# Dermatitic Effect of Nonionic Surfactants II

## Changes in Phospholipid and in Deoxyribonucleic Acid Content of Rabbit Epidermis *In Vivo*

By MICHAEL MEZEI* and R. W. SAGER

A hypothesis, namely, that the site of action of surfactants is in the biological membranes, was investigated with an indirect method. One representative member from each of 3 major types of nonionic surfactants was tested. Sorbitan trioleate, polysorbate 85, and polyoxyethylene ether 96 were incorporated, 10 percent in concentration, in white petrolatum and applied to rabbit skin daily. Measurements of lipid phosphorus indicated a considerable increase in the phospholipid content of the 0.1 mm. thick skin samples representing mainly the epidermis. Two reference standards were used: the wet weight and the deoxyribonucleic acid (DNA) content. After 4 days of treatment with polysorbate 85, sorbitan trioleate, and polyoxyethylene ether 96 preparations, the increase in phosphorus was found to be 23–56 percent, 28–73 percent, and 46–90 percent, respectively, on the basis of wet weight and 26–53 percent, 27–58 percent, and 47–81 percent, respectively, if DNA content was used as the reference standard. After 10 days of treatment, the increase in phosphorus was considerably smaller if calculated on the basis of DNA present in the sample, but did not differ much if wet weight was used as the reference. A comparison of the two reference standards demonstrated that the DNA content also increased after 10 days of treatment. The smaller spread in results indicated that DNA content may be more reliable as a reference standard, but the wet weight still can be used as a practical and useful standard of reference especially if the DNA content is also influenced by the test.

THE DERMATITIC effects of nonionic surfactants have been the subject of much research (1–7). All of these studies, however, have been limited to surfactants present in household cleaners or in aqueous solutions. Most of these investigations employed *in vitro* and/or patch test techniques, which were based upon experimental procedures that were empirical in nature and arbitrary in evaluation; they relied on gross observations and on microscopic evaluations. These evaluation methods can reveal little or nothing about the biochemical changes at the cellular level which probably initiate the morphological changes observed.

The aim throughout the present investigation has been to design and apply more definitive and rational test procedures. In contrast to the *in vitro* and patch test techniques, attempts were made to reproduce the frequent application of dermatological and cosmetic preparations, which may contain these surfactants, by the general public. Besides macroscopic and microscopic observations, attempts were made to investigate biochemical changes by standard and well-accepted assay methods. Measurements and observations regarding morphological changes (on both the macroscopic and microscopic level) and of oxygen consumption were reported as a first part of the study (8). This presentation is mainly concerned with the measurement of quantitative changes in the phospholipid content of the rabbit epidermis induced by surfactant applied to the skin in the form of an ointment. The DNA content was also determined with the intention of using a more reliable reference standard than the wet weight of sample is believed to be.

### MATERIALS AND METHODS

Thirteen New Zealand white rabbits of both sexes, 6–12 weeks old, were used as experimental animals. They were housed in hanging screen-bottom cages and were maintained on Purina rabbit chow and water *ad libitum*. The trunk of each rabbit was clipped free of hair with an electric hair clipper (Oster model A2, size 40), and was divided into 6 areas. Harness-type restrainers (9) were used for restraining rabbits with a minimum amount of stress.

One representative member from each of 3 major types of surfactant was selected. Sorbitan trioleate,[1] polysorbate 85,[2] and polyoxyethylene ether 96[3] were incorporated in white petrolatum USP in 10% concentration and were applied to the center of the designated areas of rabbit skin daily. The other three areas on the rabbits' back were reserved for controls: (*a*) untreated skin *A*, (*b*) untreated skin *B*, and (*c*) skin treated with the ointment base only. Untreated skin *B* was used as a control to determine

