Dermatitic Effect of Nonionic Surfactants III: Incorporation of ^{32}P into Phospholipids and Acid Soluble Material of Normal and Surfactant-Treated Rabbit Skin *In Vitro*

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Abstract □ The site of action of surfactants incorporated in ointment bases was investigated with the aid of radioactive tracer techniques. Representative samples of the two major types of nonionic surfactants, polysorbate 85 and polyoxyethylene (10) oleyl ether were incorporated into white petrolatum and applied daily to rabbit skin clipped free of hair. After 4 days of treatment the excised epidermis was analyzed for phospholipids, nucleic acids, and the *in vitro* incorporation of ^{32}P into phospholipids and the trichloroacetic acid (TCA)-soluble fractions. Results indicated that the surfactant treatment induced an increase in the epidermal phospholipid content, and a decrease in the phosphorus content of the TCA-soluble fraction. The incorporation of ^{32}P into phospholipids and the TCA-soluble fraction was approximately 2–2.5 times greater in the surfactant-treated skin samples than in control samples. These and other available data suggest that the site of action of surface active agents is in the biological membranes. These surfactants may interact with membranes by either rupturing them, replacing certain phospholipids present in the lipid micelles, or inducing configurational changes in these micelles.

Keyphrases □ Dermatitic effect—nonionic surfactants □ ^{32}P incorporation—epidermal phospholipids, acid soluble materials □ Surfactants in ointments—absorption effect □ Scintillometry—analysis

The results of the authors' previous experiments (1, 2) indicated that nonionic surface-active agents, incorporated into ointment bases have a distinct potential to irritate rabbit skin and cause histological and biochemical changes in the skin to which they are applied. On the basis of these results and other reports (3–7) it was postulated that the site of action of the tested surfactants is in the biological membranes. It is, unfortunately, difficult to devise experiments that would unambiguously prove the above hypothesis since there is no way, at present, to study membrane structures at the cellular or molecular level. There are several, often controversial, theories concerning the structure and function of biological membranes. Recent reviews (8–14) describe most of these theories. All of these descriptions are highly speculative in nature: there is no proven theory for the exact structural configuration of membranes at the molecular level. On the basis of the presently accepted concepts of biological membranes, it can be stated, however, that a qualitative and/or quantitative change in lipid (particularly phospholipid) composition of a tissue might indicate structural changes, and consequently, functional changes in the membranes. Results of the determination of quantitative changes of phospholipids, indicated an increase in the phospholipid content of the surfactant-treated skin (2). 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 are measured along with the newly formed molecules during the analysis of skin tissue. To repair the damage, a higher rate in the biosynthesis of certain phospholipids should be expected, in order to regenerate the original membrane structure.

To substantiate this assumption, *in vitro* tests measuring the rate of ^{32}P incorporation into phospholipids and their precursors were carried out. This report presents the results related to the rate of biosynthesis of phospholipids in the control and treated skin samples.

