synthesis and evaluation of compounds of Type I are described at this time.

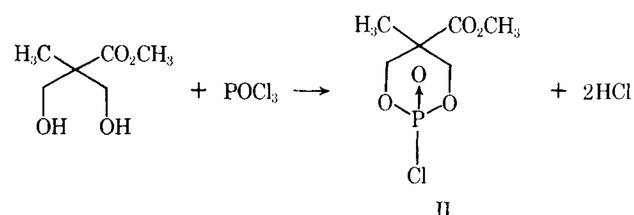


Y = —Cl, —NHR, —NR$_2$, —NHAr, —OH, -amine salts and the pyrophosphate

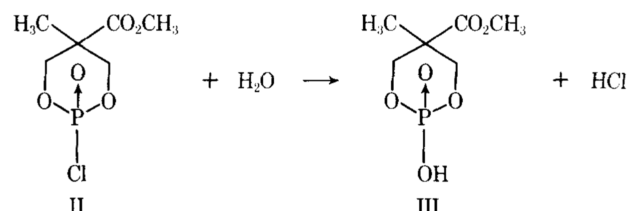
The starting point for the preparation of these compounds was the phosphochloridic acid II, which was prepared according to Scheme I. Compound II can formally be considered as an acid chloride analogous to the more commonly encountered carboxylic acid chlorides; as such, one would expect that it might exhibit many of the same types of reactions as the acyl chlorides. Indeed, many of those reactions have been observed and used to advantage. Schemes II–IV are representative of those used for the preparation of the compounds cited in this paper.

The salts of the acid were prepared for two basic reasons. Since the majority of these compounds are relatively water insoluble, it was thought that a compound with considerably greater water solubility might exhibit a greater degree of antitumor activity. Also, by incorporating amines that exhibit biological activity, one could compare the activity of the salt with that of the free amine. At this point, insufficient data have been returned to clarify either of these points.

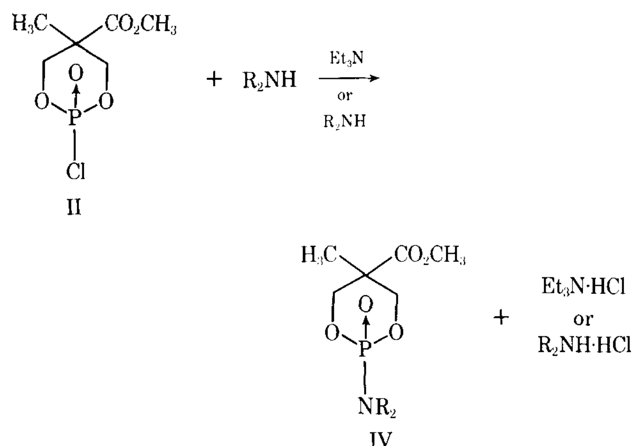
Tables I and II contain the data pertinent to the structures of the compounds under consideration.



Scheme I



Scheme II



R = alkyl, aryl, or hydrogen

Scheme III