Received April 3, 1967, from the School of Pharmacy, Oregon State University, Corvallis, OR 97331
Accepted for publication August 15, 1967.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Previous paper: Mezei, M., Sager, R. W., Stewart, W. D., and deRuyter, A. L., *J. Pharm. Sci.*, **55**, 584(1966).
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[1] Marketed as Span 85 by Atlas Chemical Industries, Inc., Wilmington, Del.
[2] Marketed as Tween 85 by Atlas Chemical Industries, Inc., Wilmington, Del.
[3] Marketed as Brij 96 by Atlas Chemical Industries, Inc., Wilmington, Del.

whether the surfactant which penetrated into the skin influenced the extraction of lipids and DNA, which could also be a reason for finding different amounts of lipids and perhaps DNA in control and treated skin. To this control area one of the surfactant preparations was applied only once: 15 min. before the sample was taken. This single application provided some surfactant in the skin homogenate, but did not induce any measurable biological changes. The sites of control and treated areas were randomly selected from different animals to exclude any influence of body area. After 4 or 10 days of treatment, the animals were killed by fracturing the neck. The test areas were quickly washed with cotton soaked in ether to remove not only the substances applied previously, but also the surface lipids. Skin samples were taken with the aid of the Castroviejo keratotome (10) set to cut a 0.1-mm. thick skin slice. The samples were immediately dipped in liquid nitrogen, and kept there until the wet weight was determined on an analytical balance. The weight of each sample was around 100 mg. The elapsed time between removing and weighing the samples was not more than 20–30 min. Immediately after weighing, the skin samples were homogenized in a glass homogenizer and extracted with 2.0 ml. of chloroform–methanol (2:1) after the method of Folch *et al.* (11). The residue was set aside for DNA extraction. Nonlipid contaminants were removed from the chloroform–methanol extracts with 0.2 vol. of 0.05% $CaCl_2$ solution. The phospholipid content of the lipid extracts was determined by the method of Bartlett (12). The residue obtained after the extraction of lipids was extracted for the determination of DNA content by the method of Santen and Agranoff (13).

## RESULTS AND DISCUSSION

In studies involving skin lipid analyses the greatest problem has been to provide skin samples in chemically unaltered form with well-defined anatomical structure. Skin lipid samples have usually been divided into 3 categories (14): (*a*) epidermal lipid samples; (*b*) lipid samples from various sebaceous type glands, or from the dermis and, (*c*) lipid samples from the skin surface.

There have been only indirect methods reported for the determination of lipid composition of human epidermis (14–18). In these studies the separation of epidermis from the dermis was achieved by chemical and physical methods (19, 20). In the present investigation, in order to avoid any artifact induced by these separation techniques, 0.1 mm. thick skin slices were cut from rabbits. Microscopic examinations revealed that these samples contained mainly the epidermis, and occasionally a trace of dermis (Fig. 1). The surface lipids were extracted with ether before cutting off the skin slices. This technique provided a well-isolated and chemically unaltered epidermis sample for lipid analysis.

The total phospholipid content of control and treated rabbit skin after 4 days of treatment is shown by Tables I and II. Using DNA content as the reference standard, it was found that the phosphorus (P) content of the control skin samples (untreated skin *A* and *B*) was within the range of 7.44–10.84 mcg. P/100 mcg. of DNA. In skin samples which were treated with the ointment base only,

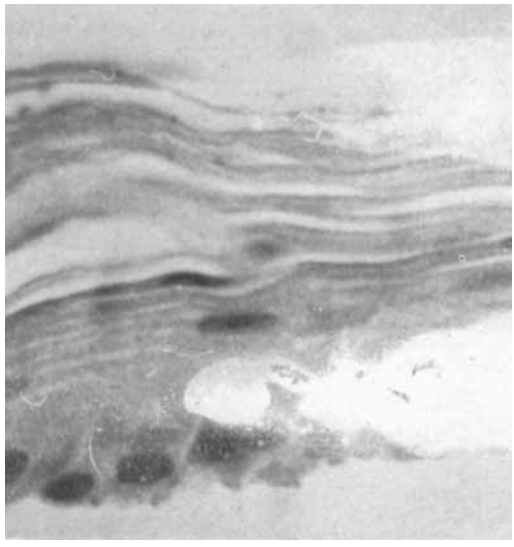

*Fig. 1—Photomicrograph of skin sample obtained with the aid of the Castroviejo keratotome set to cut 0.1 mm. thick skin slices.*

which were also considered as controls, the range was somewhat higher, 9.26–11.24 mcg. P/100 mcg. DNA. Among the treated skin samples the one which was treated with polyoxyethylene ether 96 (10% in petrolatum) showed the largest increase in phosphorus content; the range was 11.52–17.31 mcg. P/100 mcg. DNA, which represented a 47–81% increase as illustrated by Fig. 2.