MATERIALS AND METHODS

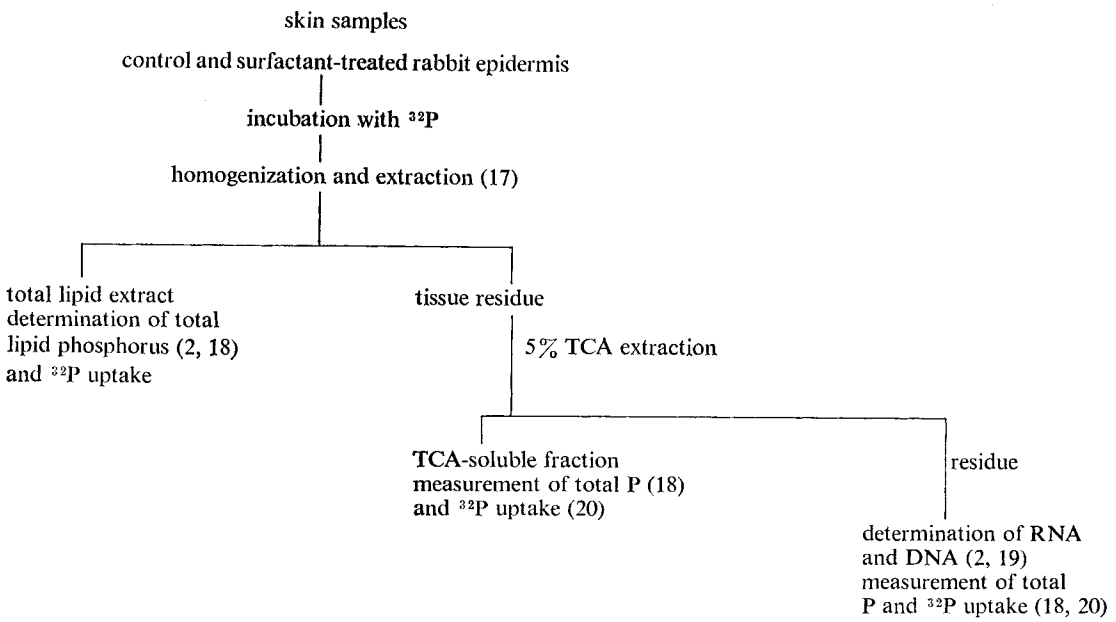
Preparation, Isolation, and Incubation of Skin Samples—New Zealand white rabbits of both sexes, 3 to 4 months old, were used as experimental animals. They were housed in hanging screen-bottom cages and were maintained on Purina rabbit chow and water *ad libitum*. The trunk of each rabbit was clipped free of hair with an electric hair clipper (Oster model A2, size 40), and was divided into three areas. Harness-type restrainers (15) were used for restraining rabbits with a minimum amount of stress.

Polysorbate 85[1] and polyoxyethylene ether 96[2] were incorporated into white petrolatum USP in 10% concentration and were applied to the center of the designated areas of rabbit skin daily. The third area on the rabbits' back was reserved for skin treated with the ointment base only, which served as control. The sites of control and treated areas were randomly selected from different animals to exclude any influence of body area. After 4 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 (16) set to cut a 0.1-mm. thick skin slice. The weight of each sample was around 100 mg. The elapsed time between removing and weighing the samples was not more than 1–2 min. Immediately after weighing, the skin samples were incubated at 37° in 50-ml. beakers in a metabolic shaking incubator for 30 and 60 min. The incubation media, 4.0 ml. for each sample, contained 56 m*M* glucose, 5 m*M* KCl, 147 m*M* NaCl, and approximately 40 μc. ^{32}P (40 μc. ^{32}P/2-μmole Na_2HPO_4). After incubation the tissues were placed on a wire screen and washed with 25 ml. of each of the following solutions: 2% Na_2HPO_4, 1% Na_2HPO_4, and distilled water at 4°. The samples were then homogenized in a glass homogenizer and extracted with 2.0 ml. of chloroform–methanol (2:1) according to the method of Folch *et al.* (17). Nonlipid contaminants were removed from the chloroform–methanol extracts with 0.2 vol. of 0.05% $CaCl_2$ solution.

Radioactive and Quantitative Determination of Phospholipids, their Precursors, and Nucleic Acids—Aliquots of the chloroform–methanol extracts, containing the phospholipids, were used to determine the lipid phosphorus content and the ^{32}P incorporation into phospholipids. The phosphorus content of lipid extracts, TCA-

[1] Marketed as Tween 85 by Atlas Chemical Industries, Inc., Wilmington, Del.

[2] Marketed as Brij 96 by Atlas Chemical Industries, Inc., Wilmington, Del.



