Biocatalytical Synthesis and Monolayer Studies of Multiple Hydroxylated Wax Esters

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ABSTRACT: The continuous production of aleuritic acid decyl ester has been investigated with Lipozyme™ as biocatalyst in a fixed-bed reactor. To avoid the limiting effects of mean substrate solubility, the direct synthesis without organic solvents was chosen, whereby a yield of 90% was obtained depending on the residence time. A comparison of different reaction paths to synthesize a centrally dihydroxylated decyl ester indicated a preference for transesterification of the methyl ester. Under external compression, the interfacial behavior of several enzymatically produced hydroxy wax esters was determined with a Langmuir film balance to find a structure/efficiency relationship. Brewster-angle microscopy allowed direct visualization of a wax ester monolayer at the air/water interface.
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KEY WORDS: π/A-isotherms, aleuritic acid, biocatalysis, Brewster-angle microscopy, enzyme fixed-bed reactor, esterification, hydroxy wax esters, Langmuir film balance, lipase, transesterification.

Long-chain common wax esters have a wide application in industry as lubricants, plasticizers, cosmetics, and pharmaceuticals. Current manufacture of these esters is realized by reacting a fatty acid and an alcohol at high temperatures in the presence of a tin, titanium, or sulfuric acid catalyst for up to 20 h. This high-temperature process can lead to degradation of the ester and undesired side reactions; additionally, the resulting energy costs are high.

Taking these aspects into consideration, biotechnological methods, such as bioconversions, become more important, e.g., lipase-catalyzed esterification is possible at moderate temperature and at neutral pH under normal pressure in simple reaction vessels. Different lipases, such as the commercially available immobilized *Rhizomucor miehei* lipase (Lipozyme™; Novo Nordisk, Bagsvaerd, Denmark), are able to catalyze the synthesis and interesterification of various classes of esters in organic solvents.

Based on previous investigations of a batch reaction method without organic solvents (1,2), this study sets a focal point in developing a reactor for continuous synthesis of aleuritic acid decyl ester. Comparable studies yielded increasing product contents of octyl oleate (3) and fructose oleate (4). Subsequently, several hydroxy wax esters were investigated to correlate molecular structure and surface properties.

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EXPERIMENTAL PROCEDURES

Materials. The immobilized lipase of *R. miehei* (Lipozyme™; activity 23.2 BIU/g) was used as biocatalyst. The *erythro* aleuritic acid and 1-decanol were purchased from Fluka (Neu-Ulm, Germany), 9,10-dihydroxy-octadecanoic acid from Sigma (Deisenhofen, Germany), 1,10-decanediol from Aldrich (Milwaukee, WI). 15,16-Dihydroxy-hexadecanoic acid methyl ester was isolated from the extract of the microbial fermentation of *Ustilago maydis* (ATCC 14826) by alkaline saponification and subsequent acidic methanolysis (5). 9,10-Dihydroxy-octadecanoic acid methyl ester was obtained from the group of Prof. Dr. S. Warwel (Institut für Biochemie und Technologie der Fette, Münster, Germany). In this case, oleic acid methyl ester was oxidized regiospecifically with H_2O_2 and the aid of Re_2O_7 as catalyst (6). The isomeric mixture of 9,12(10,13)-dihydroxy-octadecanoic acid methyl ester was prepared by the group led by Prof. Dr. H.-J. Schäfer (Organisch-Chemisches Institut, Münster, Germany) *via* hydroboration of linoleic acid methyl ester and subsequent oxidative reaction (7,8).

Wax ester synthesis. Previous studies had shown that lipase-catalyzed esterification of fatty acids and transesterification of methyl esters could be achieved alternatively with or without organic solvents (9,10). In general, the alcohol was used at a molar excess of 3:1 in 50-mL stoppered Erlenmeyer flasks. Depending on their polarity, the substrates were alternatively dissolved in 20 mL solvent or applied without solvent. The bioconversion was started by addition of 100 mg Lipozyme™, and the reaction temperature range was extended from 37 to 60°C. This reaction enforcement was realized, e.g., by the direct comparison of esterification and transesterification to produce the 9,10-dihydroxy-octadecanoic acid decyl ester. See the Results and Discussion section for variations of the reaction conditions.