Treatment with polysorbate 85 and sorbitan trioleate also produced a considerable increase in phosphorus content, 26–53% and 27–58%, respectively. If the phosphorus content is referred to the wet weight of the sample, it is found that the same pattern exists (see Fig. 3 and Table II). The phosphorus content of treated skin samples also showed an increase, but the spread in the results was somewhat larger. The increase of phosphorus, calculated according to the wet weight for samples treated with petrolatum, polysorbate 85, sorbitan trioleate, and polyoxyethylene ether 96, was within the range of 1–15%, 23–56%, 28–73%, and 46–90%, respectively.

Tables III and IV show the results of a similar treatment but for a longer period of time. After 10 days of treatment with polysorbate 85, sorbitan trioleate, and polyoxyethylene ether 96, the increase in phosphorus was found to be 19–38%, 18–35%, and 22–33% (Fig. 2), respectively, on the basis of DNA content, and 40–66%, 24–67%, and 46–76%, respectively, on the basis of wet weight of sample (Fig. 3). The increase of phospholipid phosphorus was considerably smaller than that found after 4 days of treatment, if calculated on the basis of DNA present in the sample, but did not differ much if wet weight was used as the reference. A probable explanation could be that the DNA content was also increased; consequently, the reference standard became greater in the case of DNA, and probably changed nothing in case of the wet weight.

The wet weight is generally used with caution as a reference standard in biological studies, especially

TABLE I—PHOSPHOLIPID CONTENT OF RABBIT EPIDERMIS (mcg. P/100 mcg. DNA) AFTER 4 DAYS OF TREATMENT

| Substance | Rabbit No. 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 |
|---|---|---|---|---|---|---|---|---|---|
| None (untreated skin *A*) | 8.56 | 8.60 | 8.83 | 10.24 | 9.01 | 8.45 | 7.86 | 7.84 | 7.44 |
| None (untreated skin *B*) | 9.73 | 8.23 | 7.62 | ... | 10.84 | ... | ... | ... | ... |
| Petrolatum | 9.59 | 9.86 | 9.26 | 11.24 | 9.80 | ... | ... | ... | ... |
| Polysorbate 85 | 10.78 | 12.07 | 12.89 | 14.30 | 12.64 | 12.5 | 11.16 | 11.52 | 11.41 |
| Sorbitan trioleate | 13.17 | 13.54 | 13.97 | 13.67 | 13.48 | 11.71 | 9.98 | 11.42 | 11.20 |
| Polyoxyethylene ether 96 | 14.54 | 15.20 | 15.04 | 17.31 | 14.51 | 15.28 | 11.52 | 11.80 | 11.67 |

TABLE II—PHOSPHOLIPID CONTENT OF RABBIT EPIDERMIS (mcg. P/100 mg. WET WEIGHT) AFTER 4 DAYS OF TREATMENT

| Substance | Rabbit No. 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 |
|---|---|---|---|---|---|---|---|---|---|
| None (untreated skin *A*) | 22.46 | 20.84 | 20.89 | 26.20 | 27.55 | 26.18 | 25.74 | 26.08 | 23.85 |
| None (untreated skin *B*) | 22.26 | 22.98 | 18.0 | ... | 27.11 | 27.98 | ... | ... | ... |
| Petrolatum | 25.61 | 21.13 | 23.79 | 27.88 | 28.98 | 30.07 | ... | ... | ... |
| Polysorbate 85 | 32.43 | 25.67 | 26.99 | 34.51 | 33.71 | 37.16 | 40.05 | 39.67 | 36.35 |
| Sorbitan trioleate | 34.78 | 28.58 | 26.86 | 33.54 | 46.78 | 42.41 | 44.56 | 35.95 | 34.74 |
| Polyoxyethylene ether 96 | 35.51 | 32.25 | 30.49 | 44.86 | 43.50 | 49.72 | 44.20 | 38.37 | 36.76 |