Scheme I. Representation of methods used

soluble, RNA, and DNA fractions were determined by the method of Bartlett (18). Radioactivity of phospholipid extract was measured by a liquid scintillation counter (Unilux I. Nuclear Chicago) using toluene counting solution (OMNIFLUOR). The RNA and DNA content was determined by the method of Santen and Agranoff (19). The radioactivities of TCA-soluble, RNA, and DNA fractions were measured in the above-mentioned liquid scintillation counter without the use of scintillator as described by Clausen (20). The radioactivities are expressed as counts per minute per 100 mcg. phosphorus present in the sample measured. Scheme I summerizes the methods used.

RESULTS AND DISCUSSION

The initial tests were designed to find the optimal experimental conditions regarding the amount of epidermal tissue, incubation media, and labeled phosphorus in the incubation mixture, as well as techniques to remove the adsorbed ^{32}P from the skin tissue to be homogenized.

Figure 1 illustrates the results of further experiments using ten rabbits under the experimental conditions outlined above. The ^{32}P incorporation into epidermal phospholipids is considerably higher in the surfactant-treated skin samples than in the skin samples treated with the ointment base only. Figures 2 and 3 demonstrate the change in phospholipid content and the increased rate of ^{32}P incorporation into the epidermal phospholipids of treated skin samples. It can be seen from these figures that the phospholipid content of treated skin samples is increased and that the ^{32}P incorporation into phospholipids is much greater in skin samples treated with surfactant-containing ointments. When data of control samples are taken as 100%, the average percent increase in the rate of ^{32}P incorporation into epidermal phospholipids is 221 and 230% in the skin samples treated with ointments containing polysorbate 85 and polyoxyethylene ether 96, respectively. It was postulated (2) that a probable reason for the increase of phospholipids is that the surfactants damaged the biological membranes

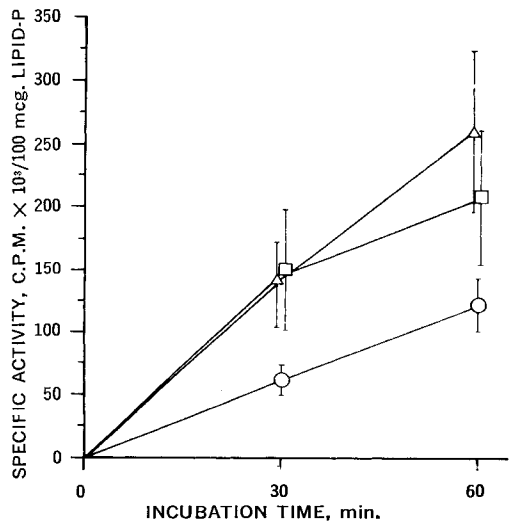


Figure 1—*Incorporation of ^{32}P into epidermal phospholipids expressed as counts per minute (C.P.M.) per 100 mcg. lipid phosphorus (lipid-P) present in the tested sample. Skin samples treated with Key: O, petrolatum (control); △, polysorbate 85, 10%; and □, polyoxyethylene ether 96, 10%. Brackets over each point indicate SD (N = 10).*

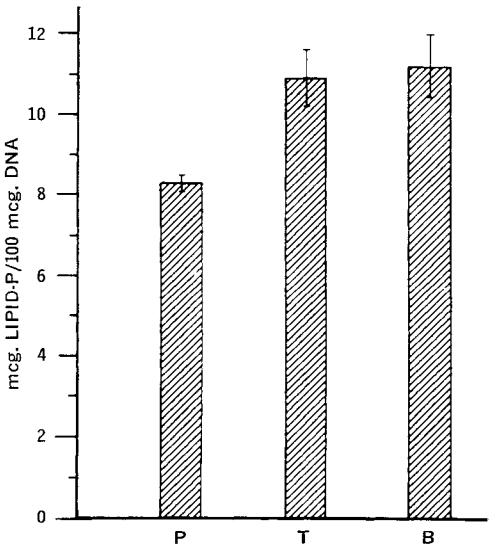


Figure 2—*Epidermal phospholipid content measured as mcg. lipid phosphorus per 100 mcg. DNA content of each sample. Each column represents the mean of results from experiments with ten rabbits (N = 10) with standard deviation. Skin samples treated with Key: P, petrolatum; T, polysorbate 85, 10%; and B, polyoxyethylene ether 96, 10%.*

