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ANTIMICROBIAL EFFECT OF SIMPLE LIPIDS AND ITS RELATION TO SURFACE FILM BEHAVIOUR—I

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Abstract—*Iso*-branched fatty acids possess fungistatic and bacteriostatic properties. It has been found that they are also able to strongly enhance the effect of conventional antimicrobial agents, which act inside the cell membrane. In a comparative study of various lipids a relation between this biological effect and the collapse properties of the corresponding monomolecular surface film on water has been observed. The mode of action has been investigated by measurements of the interaction with membrane lipids in a biological test system as well as in a membrane model system. It is shown that this type of membrane-active compounds increase the permeability of the lipid layer of the plasma membrane so that the protective barrier function is reduced and a molecular mechanism for this effect is proposed on the basis of the surface film results.

IN CONNECTION with physical and chemical studies of membrane lipids (cf. Ref. 1) an investigation of the antimicrobial effect of simple lipids was started. In order to obtain information on the molecular properties to which this biological effect can be attributed, the activity measurements have been correlated with the effect on a surface film model of a biomembrane.

Weitzel and co-workers have earlier reported studies on antibiotic effect and surface film properties of branched fatty acids (cf. Ref. 2). However, the enhancement of the effect of other active agents present in the system, which appears to be the fundamental antibiotic function of this type of lipids, was not then realized.

The lipids used in this work were selected successively during the course of the investigation with guidance from the results. A first natural step was to examine different homologues of the *iso* fatty acid series. A comparison then with *normal* fatty acids showed that the branched isomers possess much higher biological effect. The effect of branches of different size and in different positions was therefore studied. When it was realized that the *iso* configuration gave the highest biological effect, an *iso*-compound with larger polar end group (a monoglyceride) was tested. Finally, when a relation between the biological effect and the surface film behaviour had been revealed, two lipids (a keto fatty acid and a diglyceride), which according to surface film behaviour should be expected to exhibit similar properties, were investigated with respect to their inhibitory effect on germination of fungi.

In order to obtain information on the mode of action of these lipids on the plasma membrane, their effect on a model membrane constituted by an interfacial lipid film was also studied.

MATERIAL AND METHODS

Preparation of lipids

As a few of the lipids used in this work have not been described earlier and the others are very unusual, a full description of their preparation is given here.

12-*Methyltridecanoic acid.* This previously known acid[3–6] was prepared via a mixed anodic coupling (Kolbe electrosynthesis) of 4-methylpentanoic acid (Fluka AG, Buchs, Switzerland) and methyl hydrogen decan-1,10-dioate.[19] Sixty-four g (0·296 mole) of the half ester (b.p. 155°, 0·3 mm) was mixed with 34·4 g (0·296 mole) of 4-methylpentanoic acid. Sodium [0·68 g (0·030 mole)] was allowed to react with 200 ml of methanol. The solution of sodium methylate in methanol was added to the above mixture and the acids electrolyzed at pH 5·5 (50 V). After 6·5 hr the pH in the reaction mixture had risen to 7 and the electrolysis was stopped. The solvent was removed and the residue triturated with 350 ml of ether. The filtrate was evaporated and the residue distilled. Methyl 12-methyltridecanoate boiled at 136–140°, 1 mm. Yield 13·5 g (19·2 per cent). The methyl ester was hydrolyzed by refluxing overnight with a solution of 13·5 g of potassium hydroxide in 13 ml of water and 25 ml of ethanol. The free acid was recrystallized from light petroleum (b.p. 60–85°). Yield 10·7 g, m.p. 52·2–52·8°.

