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Inhibitory Effect of Cholesteryl Nitrate on Interaction of Nitrogen Dioxide with Cholesterol in Monomolecular Films

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Abstract □ Cholesterol monomolecular films exhibit a loss in surface pressure on exposure to nitrogen dioxide due to the formation and subsequent desorption of cholesteryl nitrate. The cholesterol–nitrogen dioxide interaction is inhibited by the prior addition of cholesteryl nitrate, and the degree of inhibition is a direct function of the mole fraction of the nitrate ester. Total inhibition occurs when this mole fraction is about 0.75. The differential equations developed to describe the sequence of events involved in this process were programmed for solution on an analog computer. This simulation indicated that at least six molecules of cholesteryl nitrate are required for each remaining cholesterol molecule to produce a total inhibition of the nitrogen dioxide–cholesterol reaction.

Keyphrases □ Cholesteryl nitrate, inhibitory effect—nitrogen dioxide–cholesterol interaction, monomolecular films □ Monomolecular films, nitrogen dioxide–cholesterol interaction—inhibitory effect of cholesteryl nitrate

Recently, it was reported that cholesterol monomolecular films, when exposed to approximately 175 p.p.m. of nitrogen dioxide for 60 min., exhibited a condensation effect (*i.e.*, a decrease in surface area at all surface pressures tested) that corresponded to a loss of about 75% of the cholesterol (1). Moreover, continued exposure did not result in any further loss of cholesterol (1). Other studies demonstrated that the formation and subsequent desorption of cholesteryl nitrate were responsible for this loss of cholesterol from the film and the concurrent condensation effect (2).

Similarly, Bergström and Wintersteiner (3) noted that the extent of autoxidation of cholesterol dispersed in an aqueous soap solution did not exceed 70%, even after extended exposure to air. These workers demonstrated that this limiting effect was caused by the build-up of the reaction products. Furthermore, their data indicated that this effect was not due to reversibility of the reaction but rather to a physical phenomenon related to the state of the system. The accumulation of the oxidation products at the surface of the soap–cholesterol micelles apparently served to protect the remainder of the cholesterol from oxidation (4).

It is also very likely that the reaction between cholesterol and nitrogen dioxide, which leads to the formation of cholesteryl nitrate, is irreversible. Under such conditions, then, the limiting effect observed after a loss of 75% of the cholesterol on exposure to nitrogen dioxide could be due to a physical interaction between the cholesteryl nitrate and the unreacted cholesterol.

This paper presents data which support this postulation and presents a reaction scheme that accounts for the experimental observations.

EXPERIMENTAL

Materials—Cholesterol[1] and cholesteryl nitrate[2] were used for the film studies. Their purity was verified by TLC using samples of 250 mcg. Single spots were observed in both cases. Solutions of these lipids were prepared in spectroscopic grade hexane. All other chemicals were of reagent grade. The water used for the subphase was first deionized and then distilled from an all-glass still just prior to use.

A mixture of 0.5% nitrogen dioxide[3] (99.5% pure) and 99.5% prepurified grade nitrogen[3] was used as the source of nitrogen dioxide.

Apparatus and Methods—The gas mixture was allowed to pass through a flowmeter at a rate of 100 ml./min. and directed into a short length of perforated Teflon tubing, which was fixed to the underside of a Lucite trough cover, as previously described (5). This served to maintain the desired gaseous atmosphere over the film.

The film balance used to study the surface pressure–surface area (π–A) characteristics of the film was described previously (5). Surface pressures were measured by the Wilhelmy plate method (6).

An EAI TR20 analog computer[4] was used to simulate possible models descriptive of the experimental results.

Solutions of the lipids in hexane were spread on a 0.065 M phosphate buffer at pH 7.0, and the gas was allowed to flow over the film for 60 min. At the end of this time, the gas flow was discontinued; any nitrogen dioxide remaining over the surface was removed by use of the exhaust fan in the hood in which the film-balance unit was set. Manual compression of the film was then initiated, and surface pressure readings were obtained at various film areas.

[1] Mann Research Laboratories, New York, N. Y.
[2] Eastman Organic Chemicals, Rochester, N. Y.
[3] Matheson Co., East Rutherford, N. J.
[4] Electronic Associates, Inc., Long Branch, N. J.