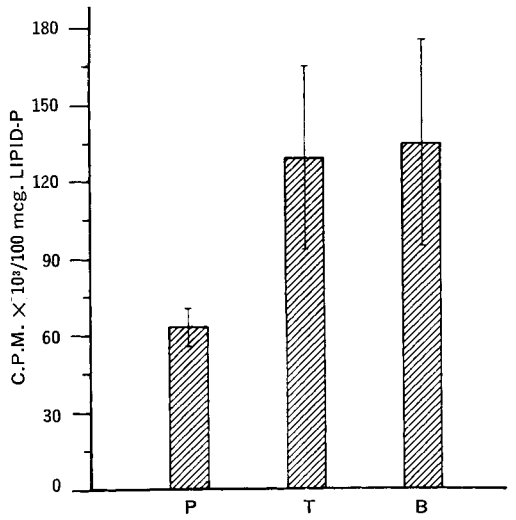


Figure 3—*The rate of ^{32}P incorporation into epidermal phospholipids (30 min. incubation) expressed as specific radioactivity of phospholipids; C.P.M. per 100 mcg. lipid phosphorus present in the tested sample. Each column represents the mean of results from experiments with ten rabbits (N = 10) with standard deviation. Skin samples treated with Key: P, petrolatum; T, polysorbate 85, 10%; and B, polyoxyethylene ether 96, 10%.*

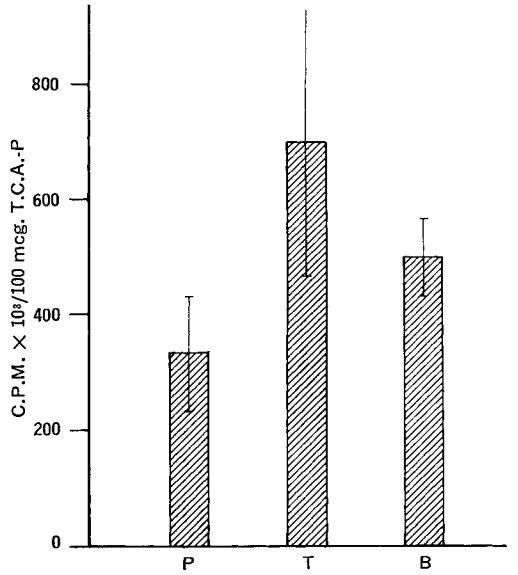


Figure 5—*The rate of ^{32}P incorporation into TCA-soluble fraction (30 min. incubation) expressed as specific radioactivity; C.P.M. per 100 mcg. phosphorus present in the TCA-soluble fraction. Each column represents the mean of results from experiments with six rabbits (N = 6) with standard deviation. Skin samples treated with Key: P, petrolatum; T, polysorbate 85, 10%; and B, polyoxyethylene ether 96, 10%.*

by either rupturing the membranes or replacing certain 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 (2). The higher rate of ^{32}P incorporation into phospholipids in the case of treated skin samples indicates that the rate of biosynthesis of phospholipids in the treated skin samples is greater. Other supporting data for different rates of synthesis of phospholipids can be seen in Figs. 4 and 5. The phosphorus content of the TCA-soluble fraction, which contains precursors of phospholipids, is lower in the treated skin samples than in the control areas. The specific radioactivity of these precursors, on the other hand, shows an opposite pattern, meaning that the utilization rate of these precursors for the synthesis of phospholipids is much higher in the treated skin samples; the phospholipid pool is lowered, and the ^{32}P incorporation rate is greater in the surfactant-treated skin samples than in the untreated samples. When comparison is made between the phospholipid content and specific activity of the phospholipids, and of the TCA-soluble fraction, it may be concluded that the epidermal phospholipids in the surfactant-treated skin samples are synthesized and metabolized at a higher rate than in the controls.

