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Keyphrases

Testosterone micellar solubilization
Ionic surfactants—testosterone solubilization
Z values—determination
Surfactant concentration effect—*Z* values

Interaction of NO_2 with Monolayers of Phospholipids Extracted from *E. coli* at 15 and 37°

By N. D. WEINER, M. AMANAT, D. BLONDO, R. CAPRIOLI, N. DINERMAN, and A. FELMEISTER*

Phospholipids were extracted from *E. coli* grown at 15 and 37°. The fatty acid residues of the 15° phospholipids were found to be considerably more unsaturated than the 37° phospholipids. These phospholipids were spread as monomolecular films and exposed to NO_2-containing atmospheres. Whereas the 37° phospholipid films showed no interaction, NO_2 was found to expand considerably the 15° phospholipid films. The results demonstrate that simple changes in environmental conditions may affect markedly the interaction of air pollutants such as NO_2 with biological membranes.

Isolation and characterization of membrane components from a wide variety of organisms indicate that the compositions of the membrane phospholipids differ significantly, not only from organism to organism, but even from tissue to tissue within a single organism (1). The phospholipid composition of the membranes of a number of microorganisms has also been shown to vary considerably with varying growth conditions. For example, Engleman, Terry, and Morowitz (2) point out that the fatty acid residues of the membrane phospholipids of *Mycoplasma laidlawii* are a function of the fatty acids included in the growth medium. Marr and Ingraham (3) demonstrated that the degree of unsaturation of membrane fatty acids of *E. coli* is dependent on growth temperature. These latter workers pointed out that the increase in unsaturation of the fatty acids of *E. coli* grown at lower temperatures is part of an expected adaptive process. The degree of fluidity exhibited at low temperatures by these unsaturated acids compares to that of their saturated counterparts at normal growth temperatures. Thus, the organism is able to maintain the level of membrane transport and other essential processes even at temperatures well below the normal optimum level.

In previous studies of the interaction of air pollutants with monomolecular films, it was noted that NO_2 expanded monolayers of egg lecithin (where 50% of the fatty acid groups contain at least one unsaturated bond), but did not affect monolayers of synthetic dipalmitoyl lecithin. The expansion apparently is the result of a chemical interaction of NO_2 with the double bonds of the unsaturated fatty acid groups of egg lecithin rather than a simple physical penetration into the film (4).

In view of these latter results and Marr and Ingraham's report, it was of interest to determine whether the membrane phospholipids extracted from *E. coli* grown at 15 and 37°, respectively, would exhibit similar differences in their interaction with an air pollutant such as NO_2.

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EXPERIMENTAL

All inorganic salts were reagent grade; the glucose was USP quality. All organic solvents were spectrograde and distilled prior to use.

The growth media used for both the 15 and 37° studies consisted of: 3 g. glucose; 2 g. NH_4Cl; 6 g. Na_2HPO_4; 3 g. KH_2PO_4; 3 g. NaCl; 85 mg. $MgCl_2 \cdot 6H_2O$; 115 mg. Na_2SO_4; 1,000 ml. distilled water.

E. coli (ASTM No. 9637) was inoculated into a flask containing 1,000 ml. of media and the flask was incubated at 37° until the absorbance of the mixture at 600 mμ was about 0.5. Five-hundred milliliters of the resultant mixture was then added to each of two flasks containing 950 ml. of media. The flasks were then placed in water baths at 15 and 37°, respectively. Incubation was allowed to proceed, in the presence of continuous aeration, until the absorbance of the mixture at 600 mμ was 0.7–0.8. The time required to reach this absorbance was about 72 hr. for the 15° batch and about 24 hr. for the 37° batch.

The cells were harvested by centrifugation at 5,000 r.p.m. at 4° for 30 min. The lipids were extracted from the bacterial cells by a modification of the procedure of Kanfer and Kennedy (5). The cells were added to flasks containing 300 ml. of chloroform–methanol (2:1, v/v), and the contents were stirred with a magnetic stirrer for 24 hr. The mixture was then filtered through a thick plug of glass wool to remove most of the cellular debris. The filtrate was then washed three times with equal volumes of 2 *M* KCl and once with an equal volume of water. The solvent was then evaporated by the use of a flash evaporator until a viscous slurry was obtained. The slurry was then redissolved in a minimum amount of chloroform–methanol (2:1, v/v).