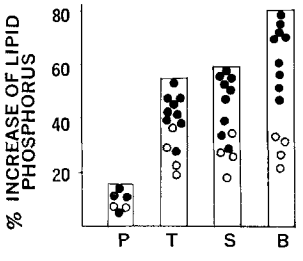


*Fig. 2—Percent increase of lipid phosphorus calculated on the basis of DNA content of skin sample. Skin samples treated with* P, *petrolatum;* T, *polysorbate 85, 10%;* S, *sorbitan trioleate, 10%;* B, *polyoxyethylene ether 96, 10%;* ●, *after 4 days of treatment;* ○, *after 10 days of treatment. The percent increase of lipid phosphorus was calculated individually for each rabbit. Example: rabbit 51 (Table I)—untreated skin, 8.56 mcg. P/100 mcg. DNA; skin treated with polysorbate 85, 10.78 mcg. P/100 mcg. DNA; increase in lipid phosphorus, 2.22 mcg.; percent increase, 2.22/8.56 × 100 = 25.93%.*

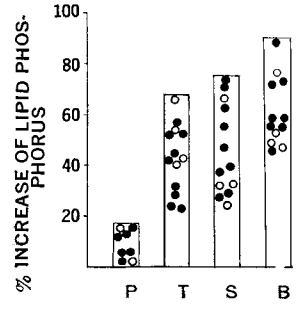


*Fig. 3—Percent increase of lipid phosphorus calculated on the basis of wet weight of skin sample. Skin samples treated with* P, *petrolatum;* T, *polysorbate 85, 10%;* S, *sorbitan trioleate, 10%;* B, *polyoxyethylene ether 96, 10%;* ●, *after 4 days of treatment;* ○, *after 10 days of treatment.*

TABLE III—PHOSPHOLIPID CONTENT OF RABBIT EPIDERMIS (mcg. P/100 mcg. DNA) AFTER 10 DAYS OF TREATMENT

| Substance | Rabbit No. 60 | 61 | 62 | 63 |
|---|---|---|---|---|
| None (untreated skin) | 8.24 | 7.26 | 8.25 | 8.25 |
| Petrolatum | 8.83 | 7.85 | ... | ... |
| Polysorbate 85 | 10.64 | 8.6 | 10.04 | 11.42 |
| Sorbitan trioleate | 11.12 | 9.30 | 9.76 | 10.50 |
| Polyoxyethylene ether 96 | 10.74 | 8.88 | 10.41 | 11.0 |

TABLE IV—PHOSPHOLIPID CONTENT OF RABBIT EPIDERMIS (mcg. P/100 mg. WET WEIGHT) AFTER 10 DAYS OF TREATMENT

| Substance | Rabbit No. 60 | 61 | 62 | 63 |
|---|---|---|---|---|
| None (untreated skin) | 20.37 | 26.0 | 34.63 | 36.50 |
| Petrolatum | 23.56 | 26.54 | ... | ... |
| Polysorbate 85 | 33.76 | 39.40 | 48.57 | 51.58 |
| Sorbitan trioleate | 25.14 | 43.34 | 44.78 | 47.92 |
| Polyoxyethylene ether 96 | 35.79 | 39.77 | 51.54 | 53.20 |

when the tissue studied is heterogeneous in nature. An attempt was made in this study to use samples with uniform structure. Skin samples of 0.1-mm. thick slices could, however, contain different proportions of the four layers of epidermis (stratum germinativum, stratum spinosum, stratum granulosum, and stratum corneum). In order to find a more reliable reference standard than the tissue wet weight is believed to be, the DNA content of each sample was determined. DNA content is used to indicate the number of viable cells. On the basis of DNA content the results of lipid phosphorus determinations show patterns similar to those found when the wet weight was used as a reference. There were, however, 2 distinct differences between the results, calculated on the basis of the two refer-