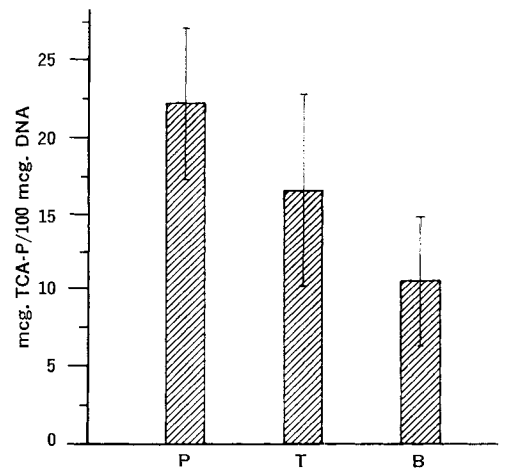


Figure 4—*Phosphorus content of TCA-soluble fraction (TCA-P) of control and surfactant-treated rabbit epidermis expressed as mcg. of phosphorus per 100 mcg. DNA present in the sample. Each column represents the mean of results obtained from experiments with six rabbits (N = 6) with standard deviation. Skin samples treated with Key: P, petrolatum; T, polysorbate 85, 10%; and B, polyoxyethylene ether 96, 10%.*

At present the authors are not able to provide a reliable explanation for the above results. They agree with Dawson (21) that "The puzzle of phospholipid turnover still largely remains a challenge to the genius and ingenuity of future investigators." However, some reasonable explanations and interpretations of the authors' experimental data to specify the primary site of action of surfactants and to project further objectives is presented.

The synthesis of phospholipids in skin may be required mainly for the steady turnover of structural elements and the regeneration of the surfactant-damaged membranes. It may also be needed in net growth—the authors reported hyperkeratinization and hyperplasia of the epidermis as the effects of surfactants applied regularly to the skin (1). The latter type of synthesis might be in excess of that required for the turnover or regeneration of structural elements. To eliminate the possibility of misinterpretation of the increased rate of synthesis, the DNA content of each sample was determined, which should reflect the cell number of that sample. The changes in specific activities of epidermal phospholipids of treated and control skin samples are demonstrated by Fig. 6 in which DNA has been used as the standard of reference. It is generally believed that the turnover or synthesis of phospholipids is very slow in tissues where phospholipid secretion or net new growth is negligible (21). In other tissues, liver, lung, and endocrine tissues, the synthesis of phospholipids is much greater, where the phospholipid may be needed for membrane structural requirements as well as for phospholipid-containing secretions (bile and plasma proteins secreted by the liver cells, lipoprotein surfactants from the lungs), or it may be required in net growth. According to data shown by Fig. 6 the increased turnover rate of phospholipids cannot be due only to net growth induced by the surfactant treatment. The specific activity of epidermal phospholipids in the treated skin samples is still higher when related to DNA content (cpm./mcg. P/mcg. DNA). If the content and the biosynthesis of phospholipids were increased because of net growth, the DNA content should have increased proportionally to the net growth, and consequently should lower the values of specific activity of phospholipids in the surfactant-treated samples to that of the control samples. The DNA content also increased as a result of treatment with surfactants (2) but this increase was not in proportion with the increase of phospholipid content and biosynthesis of phospholipids. On the basis of DNA content, the specific activity of epidermal phospholipids of