Wax ester detection, purification, and identification. Details of these processes were described recently (11). The con-

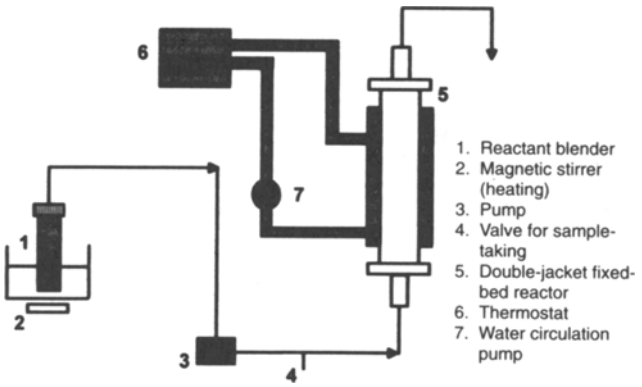
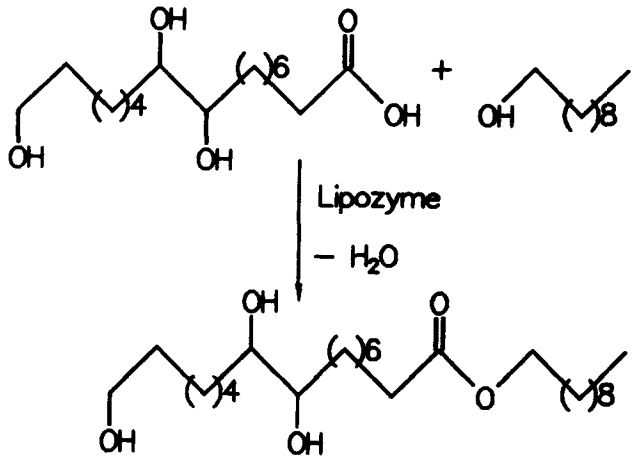


FIG. 1. Scheme of the enzyme fixed-bed reactor and associated equipment.



SCHEME 1

versions were determined by quantitative thin-layer chromatography (TLC) with the aid of a TLC-scanner (Model CD 60; Desaga, Heidelberg, Germany) after coloring with detecting agents. The polar wax esters were purified by crystallization in *n*-hexane, the more unpolar products in CH_3OH/H_2O mixtures. The molecular structures of the purified wax esters were determined by ^1H, ^{13}C nuclear magnetic resonance and gas chromatography/mass spectrometry measurements.

Enzyme fixed-bed reactor. The fixed-bed reactor is schematically shown in Figure 1. The reactor was a 50-cm long double-jacket glass column with an internal diameter of 1 cm, which enabled water circulation at a controlled temperature. The catalyst bed was 27-cm high, corresponding to 8.0 g Lipozyme™. The total volume of the column was 28 mL; the volume available to substrate solution was 15 mL. The reactant blend was fed into the column reactor by a micro-dosage pump (Pro minet mikro, Heidelberg, Germany).

The production of aleuritic acid decyl ester in the fixed-bed reactor was achieved alternatively in the presence or in the absence of organic solvents. For detailed reaction conditions, including temperatures, see the figure captions.

Determination of surface film pressure. The film pressure/area isotherms were measured on an aqueous subphase during a period of 15 min at 25°C with a Langmuir film balance (Lauda FW 2; Lauda Königshofen, Germany).

Visualization of the monolayers. The formation of a monolayer of a wax ester at the air/water interface, induced by increasing pressure on a Langmuir film balance, is directly visualized by Brewster-angle microscopy (12,13). The monolayer was irradiated with laser-light at the Brewster angle of 53° for air/water interfaces. The light reflected by the surface molecules was observed by a microscope connected to a computer-controlled video camera. This method enabled us to record areas of different brightness in the monolayer due to different refraction index and/or thickness.

RESULTS AND DISCUSSION

Continuous aleuritic acid decyl ester synthesis. The general reaction scheme for biocatalytical production of an aleuritic acid ester is shown in Scheme 1. Previous investigations with preparative batch attempts of this conversion resulted in large-scale purification and loss of the activity of the biocatalyst (1). For this reason, a fixed-bed reactor without catalyst exchange was developed for continuous synthesis in the presence or absence of diisopropyl ether (DIPE), respectively.

Operating the reactor as a closed circuit, Figure 2 presents the molar product contents of the submitted aleuritic acid after a reaction time of 120 h by an optimized constant flow of 1.5 mL/min in DIPE. The low solubility of aleuritic acid in organic solvents, the formation of side products (lactonization), and the low yield of only 40% wax ester were unsatisfactory.