TABLE V—DNA CONTENT RELATED TO WET WEIGHT (mcg. DNA/100 mg. WET WEIGHT)

| Substance | 4 Days Treatment | | | | | | 10 Days Treatment | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Rabbit No. | | | | | | | | | | | |
| | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 | 63 |
| None (untreated skin *A*) | 232 | 236 | 256 | 306 | 309 | 340 | 342 | 325 | 247 | 358 | 420 | 440 |
| None (untreated skin *B*) | 279 | 241 | ... | 250 | 244 | 346 | ... | ... | ... | ... | ... | ... |
| Petrolatum | 214 | 257 | 248 | 296 | 261 | ... | ... | ... | 291 | 310 | ... | ... |
| Polysorbate 85 | 213 | 209 | 241 | 267 | 296 | 359 | 343 | 315 | 317 | 458 | 462 | 473 |
| Sorbitan trioleate | 211 | 192 | 245 | 347 | 362 | 429 | 315 | 310 | 227 | 466 | 459 | 445 |
| Polyoxyethylene ether 96 | 212 | 203 | 359 | 427 | 325 | 469 | 325 | 315 | 330 | 452 | 464 | 486 |

ence standards. Using DNA as a reference, the range of these percentages is narrower than if wet weight is used as a reference standard. The second difference is apparent with the results of the 10 day treatment. Here not only the range is narrower but the increase of phosphorus is also smaller when using DNA as a reference. The smaller spread in results indicates that DNA may be more reliable as a reference standard, but the wet weight still can be used as a practical and useful standard of reference, especially if the DNA content is also influenced by the test.

Table V shows the comparison of the 2 reference standards. DNA content, as related to wet weight of the sample, shows an increase after 10 days of treatment. This might be due to irritation of the skin, caused by the surfactant treatment which could initiate a higher metabolic and mitotic activity. An increased rate in cell proliferation, and hyperplasia of the epidermis, are often paralleled with an increased rate of DNA synthesis and an increase in DNA content (21–24).

It was postulated (8) that the effect of nonionic surfactants on the respiratory metabolism of rabbit skin is partly an indirect one and may be due to permeability changes of cell membranes.

The structure of biological membranes is based upon a lipid–protein positional relationship. The classical Danielli-Davson (25) model of the membrane and the more recent "unit membrane" hypothesis of Robertson (26) have suggested a bimolecular phospholipid leaflet as the backbone of membrane structure. Recent reports and reviews (27–32) describe the structure and function of membranes in a more detailed manner. All of these descriptions are highly speculative in nature; there is no proven theory for the exact nature of membranes at the molecular level.

On the basis of these hypotheses it can be stated, however, that a qualitative and/or quantitative change in the lipid composition of a tissue might indicate structural changes in the membranes, especially if these changes were induced by substances which more likely act on the membranes.

Surfactants, due to their hydrophilic–lipophilic character, generally possess a particular affinity for membranous structures. As a result of this affinity, a nonionic surfactant can (*a*) interact by hydrophobic bonding, being a macromolecule itself; (*b*) interact by hydrogen bonding, because of the strong hydrogen bonding affinity of the ether oxygens of the polyoxyethylene chains; (*c*) disturb the micellar structure of phospholipids, forming micelles, or replacing phospholipid molecules in the continuous lipid micelles; and (*d*) interact with water molecules participating in these micelles. These are only a few possibilities which may result in alterations of membrane structure and, consequently, functional changes and metabolic disorders.

On the basis of the postulation that the primary site of action of surfactants is in the biological membranes, it was decided to seek ways to test this possibility. It was apparent that proving this hypothesis with direct methods would be difficult, since there is no way at the present time to study membrane structures at the molecular level. The determination of quantitative changes of phospholipids, an indirect method, was intended to investigate and to support this hypothesis.