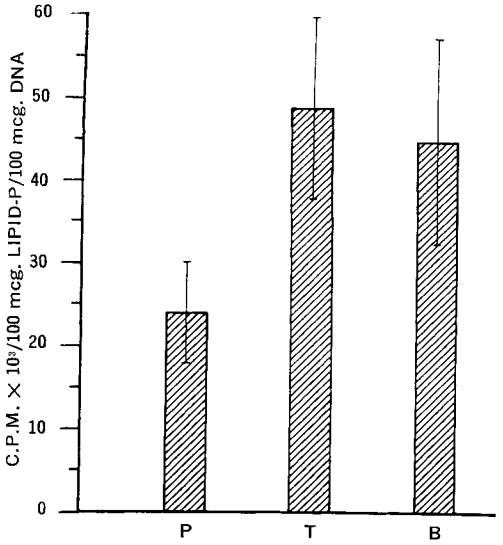


Figure 6—*Specific activity of epidermal phospholipids on the basis of DNA content (30 min. incubation) expressed as C.P.M. per 100 mcg. lipid phosphorus per 100 mcg. DNA content of the sample. Each column represents the mean of results from experiments with ten rabbits* (N = *10*) *with standard deviation. Skin samples treated with Key: P, petrolatum; T, polysorbate 85, 10%; and B, polyoxyethylene ether 96, 10%.*

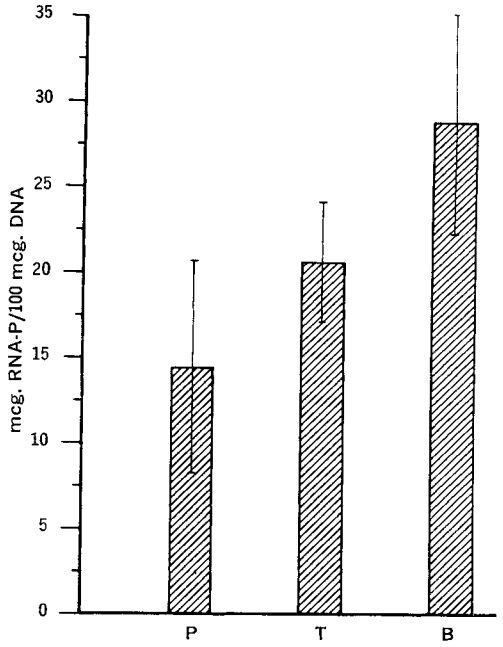


Figure 7—*The epidermal RNA content of the control and surfactant-treated rabbit skin, expressed as RNA phosphorus per 100 mcg. DNA content of the sample. Each column represents the mean of results from experiments with six rabbits* (N = *6*) *with standard deviation. Skin samples treated with Key: P, petrolatum; T, polysorbate 85, 10%; and B, polyoxyethylene ether 96, 10%.*

the surfactant-treated samples is still almost twice as high as that of the control.

It may be questioned whether or not the synthesis and breakdown of phospholipids in epidermis can always be an essential factor for maintaining the structural integrity of epidermal (barrier) membranes. It is possible that the considerable synthesis or catabolism of phospholipids may occur during the movement and alteration of the membrane structure during cellular activity, which is influenced by the surfactant treatment.

A higher turnover rate of phospholipids in treated skin samples might also be explained by presuming that lipid micelle-surfactant interactions changed the packing and the alignment of phospholipid molecules in the membranes, exposing them to greater enzyme attack. It has been realized recently that the rate and extent of the attack of phospholipases on phospholipids is controlled, not only by the chemical nature of the substrate, but also by the area and physical nature of its substrate (21). Each phospholipid in the membranes has a surface area available for enzymatic attack depending on the micellar configurations which may present a better approach and orientation of an attacking enzyme molecule. This leads to a higher rate of metabolism of phospholipids and, consequently, to changes in membrane structure. Lucy (12) has stated recently that the proportion of lipid molecules in the micellar configuration may vary, not only from one membrane to another, but also within any one membrane, depending on the micro-environment and on the chemical constitution of the membranes. The surfactants may change the micellar structure of membrane lipids by several mechanisms: (*a*) it may replace one lipid component in the continuous lipid micelles, (*b*) it may interact with the water molecules participating in the lipid micelles, since surfactants can also form micelles in aqueous media; and/or (*c*) it may act by hydrogen or hydrophobic bondings. Reversible and irreversible transitions in micellar arrangements are very important in both physiological and pathological phenomena.