The results described here forced direct synthesis with a significant excess of the alcohol compound (molar excess of 35:1) and an increase of the reaction temperature to reduce the viscosity of the mixture. Figure 3 shows aleuritic acid decyl ester formation without any organic solvents as a function of the average residence time in the fixed-bed reactor. By increasing the residence time—which means extended contact between enzyme and substrates—an increase of product formation and a decrease of acid content could be detected.

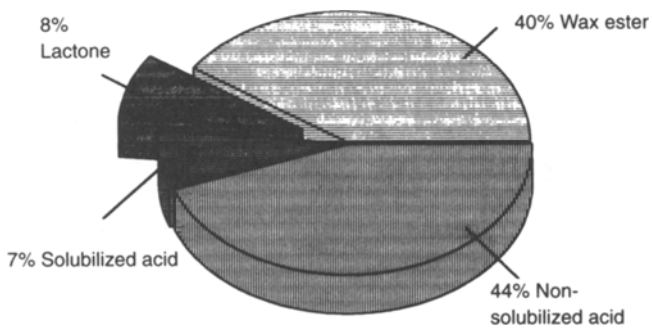


FIG. 2. Molar product contents of the submitted aleuritic acid in diisopropyl ether, produced after a reaction time of 120 h in the enzyme fixed-bed reactor with a constant flow of 1.5 mL/min. Conditions: 1.25 g aleuritic acid, 2.5 mL 1-decanol, 500 mL diisopropyl ether, temperature at 60°C.

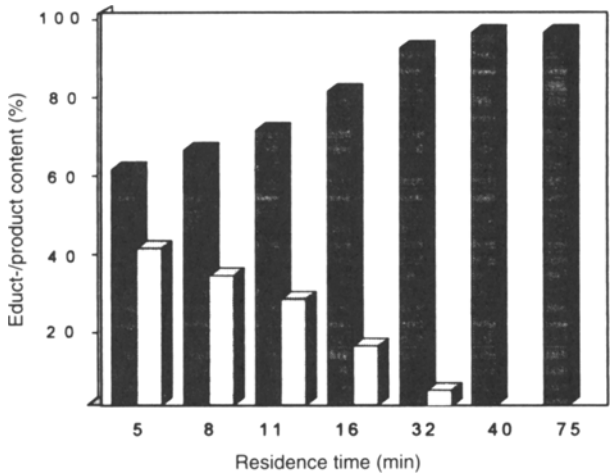


FIG. 3. Wax ester formation without organic solvents as a function of the average residence time in the enzyme fixed-bed reactor. Conditions: 20 g aleuritic acid in 450 mL 1-decanol, 80°C. Black bars, wax ester formation; grey bars, acid content.

At an average residence time of 40 min, the decyl ester yield was higher than 90%; formation of lactone did not occur.

Comparison of the kinetics of esterification and transesterification. Because of the low solubility of aleuritic acid in organic solvents, a change of the acidic compound to 9,10-dihydroxy-octadecanoic acid and its corresponding methyl ester was made. In this study, common submission of the free acid and the methyl ester was effected to make an evaluation of the substrate specificity possible. Both acidic components were added at the same time to the alcohol compound in a molar portion of 1:6. DIPE was the reaction medium. The kinetics of the decyl ester formation (see Fig. 4) show a limiting value approximation of nearly 90%; the methyl ester decrease was faster than that of the free acid; after 6.5 h, no methyl ester could be detected. That means, under the chosen conditions, transesterification of the methyl ester to the decyl ester is preferred over esterification of the free acid.

Film pressure behavior of hydroxylated wax esters. Figure 5 represents π/A-isotherms of three decyl esters, synthesized from different hydroxylated fatty acids and measured in a Langmuir film balance. The different number and locality of the polar anchors within the wax ester molecules result in distinctive differences in the physicochemical behavior of the monolayers upon external compression. The decyl ester of aleuritic acid and of 9,10-dihydroxyoctadecanoic acid exhibit gaseous-like behavior at high areas/molecule and show a point of inflection nearly at the same molecular area (80–90 $Å^2$/molecule). This behavior can be correlated to a transition state from a liquid-expanded to a liquid-condensed phase of a π/A-isotherm. The collapse point of the aleuritic acid decyl ester monolayer was reached at a surface pressure above 60 mN/m at nearly 20 $Å^2$/molecule (cross-section of a vertically oriented *n*-alkyl chain). The centrally functionalized 9,10-dihydroxyoctadecanoic acid decyl ester does not show a rapid increase at small areas; this indicates that the point of inflection is identical with the collapse point of the monolayer. The π/A-isotherm of the 15,16-dihydroxy-hexadecanoic acid decyl ester does not exhibit a transition state, but a stable condensed film is formed with high surface pressure at a molecular area of 25 $Å^2$.