The probable reason for the increase of phospholipids is 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. To repair this damage, a higher rate in the biosynthesis of certain phospholipids should be expected, in order to regenerate the original membrane structure. Phospholipid molecules present in ruptured membranes and those that are possibly replaced by surfactants are measured along with the newly formed molecules during the analysis of skin tissue.

An investigation of the effects of polysorbate 80 on the *in vitro* metabolism of cells, reported by Kay (33), should be mentioned here to support this hypothesis. Treatment with polysorbate 80 induced a marked increase in cell permeability in preparations of Ehrlich-Lettré ascites cells. An extraordinary increase (up to almost 300%) in the incorporation of $^{32}P$ into phospholipid fractions was also observed in cell preparations containing 1% polysorbate 80.

The above phenomena might also be explained by the assumption that the increase in permeability is due to structural changes (disruption of membranes). The rapid incorporation of $^{32}P$ into phospholipids after the surfactant treatment suggests a reconstitution of cell membrane material.

A further study of the rate of biosynthesis of phospholipids in rabbit and in human skin treated with surfactant preparations should prove the above hypothesis.

Another well-known phenomenon, namely, that surfactants generally increase percutaneous absorption of various substances (34), could be explained partly by permeability changes, due to structural changes in skin "barrier" membranes.

The results reported here are in accordance with the authors' previous findings regarding gross and microscopic changes in rabbit skin induced by surfactants. It is apparent that the polyoxylene ether-type surfactants have the highest capacity to pro-

duce dermal reactions: severe erythema, hyperkeratinization, and desquamation with marked induration and fissuring on the macroscopic level; inflammation with a number of polymorphonuclear and round cells in the dermis and acanthosis with hyperplasia and various degrees of necrosis in the epidermis on the microscopic level; and the highest increase in lipid phosphorus content.

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# Reaction Gas Chromatography III

## Recognition of Tropane Structure in Alkaloids

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**The technique of "reaction gas chromatography" involving dehydrogenation of heterocyclic compounds by a platinum-firebrick catalyst was applied to alkaloids possessing the tropane moiety in their structures. The reaction chromatogram of 3-tropanol, from which most of these alkaloids may be considered to be derived, exhibited several peaks, among which were those due to pyrrole, methylpyrrole, pyrrolidine, pyridine, piperidine, and toluene. These products were also produced by atropine, homatropine, scopolamine, ecgonine, and cocaine when these alkaloids were examined under similar experimental conditions. Atropine, homatropine, and scopolamine exhibited some additional peaks which were correlated with their structures.**

APPLICATIONS OF reaction gas chromatography involving catalytic dehydrogenations, to alicyclic and heterocyclic compounds in general and to monoterpenoids in particular, were described in earlier publications from this laboratory (1, 2). The success achieved in experiments with heterocyclic compounds (1) prompted the authors to extend the technique to bases belonging to the tropane group of alkaloids. The present communication reports the results of the investigations. It was observed that these alkaloids as well as tropine, the alcohol from which they are derived, suffered pyrolytic dehydrogenation when they were passed through a reactor packed with 5% platinum on base-washed firebrick. Structures of the substances examined could be meaningfully correlated with the products of reaction and the technique may be employed for the detection of tropane moiety in natural and synthetic alkaloids.

Received April 5, 1967, from the Research Laboratories, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada.
Accepted for publication August 8, 1967.
Presented to the Thirteenth Canadian Conference on Pharmaceutical Research, Saint John, New Brunswick, Canada, August 16, 1966.
The authors are thankful to Dr. Leo Levi for his interest in these investigations.
Previous paper: Mizrahi, I., and Nigam, I. C., *J. Chromatog.*, **25**, 230(1966).

## EXPERIMENTAL

**Apparatus**—*Gas Chromatograph*—Burrell Kromotog K-2 equipped with thermal conductivity detector and gas valves for directing the carrier gas to the chromatographic column directly or *via* the reactor, as required.

*Column*—Glass tube (225 cm. long, 6 mm. i.d.)