Data illustrated by Fig. 7 can also supplement the above findings. An increase in the content of RNA indicates that the RNA synthesis is also stimulated by the surfactant treatment. This may be due to an increased synthesis of messenger RNA's which are responsible for the synthesis of (lipid and protein) components of the altered membranes (4). Another explanation for the increase in RNA content could be that the membrane-bound RNA which is bound to the damaged membrane is regenerated simultaneously with the new membrane (22).

Comparing the results of control and treated skin the authors can see a definite change in the content and the rate of synthesis of phospholipids of the treated skin samples. Considering the physicochemical properties of the surfactants and the above results, it is concluded that the above changes are mainly due to structural changes in the membranes. The membrane might be damaged by the surfactant as a result of hydrophobic or micellar interactions, or by hydrogen bonding; therefore, the rate of synthesis should be increased to regenerate the original membrane structure. *In vivo* experiments to further investigate the above speculations are in progress.

REFERENCES

(1) M. Mezei, R. W. Sager, W. D. Stewart, and A. L. deRuyter, *J. Pharm. Sci.*, **55,** 854(1966).

(2) M. Mezei and R. W. Sager, *ibid.*, **56,** 1604(1967).

(3) A. D. Bangham and R. W. Horne, *J. Mol. Biol.*, **8,** 660 (1964).

(4) E. R. M. Kay, *Cancer Res.*, **25,** 764(1965).

(5) G. Weissmann, G. Sessa, and S. Weissmann, *Biochem. Pharmacol.*, **15,** 1537(1966).

(6) T. Kondo and M. Tomizawa, *J. Colloid Interface Sci.*, **21,** 224(1966).

(7) A. C. Allison, *Brit. Med. Bull.*, **24,** 135(1968).

(8) D. E. Green and A. Tzagoloff, *J. Lipid Res.*, **7,** 587(1966).

(9) E. D. Korn, *J. Gen. Physiol.*, **52,** 257(1968).

(10) V. P. Whittaker, *Brit. Med. Bull.*, **24,** 101(1968).

(11) A. D. Bangham and D. A. Haydon, *ibid.*, **24,** 124(1968).

(12) J. A. Lucy, *ibid.*, **24,** 127(1968).

(13) A. Rothstein, *Ann. Rev. Physiol.*, **30,** 15(1968).

(14) D. F. H. Wallach and A. Gordon, *Federation Proc.*, **27,** 1263(1968).

(15) E. A. Newmann, *Lab. Animal Care*, **13,** 207(1963).

(16) H. Blank, E. W. Rosenberg, and I. Sarkany, *J. Invest. Dermatol.*, **36,** 303(1961).

(17) J. Folch, M. Lees, and G.H. Sloane-Stanley, *J. Biol. Chem.*, **226,** 497(1957).

(18) G. R. Bartlett, *ibid.*, **234,** 466(1959).

(19) R. J. Santen and B. W. Agranoff, *Biochem. Biophys. Acta*, **72,** 251(1963).

(20) T. Clausen, *Anal. Biochem.*, **22,** 70(1968).

(21) R. M. C. Dawson in "Metabolism and Physiological Significance of Lipids," R. M. C. Dawson and D. N. Rhodes, Eds., John Wiley and Sons, Ltd., London England, 1964 p. 179.
(22) J. R. Tata, *Nature*, **219**, 331(1968).