To describe this physicochemical behavior, Figure 6 shows (following the proposals of Ref. 14) a schematic representation of hypothetical monolayer states of aleuritic acid decyl ester during the course of its compression. After spreading on the aqueous subphase, most of the wax ester molecules are distributed prone on the surface a. As external pressure is applied, the lipophilic hydrocarbon-chain at the end of the molecule begins to be forced out of the water, and a resultant bowing of the chains between the three polar anchors can be observed at point b. At the first point of inflection of the isotherm (90 $Å^2$/molecule), the weaker polar ester group is forced out of the water (point c). This transition state is fin-

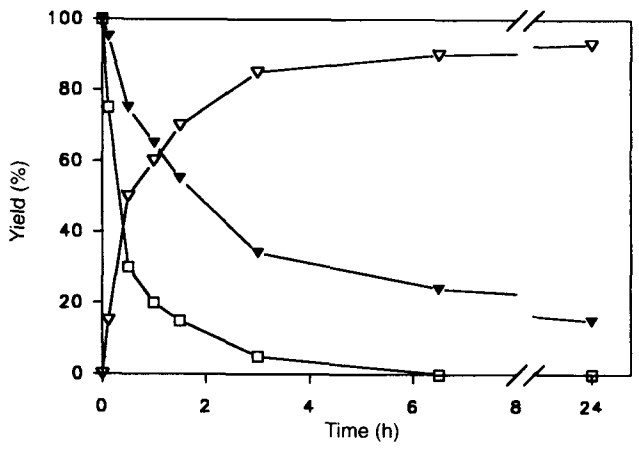


FIG. 4. Direct comparison of the formation of 9,10-dihydroxy-octadecanoic acid decyl ester *via* esterification and transesterification by common submission of the acidic compounds. Conditions: 0.082 mmol acid and methyl ester, 0.492 mmol 1-decanol, 20 mL DIPE, 25 mg Lipozyme™ (Novo Nordisk, Bagsvaerd, Denmark). ▽, Wax ester formation; ▼, acid decrease; □, methyl ester decrease.

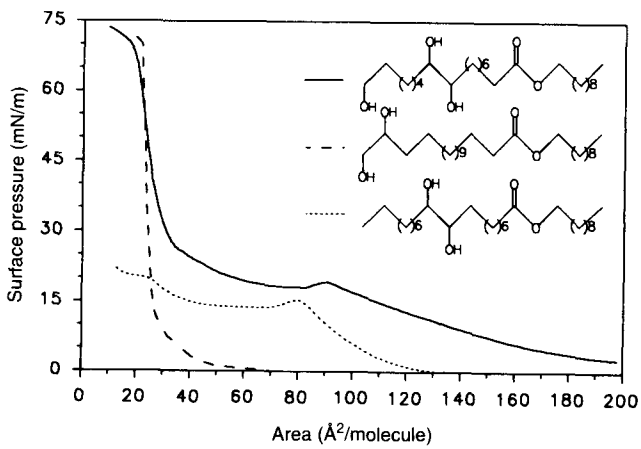


FIG. 5. π/A-isotherms of decyl esters from different hydroxy fatty acids at 25°C.

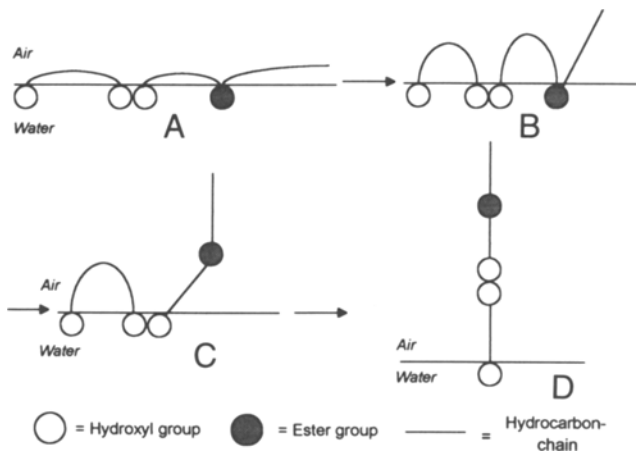


FIG. 6. Schematic representation of possible molecular conformations during the various monolayer states of aleuritic acid decyl ester by increasing pressure.

ished at a second point of inflection, where the 16-hydroxy group is the sole hydrophilic anchor in the subphase d, and all molecules are aligned vertically in a close-packed structure by further increasing pressure. Similar phases of packing for other bipolar compounds, including ester groups, have been reported (15).