The resultant yellow solution was fractionated by column chromatography using a modification of the method of Huston and Albro (6). A slurry of 60 g. of silicic acid (Mallinckrodt, 100/200 mesh) in *n*-hexane was packed into a 2 × 40 cm. column, and the flow rate was adjusted to 1.5 ml./min. The column was then washed with 50-ml. portions each of acetone, ethyl ether, *n*-hexane, and chloroform. The sample, contained in 2–3 ml. of solvent, was then added to the column.

The crude hydrocarbon fraction was eluted with 150 ml. of *n*-hexane–benzene (49:1, v/v), and the simple lipids were eluted next with 300 ml. of chloroform. The complex lipids were next removed by eluting with 300 ml. of chloroform–methanol (1:1, v/v), followed by 100 ml. of methanol. This complex lipid fraction was collected in increments of 10 ml. Each of these 10-ml. fractions was tested with ninhydrin reagent, and only the first 10 fractions, *i.e.*, the first 100 ml. of chloroform–methanol (1:1, v/v) eluate, yielded positive ninhydrin tests. The next five fractions, when concentrated by partial evaporation, also yielded positive ninhydrin tests, while all the remaining fractions gave negative tests. It was therefore decided to use the first 150 ml. of the chloroform–methanol (1:1, v/v) eluate as the total phospholipid fraction.

The identification and purity of this phospholipid fraction was determined by a modification of the method of Parker and Peterson (7). Both 15 and 37° fractions were spotted on Eastman Chromogram sheets (Silica Gel G) and developed using the Eastman Chromogram developing apparatus.

The solvent system of Parker and Peterson proved unsatisfactory in that the phospholipids traveled with the solvent front. A modified solvent system of chloroform–methanol–acetic acid–water (65:10:4:2) proved quite satisfactory. The samples were spotted 2.5 cm. from the bottom of the sheet and the solvent front was allowed to rise to within 5 cm. from the top of the sheet. The average running time was 4–5 hr.

The 15 and 37° samples, as well as a sample of bovine phosphatidyl ethanolamine (Applied Science Laboratories), gave single spots with identical R_f values as determined by development with ninhydrin reagent. No other spots were detected upon development with iodine vapors and concentrated sulfuric acid spray.

Qualitative iodine determinations of the 15 and 37° phospholipids demonstrated that the 15° samples were significantly more unsaturated than the 37° samples. This was confirmed by the method described by Weiner, Felmeister, and Amanat (8) where the surface-pressure increase of monolayers of phospholipids spread on an iodine solution subphase over that of an aqueous subphase was used to measure the degree of unsaturation.

A phosphorous determination according to the method of King (9) was performed on both the 15 and 37° solutions. On the basis of the molecular weight of phosphatidyl ethanolamine, it was calculated that the authors' extraction procedure yielded approximately 80 mg. of phospholipid per 10 l. of *E. coli*, grown at both 15 and 37°.

Both the 15 and 37° samples were then spread as monolayers and subjected to standard and NO_2-containing atmospheres by the method previously described by Felmeister *et al.* (4). The gases were metered by individual flowmeters into a glass tube equipped with baffles, which served as a premixing tube. Mixing was completed by passing the gases into a 500-ml. round-bottom flask. The gas mixtures were then led through a 0.9-m. length of glass tubing connected to a short length of Teflon tubing. The latter was affixed to the underside of the Lucite trough cover which served to maintain the desired gaseous atmosphere over the film. The Teflon tubing within this enclosure was formed into a loop, and a series of small perforations was made in the wall of the tubing. The shape of the loop and the positions of the perforations gave a uniform flow of the gases over the film surface.

The gas mixtures were permitted to flow through the system for at least 4 hr. before the start of each experiment to ensure steady-state conditions.