13-*Methyltetradecanoic acid.* This previously known acid[5] was prepared from 12-methyltridecanoic acid by chain-lengthening (Arndt–Eistert reaction). Five g (0·022 mole) of 12-methyltridecanoic acid was refluxed for 3 hr with 20 ml of thionyl chloride. The excess of thionyl chloride was distilled off at reduced pressure. Two 15-ml portions of benzene were added and subsequently distilled off. The crude acid chloride (5·8 g) was dissolved in 15 ml of dry ether and the solution added dropwise to a stirred, cold (−5°) solution of 2·5 g of diazomethane in 95 ml of ether. The stirring was continued for 2 hr. After evaporation, 5·9 g of the corresponding crude diazoketone was obtained. This was dissolved in 75 ml of methanol and heated to 70° with stirring. Two g of commercial silver oxide was added in portions. The stirring and heating was continued overnight and the suspension subsequently filtered. Ether and water were added to the filtrate and the organic phase washed three times with water, dried ($MgSO_4$) and evaporated. The crude methyl 13-methyltetradecanoate (5·1 g) was chromatographed on 50 g of silicic acid (Mallinckrodt 100 mesh) with ether–light petroleum (b.p. 40–60°) (1:50, v/v). Yield 4·5 g.

The methyl ester was hydrolyzed as described. The free acid (2·1 g) was crystallized from light petroleum (b.p. 40–60°). The pure acid had m.p. 49·7–50·3°.

17-*Methyloctadecanoic acid* (cf. Refs. 7, 8) was prepared via a mixed anodic coupling of 12-methyltridecanoic acid (1·6 g, 7·0 m-moles) and methyl hydrogen heptane-1,7-dioate (1·0 g, 7·0 m-moles). The latter was prepared from the corresponding dimethyl ester.[9]

The crude methyl 17-methyloctadecanoate obtained was refluxed overnight with a solution consisting of 1·4 g of potassium hydroxide in 2 ml of water and 20 ml of ethanol. Most of the ethanol was distilled off and the remaining solution distributed between water and light petroleum (b.p. 40–60°). The aqueous layer was acidified with diluted hydrochloric acid (1:3) to pH 2 and extracted twice with ether. The organic phase was washed, dried (molecular sieve type 3A) and evaporated. The residue (851 mg) was chromatographed on 5 g of neutral Al_2O_3. A forerun (77 mg) was eluted with ether–light petroleum (b.p. 40–60°) (1:10, v/v) and then 530 mg of

acidic material with acetic acid–light petroleum (b.p. 40–60°) (1:100, v/v). The acid was recrystallized from light petroleum (b.p. 60–80°). 323 mg 17-methyloctadecanoic acid (m.p. 66·2–66·6°) was obtained with a purity >99 per cent indicated by the gas chromatographic analysis of the methyl ester.

The previously known 20-*methylheneicosanoic acid* (cf. Refs. 3, 4) was prepared via a mixed anodic coupling of 2·0 g (8·8 m-moles) of 12-methyltridecanoic acid and 1·8 g (8·8 m-moles) of methyl hydrogen decan-1,10-dioate in the presence of 52·2 mg of sodium methoxide in 40 ml of methanol (1A, 70V, 105 min). The acid was worked up in the manner described. The purity was >99 per cent and the m.p. was 78·5–79·0°.

13-*Methylpentadecanoic acid* (*racemic*). Ten g (0·098 mole) of 2-methylbutanoic acid (Fluka AG, Buchs, Switzerland) was conventionally reduced with 3·2 g (0·085 mole) of $LiAlH_4$ to give 8·3 g (96·5 per cent) of 2-methylbutan-1-ol. This was converted into 1-iodo-2-methylbutane (9·3 g) which was subsequently used for alkylation of diethyl malonate. After hydrolyses and decarboxylation of the monoalkylated malonic ester 4·7 g of 4-methylhexanoic acid was obtained, b.p. 120°, 20 mm (cf. Ref. 10). For the details of this route of synthesis, cf. Ref. 11. The acid (2·0 g) was subjected to mixed anodic coupling with methyl hydrogen undecan-1,11-dioate (3·5 g) in the presence of 83 mg of sodium methoxide in 20 ml of methanol (1A, 60V, 75 min). The product was worked up in the same way as earlier described. The crude methyl 13-methylpentadecanoate, however, was purified by preparative gas chromatography on a 2-m column with 5% OV-17 on Chromosorb G (60–80 mesh) as stationary phase. The chromatography was performed at 190°, the flow of argon carrier gas was 200 ml/min and the vapours condensed in standard Perkin–Elmer cooling traps at −80°. The free racemic acid gave a m.p. of 29·6–30·3°.