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Essential Oil of *Anemopsis californica* Part II: Minor Constituents

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Abstract □ The minor constituents of the essential oil hydrodistilled from the roots and rhizomes of *Anemopsis californica* (Nutt.) Hook and Arn. (Fam. *Saururaceae*) have been isolated by gas chromatography on two stationary phases: polypropylene glycol adipate and polyethylene glycol. The isolated compounds were identified by the comparison of their IR spectra with those of pure authentic compounds. Esdragol, thymolmethylether, linalool, *p*-cymene, 1,8-cineol, *d*-limonene, camphene and α- and β-pinene have been shown to be present in the oil. In addition, the presence of two ketones, a *sec* alcohol, tentatively identified as 1-methyl-4-isopropyl-6-hydroxy-bicyclo (3:1:0) hexane, an ester, and six hydrocarbons is also indicated on the basis of the IR spectra of the corresponding fractions.

Keyphrases □ *Anemopsis californica* oil—minor constituents □ GLC—separation, identity □ IR spectrophotometry—identity □ NMR spectroscopy—identity

Methyleugenol is the major constituent of the essential oil hydrodistilled from the roots and rhizomes of *Anemopsis californica* (Nutt.) Hook and Arn. (Fam. *Saururaceae*). Thymol and piperitone have also been shown to be present in appreciable quantity (1). These three compounds together make up approximately 74% of the oil. The isolation and identification of nine minor constituents of the essential oil of *Anemopsis* is reported here.

EXPERIMENTAL

Extraction of Essential Oil—The essential oil used in the previous experiments (1) was used for the quantitative determination of the constituents. Additionally, 500 g. crude drug (1) + 3.5 l. water was also hydrodistilled in the apparatus described earlier (1). However the distillation was carried out in an interrupted manner. After the first short period of distillation, the flask was cooled and the collected oil removed and stored over anhydrous Na_2SO_4. Distillation was then resumed for another short period followed by cooling and removal of oil. A series of fractions were thus separately collected as shown in Table I.

Table I—Fractional Collection of Essential Oil of *A. californica*

Fraction No	Distillation Period, hr.	Volume Collected, ml.	Color of Fraction
1	2.5	2.5	Colorless
2	2.0	2.5	Pale yellow
3	3.75	4.0	Pale yellow
4	4.0	4.0	Light-green
5	3.75	4.0	Greenish-blue
6	7.50	8.0	Greenish-blue
7	6.0	6.0	Deep bright-blue
Total	29.25	31.0	

Gas Chromatography (GC)—The chromatograph, recorder, and column dimensions were described earlier (1). In addition, a recorder[1] with an integrator,[2] was used for quantitation of peaks. The chart-speed of this recorder was 2.54 cm. (1 in.)/3 min.

Packing—Two stationary phases polypropylene glycol adipate[3] and polyethylene glycol[4] were coated on acid-washed diatomite aggregate[5] 60–80 mesh, as previously described and packed in two separate columns. Each column was preconditioned overnight at 170° in a slow stream of helium. The operating conditions were as follows: carrier gas, helium, 75 ml./min., inlet pressure Column A, 20.5 p.s.i. and Column B, 28 p.s.i.; detector, 240°, 150 ma. filament current; injection port, 200°; outlet 180°; column temperature, Column A, 160°, isothermal and 110–170°, isothermal-linear, and Column B, 180°, isothermal and 110–190°, isothermal-linear. In the isothermal-linear mode the starting temperature of the column (110°) was maintained for 15 min. after injection of the sample and then raised to the final temperature for each column by manual programing.

GC Fraction Collection and Identification of Constituents—The effluent peaks were collected in U-shaped traps fashioned from 22.86-cm. (9-in.) lengths of 0.31-cm. (0.125-in.) glass tubing. The straight, long arm of the trap was connected to the heated chromatograph

[1] Varian Aerograph model-20, Varian Aerograph Instruments Inc., Walnut Creek, Calif.
[2] Disc Chart, model-244, Disc Instruments Inc., Santa Ana, Calif.
[3] Reoplex-400, Union Carbide Corp., New York, N. Y.
[4] Carbowax-20M, Union Carbide Corp., New York, N. Y.
[5] Chromosorb-W, Johns-Manville Products Corp., New York, N. Y.