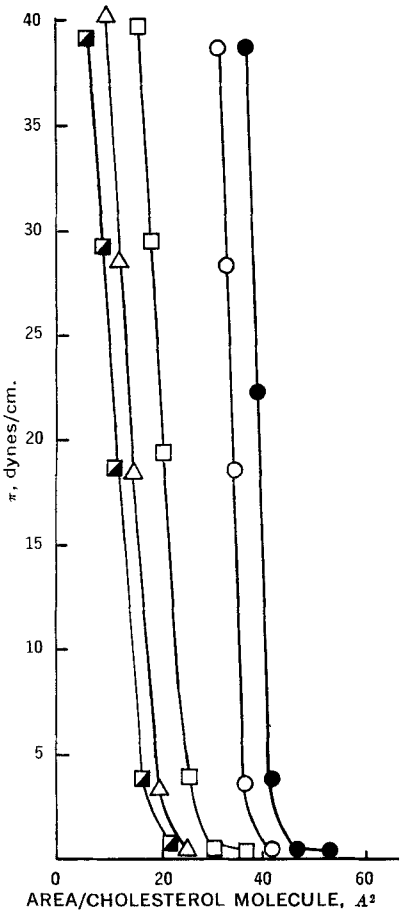

Figure 1—*Surface pressure* (π) versus *surface area per cholesterol molecule for various mole fraction mixtures of cholesterol and cholesteryl nitrate after exposure to 175 p.p.m. of nitrogen dioxide for 60 min. Key: Mole fraction of cholesteryl nitrate:* ◪, *0;* △, *0.25;* □, *0.50; and* ○, *0.75. Closed circle curve represents films of all of these mole fraction mixtures prior to exposure to nitrogen dioxide.*

The concentration of the nitrogen dioxide over the subphase during exposure was determined as follows: At periodic intervals during each experiment, 10-ml. samples of the atmosphere under the Lucite cover were drawn into a gas-tight Hamilton syringe containing 10 ml. of absorbing solution. The color of the absorbing solution was permitted to develop for 15 min., and the absorbance was measured at 550 nm. using a Spectronic-20 spectrophotometer. The concentration of nitrogen dioxide was determined from a standard nitrite curve (7).

RESULTS

As previously reported (2), cholesteryl nitrate does not form a stable monomolecular film on an aqueous subphase. Moreover, when mixtures of various mole fractions of cholesterol and cholesteryl nitrate are spread on an aqueous subphase, the π–A curves are representative only of the cholesterol fraction (2). Thus, even in the presence of cholesterol, the nitrate ester does not occupy an area-determining position in the film. Nevertheless, as seen in Fig. 1, the presence of cholesteryl nitrate does have an inhibitory effect on the nitrogen dioxide-induced condensation of cholesterol monomolecular films, and the degree of inhibition is a direct function of the mole ratio of cholesteryl nitrate.

To quantitate this inhibitory effect, an estimate of the mole fraction (F) of cholesterol that was desorbed from the film after the 60-min. exposure to nitrogen dioxide (*i.e.*, cholesterol assumed converted to the nitrate ester) was made using the following equation (1):

$$F = (1 - A/A_0) \qquad \text{(Eq. 1)}$$

where A_0 and A are the areas/molecule apparently occupied by cholesterol before and after exposure to nitrogen dioxide, respectively, at a surface pressure of 30 dynes/cm.

Equation 1 assumes that if products other than cholesteryl nitrate form during the course of the reaction, they remain in the film and occupy the same area as that of cholesterol. This is a reasonable assumption, even though some autoxidation of cholesterol will occur under these conditions (2). This is particularly so since all of the autoxidation products formed yield stable films (*i.e.*, do not desorb from the surface) and exhibit areas/molecule only slightly larger than that of cholesterol (8). Furthermore, mixed films of cholesterol with these oxidation products exhibit an average area/molecule essentially not different from that of cholesterol alone (8).

Calculated values of the fraction of cholesterol converted to the nitrate ester as a function of the mole fraction of this ester present prior to exposure are given in Table I. It is apparent that as the initial mole fraction of cholesteryl nitrate increases, the fraction of cholesterol converted (F) decreases. Furthermore, as can be seen in the third column, the limiting effect is observed when the mole fraction of the cholesteryl nitrate reaches about 0.75 and that of the cholesterol about 0.25; that is, when the system contains approximately three molecules of cholesteryl nitrate for each molecule of cholesterol, total inhibition is observed.

Table I—Estimated Mole Fraction of Cholesterol (C) Converted to Cholesteryl Nitrate (CNO_3) by NO_2[a] as a Function of the Initial Mole Fraction of CNO_3

Initial Mole Fraction, CNO_3	F (Mole Fraction C → CNO_3)	Calculated Mole Fraction after Exposure to NO_2 of: C_p[b]	CNO_3
0	0.76	0.24	0.76
0.25	0.68	0.25	0.75
0.50	0.53	0.24	0.76
0.75	0.12	0.22	0.78

[a] 60-min. exposure to 175 ± 25 p.p.m. of NO_2; 90–120-min. exposures gave essentially the same results. [b] C_p is protected cholesterol, *i.e.*, not subject to attack by NO_2.