13-*Ethylpentadecanoic acid.* Diethyl malonate was conventionally alkylated with ethyl bromide to give diethyl ethylmalonate. The monoalkylated malonic ester was distilled twice on a 0·5-m Nester/Faust Spinning band column to remove unreacted starting material. Gas chromatographically pure diethyl ethylmalonate boiled at 127·0°, 30 mm (cf. Ref. 12). This was alkylated a second time with ethyl iodide, and after distillation on the spinning band column 35·0 g pure diethyl diethylmalonate of b.p. 123·0°, 25 mm was obtained. The dialkylated malonic ester was hydrolyzed and the free malonic acid was decarboxylated (at 140°) yielding 13·7 g of crude 2-ethylbutanoic acid. This was not further purified. The acid (13·7 g) was converted to 1-iodo-2-ethylbutane (9·7 g) via the reaction sequence mentioned previously. The iodo-compound was subsequently used for conventional alkylation of diethyl malonate. After hydrolyses and decarboxylation 5·0 g of crude 4-ethylhexanoic acid was obtained. This was filtered through 30 g of silicic acid with ether–light petroleum (b.p. 40–60°). Yield 4·5 g (68·4 per cent calculated on the iodo-compound). The acid (4·5 g) was subjected to mixed anodic coupling with methyl hydrogen undecan-1,11-dioate (7·2 g) in the presence of 0·17 g of sodium methoxide in 100 ml of methanol (1 A, 60 V, 4 hr). The product was worked up as described for 13-methylpentadecanoic acid. The preparative gas chromatography was performed at 200°. The free acid had m.p. 23·0–23·6°.

2-*Methyloctadecanoic acid* (*racemic*). This previously known acid (cf. Refs. 13–16) was prepared via alkylation of diethyl methylmalonate with 1-bromohexadecane followed by hydrolyses and decarboxylation. 9·45 g (0·0391 mole) of hexadecan-1-ol

was refluxed with 200 ml of hydrobromic acid in water (48%) overnight. Water and ether were added and the organic layer carefully washed, dried ($MgSO_4$) and evaporated. The residue was filtered through 50 g of neutral Al_2O_3 with light petroleum (b.p. 40–60°), giving 6·36 g (53·5 per cent) of 1-bromohexadecane. Alkylation of 3·64 g (0·029 mole) of diethyl methylmalonate with the bromo-compound followed by hydrolysis, decarboxylation and crystallization of the product in light petroleum (b.p. 60–80°), afforded 3·08 g of 2-methyloctadecanoic acid (purity >98 per cent), m.p. 55·4–55·6°.

Preparations of 8-*oxooctadecanoic acid* and 9-*methyloctadecanoic acid* are described in Refs. 17 and 18.

The racemic 1-monoglyceride of *iso*-tetradecanoic acid and 1,3-dimyristin were prepared according to conventional methods for glyceride syntheses (cf. Refs. 19 and 20). The m.p. of the monoglyceride was 45·7–46·8° and that of the β-form of the diglyceride was 63·0°.

Preparation of the glycosphingolipid used as membrane model, sodium sulfatide, has been described elsewhere.[1]

Activity measurements

The biological activity of the various lipids was tested in germination experiments with conidia of *Fusarium roseum*. The experiments were performed as follows. Two wetted and sterilized strips (10 × 10 mm) of cellophane (PT 300) were placed on a sterile glass filter (Jena GI) in a small petri dish (diameter 6 cm) containing 3 ml of a test solution. In the control series it consisted of glucose (4·0 g), KNO_3 (2·0 g), KH_2PO_4, (2·5 g), $MgSO_4$–7 H_2O (1·25 g), yeast extract (1·0 g) and Tween 80 (0·1 ml) in distilled water (1000 ml). In the test series this nutrient solution (F-solution) was supplemented with the lipid to be examined, added in methanol solution, always giving the medium a methanol concentration of 0·05%. All experiments were run at pH 6·5.