A Film Balance (Frater Instrument Co., Corona, N. Y.) was used to study the surface pressure-surface area (π-A) characteristics of the films. The balance consists of a Teflon-coated removable trough, totally free of metal contacts. Two variable-speed reinforced rigid Teflon stirrers are set into the trough to facilitate subphase mixing and temperature control. The temperature of the subphase was maintained at 25 ± 0.1° by circulating water from a constant-temperature bath through the water jacket around the trough. The precision lead screw, which drives the reinforced Teflon barrier, allows for changes in surface area of the

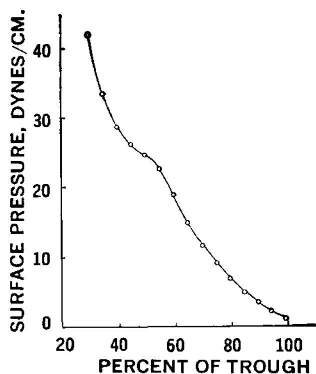

Fig. 1—π-A curve of 37° phospholipid exposed to standard atmosphere (air) and to NO_2-containing test atmosphere.

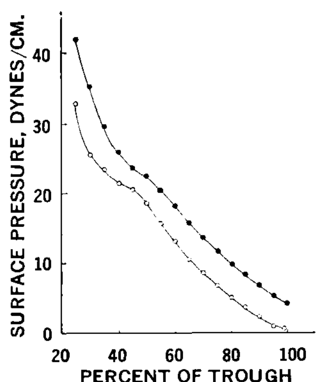

Fig. 2—π-A curves of 15° phospholipid exposed to standard atmosphere (air) (○); and to NO_2-containing test atmosphere (●).

trough as small as 0.0125 cm.². A quick disengage mechanism permits rapid sweeping of the film for cleaning the surface. The film balance was enclosed in a dust-protective cabinet.

Surface pressures were measured by the Wilhelmy plate method. A thin platinum plate, roughened to ensure complete wetting, was used.

Both the 15 and 37° samples were spread as monolayers from 1:1, v/v, methanol–chloroform solvent mixtures. It was found that when 0.05 ml. of solution was spread onto a subphase containing 0.9% roasted sodium chloride in double-distilled water, a surface pressure of about 1 dyne/cm. was observed, in the case of both the 15 and 37° samples.

These films were studied while exposed to a standard atmosphere (air flowing at the rate of 300 ml./min.) and to a test atmosphere (0.33% nitrogen dioxide in air) flowing at this same rate of 300 ml./min.

In all cases, the films were permitted to stand with the gases flowing for 1 hr. before manual compression of the film was initiated. Surface-pressure readings were then obtained at various film areas.

RESULTS AND DISCUSSION

The surface pressure-surface area (π-A) curve for the 37° phospholipid film exposed to the standard atmosphere is shown in Fig. 1. Exposure of the 37° phospholipid film to the NO_2-containing test atmosphere yielded a π-A curve identical to that of the standard (within ±0.4 dyne/cm. at all areas), indicating no film–NO_2 interaction. Figure 2 shows the π-A curves for the 15° phospholipid films exposed to the standard and test atmospheres. Exposure of 15° phospholipid films to the NO_2 test atmosphere resulted in a π-A curve that was considerably more expanded than that of the standard curve. This interaction, once it occurred, could not be reversed by replacing the NO_2 test atmosphere with the standard atmosphere.

It should be noted that all the curves in Figs. 1 and 2 show a shoulder at π values of about 20–25 dynes/cm. This effect was apparent only at very slow compression rates. Van Deenen *et al.* (10) also reported a shoulder for a synthetic phosphatidyl serine layered on a pH 4 subsolution.

It appears that the 15 and 37° materials differ from one another primarily in the degree of unsaturation of their fatty acid groups. The observed effect of NO_2 on the 15° phospholipid film is most probably the result of the interaction of NO_2 with the double bonds of the unsaturated fatty acid groups.

The results demonstrate that simple changes in environmental conditions may affect markedly the interaction of air pollutants, such as NO_2, with membranes. *In vivo* work now in progress in these laboratories on the effect of NO_2 on *E. coli* grown at different temperatures should serve to determine the relevance of this phenomenon to living systems.

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Keyphrases

Phospholipids—*E. coli* extracted
NO_2 atmosphere—phospholipid film
Film surface, pressure, area—determination
Column chromatography—separation
TLC—identity