Table II—Results of a Computer Simulation of Proposed Model for Cholesteryl Nitrate Inhibition of NO_2–Cholesterol Reaction

Initial Mole Fraction of CNO_3	Mole Fraction of C_p Determined when: $x = 1$	$x = 2$	$x = 4$	$x = 6$
0	0.25	0.25	0.25	0.25
0.25	0.23	0.245	0.25	0.25
0.50	0.18	0.215	0.24	0.25
0.75	0.10	0.135	0.17	0.205
k_2/k_1 =	0.087	0.0185	0.0055	0.003

DISCUSSION

The sequence of events on exposure of a cholesterol film to nitrogen dioxide appears to involve first the formation of cholesteryl nitrate, *i.e.*,

$$C + NO_2 \xrightarrow{\text{air}} CNO_3 \qquad \text{(Eq. 2)}$$

where C is the unreacted "free" cholesterol, and CNO_3 is cholesteryl nitrate. Since the concentration of the nitrogen dioxide is held constant throughout the reaction, the rate of disappearance of the cholesterol can be expressed by a pseudo-first-order expression.

This reaction step is followed by a rapid desorption of the cholesteryl nitrate from the film to a non-area-determining position. While this compound does not remain in the film and is not soluble in the subphase, it must nevertheless maintain a position at the interface, probably in contact with the water and in close proximity to the cholesterol film. Polycyclic hydrocarbons (*e.g.*, 1'-methyl-1,2-benzanthracene) possess similar properties and have, under certain conditions, been reported to aggregate as colloidal dispersions, without area-determining capability, below sterol monomolecular films (9).

Early in the reaction, the desorbed cholesteryl nitrate will "see" predominately cholesterol molecules and will tend to associate with them *via* van der Waal's interaction. As the reaction proceeds and more cholesteryl nitrate is formed, an accumulation of these molecules will occur around each cholesterol molecule. Eventually, as sufficient numbers of cholesteryl nitrate molecules accumulate around each remaining cholesterol molecule in the film, further attack by the nitrogen dioxide will be inhibited. The data show that this total inhibition occurs when the mole fraction of cholesteryl nitrate reaches about 0.75 (Table I).

This interaction between the "free" unreacted cholesterol and the cholesteryl nitrate can be described by:

$$C \xrightarrow{CNO_3} C_p \qquad \text{(Eq. 3)}$$

where C_p is protected cholesterol, *i.e.*, cholesterol not sensitive to attack by nitrogen dioxide. The C_p is indistinguishable from C in so far as its effect on surface pressure is concerned.

The protected cholesterol may be viewed as a probability complex involving cholesteryl nitrate. Any molecule of the nitrate ester may be associated with any molecule of the "free" unreacted cholesterol; therefore, the rate of this process becomes a function of the total amount of the cholesteryl nitrate species.

Differential equations representing Eqs. 2 and 3 can be formulated as follows:

$$-\frac{dC}{dt} = k_1(C) + k_2(C)(CNO_3)^x \qquad \text{(Eq. 4)}$$

$$\frac{d(CNO_3)}{dt} = k_1(C) \qquad \text{(Eq. 5)}$$

where x is the number of molecules of CNO_3 required to protect the unreacted cholesterol, k_1 and k_2 are the respective rate constants, and

$$C_p = C_0 - C - CNO_3 \qquad \text{(Eq. 6)}$$

where C_0 is the initial amount of cholesterol in the film. The magnitude of k_2 is a function of the rate of surface diffusion of CNO_3.

To establish the validity of the proposed mechanism, these equations were programmed for solution on an analog computer[5]. In addition, this simulation was used to determine the smallest value of x for which the amount of protected cholesterol formed would be independent of the initial amounts of cholesterol and cholesteryl nitrate.

In this simulation, k_1 was made large enough so that the reaction between cholesterol and nitrogen dioxide was completed rapidly. The ratio k_2/k_1 was adjusted so that the concentration of protected cholesterol observed in the absence of any added cholesteryl nitrate was equivalent to that observed experimentally, *i.e.*, 0.25 mole fraction.

Table II lists the mole fraction of protected cholesterol calculated at different initial mole fractions of cholesteryl nitrate for values of x from 1 to 6.

It can be seen that when the value of x is increased to 6, the mole fraction of protected cholesterol is almost independent of the initial mole fraction of the cholesteryl nitrate. This suggests that an average of at least 6 moles of the nitrate ester per mole of cholesterol is required to inhibit completely the surface reaction of nitrogen dioxide with cholesterol.

Thus, the model represented by Eqs. 4 and 5 satisfactorily reproduces the experimental observations. Furthermore, since there are only three molecules of the nitrate present for each cholesterol molecule when the limiting effect is reached, it is apparent that each molecule of cholesteryl nitrate must be associated with more than one molecule of cholesterol.

Direct complexation between the cholesterol and cholesteryl nitrate, an alternate model, is precluded on the basis of surface pressure measurements of mixed films. Under such conditions, the surface pressure of the "free" and protected cholesterol would be expected to differ.

[5] The program is available on request.

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