Measurements of the π/A-isotherms of multiple hydroxylated wax esters (Fig. 7) enabled further conclusions of the relationship between molecular structure and interfacial behavior. Continuing previous investigations (16), these wax esters were produced *via* biotransformation from various hydroxylated fatty acids and 1,10-decanediol as alcohol compound. This reaction yielded molecules with a hydroxy group at the end of the lipophilic hydrocarbon chain; the aleuritic acid 10′-hydroxy-decyl ester has polar anchors at both ends of the molecule. This wax ester was too polar to build up a stable monolayer. On the other hand, the isomeric 9,12(10,13)-dihydroxy-octadecanoic acid 10′-hydroxy-decyl ester and the 9,10-dihydroxy-octadecanoic acid 10′-hydroxy-decyl ester exhibit the typical behavior of long-chain wax esters with polar groups in the center and at the end of the molecule, analogous to the aleuritic acid decyl ester. More remarkable was the stable condensed film with high surface pressure at small areas, caused by the neighboring hydroxy-groups in the 9- and 10-positions.

The synthesis and physicochemical characterization of the film balance behavior of different hydroxylated wax esters enable a summary of structure/efficiency relationships as presented schematically in Figure 8. Based on investigations of Sackmann and Dörfler (17), four types of isotherms can be distinguished: type 1—isotherms with high compressibility at small areas (condensed films); type 2—isotherms starting at large areas and exhibiting a strong initial compressibility (expanded film region); a transition state begins at a definite point of inflection and is followed by a region with high compressibility at small molecular areas; type 3—isotherms of unstable monolayers, possibly starting at large areas and observing some transition states; the monolayer breaks down gradually without an exact determination of the collapse point; and type 4—isotherms with the typical compressibilities of expanded films, but without the formation of transition states; the isotherms are finished with the film collapse at large molecular areas.

A direct on-line visualization of the aleuritic acid decyl ester monolayer during the course of its compression is shown in Figure 9, which resulted from Brewster-angle microscopy. The photos exhibit the transition state from liquid-expanded to liquid-condensed phase by increasing formation of solid domains with characteristic shape upon further compression (Fig. 9, A,B). The solid-condensed phase is recorded before and after film collapse (Fig. 9, C,D), whereby an optical determination of the end of this isotherm was additionally enabled.

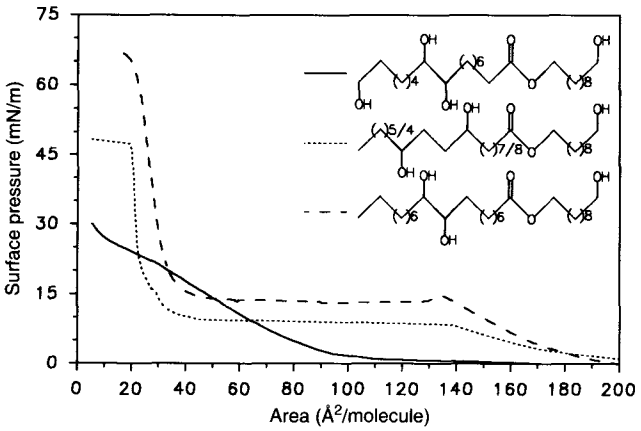


FIG. 7. π/A-isotherms of multiple hydroxylated wax esters performed by various acidic compounds and 1,10-decanediol at 25°C.

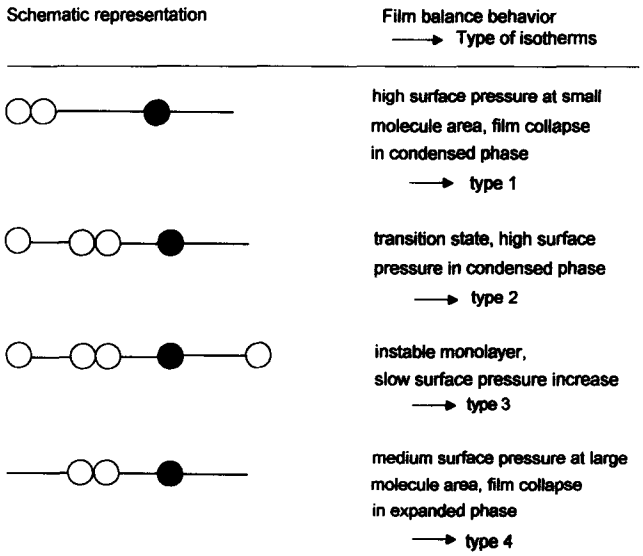


FIG. 8. Summary of the film balance behavior of different hydroxylated wax esters. For explanation of symbols, see Figure 6.

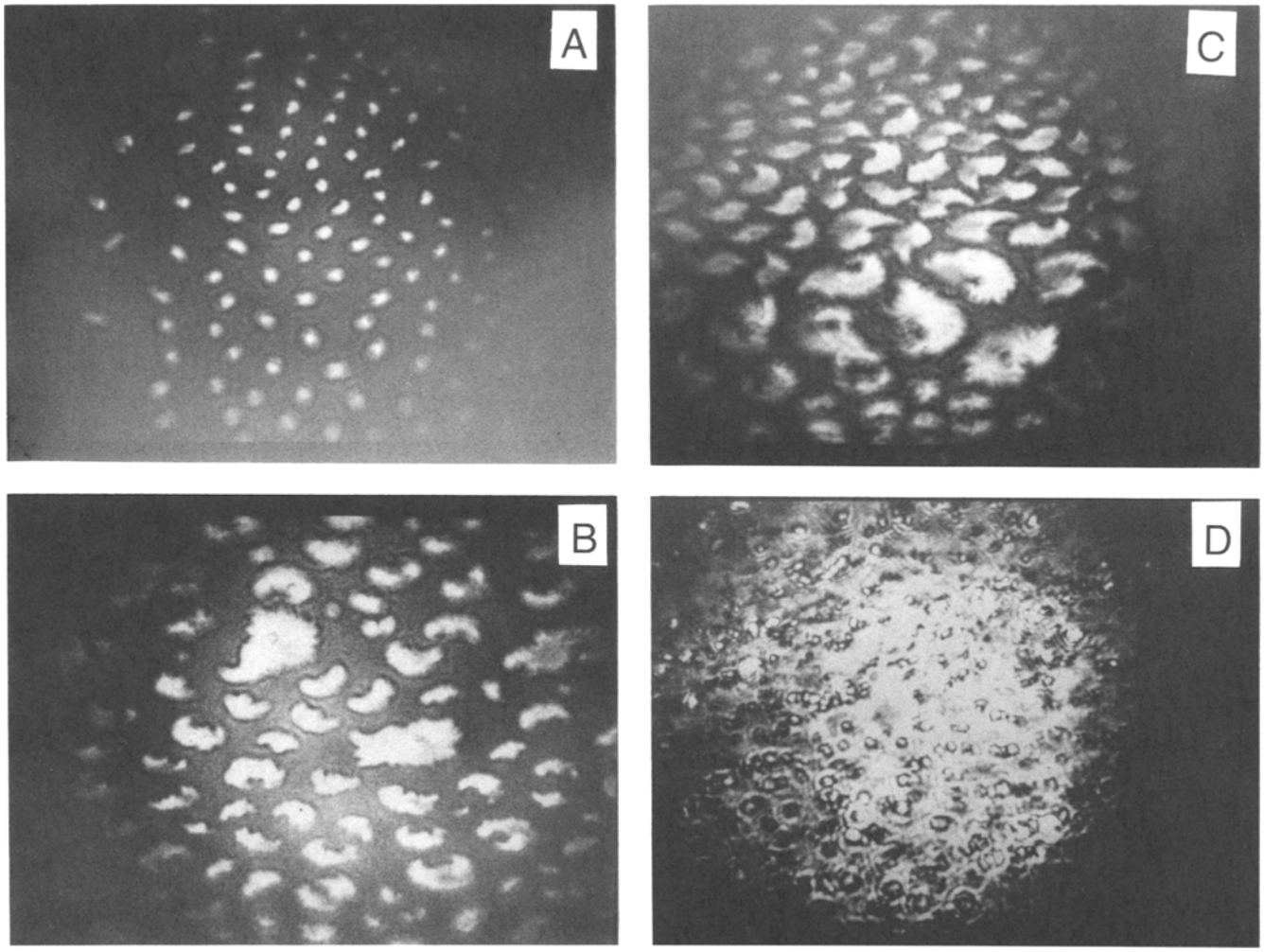


FIG. 9. Brewster-angle microscopy photos of the aleuritic acid decyl ester monolayer upon increasing pressure. Exhibition of the transition state from liquid-expanded to liquid-condensed phase (A, B) and the solid-condensed phase before and after film collapse (C, D).

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