The strips thus arranged were inoculated with conidia of *F. roseum* (40,000 conidia per strip) from cultures grown for 10 days on malt agar. After 3 and 4 hr of incubation at 25°, the strips were mounted on slides and treated with lactophenol to stain and kill the conidia. The proportions of germinated and non-germinated conidia were then determined in the microscope. In each series at least 400 conidia were examined. Every compound was tested in two or more experiments. In order to facilitate the comparisons between different experiments the value of the germination frequency of the control series was always set to 100 and that of the other series was related to this scale. The inhibition index for the substances tested was obtained by subtracting the calculated value of germination frequency of the test series from 100 (i.e. the control value).

Thiram, i.e. tetramethylthiuramdisulfide (TMTD), was used as fungicide. The values given here correspond to the inhibition obtained 4 hr after incubation. This appeared to be the most convenient time in order to demonstrate the enhancement effect of the lipid component as too strong inhibition was obtained at shorter time intervals of the same components individually.

Surface film technique

Pressure–area (π–A) isotherms were obtained using a continuously recording surface balance of Wilhelmy–Dervichian type with the booms moving symmetrically

with respect to the Wilhelmy glass plate.[21] Hexane or a hexane–ethyl alcohol (9:1) mixture was used as solvent and spread by an Agla micrometer syringe. The compression rate was usually about 6 Å^2/molecule/min, although much lower compression rates also were used in order to check that equilibrium had been obtained. The same nutrient solution as used in the biological studies was used as substrate for the surface film measurements.

The π–A curves were recorded photographically and are reproduced in Figs. 3–8.

RESULTS

Biological activity

The enhancement effect of *iso*-branched fatty acids on fungicides is demonstrated in Fig. 1. A constant amount (100 μg/ml) of 12-methyltridecanoic acid or alternatively

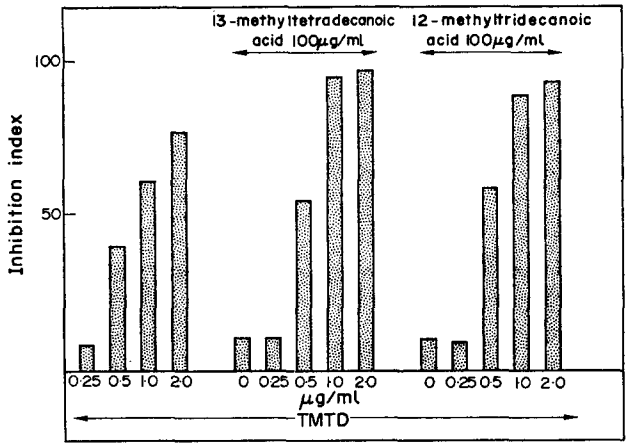

FIG. 1. Inhibition of the germination of conidia of *Fusarium roseum*, incubated for 4 hr at 25° in F-solution supplemented with 100 μg/ml of 13-methyltetradecanoic acid of 12-methyltridecanoic acid and various amounts of TMTD. Relative number of germinating conidia in F-solution (control) =100.

13-methyltetradecanoic acid was used in combination with various concentrations of TMTD. It can be seen that a minimum concentration of TMTD in the medium is needed in order to obtain inhibition. When the concentration exceeded this minimum value for TMTD, the presence of the *iso*-branched fatty acids strongly increased the inhibition of the conidia germination. At an amount of 1·0 μg/ml, TMTD alone produced an inhibition index of 61, and addition of any of the two acids then resulted in almost total inhibition of the germination.

The effect of various amounts of 13-methyltetradecanoic in combination with TMTD (0·5 μg/ml) was then analysed. The results are presented in Fig. 2. The largest enhancement effect per amount of *iso*-acid is obtained when the concentration is in in the range 50–200 μg/ml.

The inhibition effects of different lipids selected as described earlier have been studied and the results are given in Table 1. Different concentrations were used and

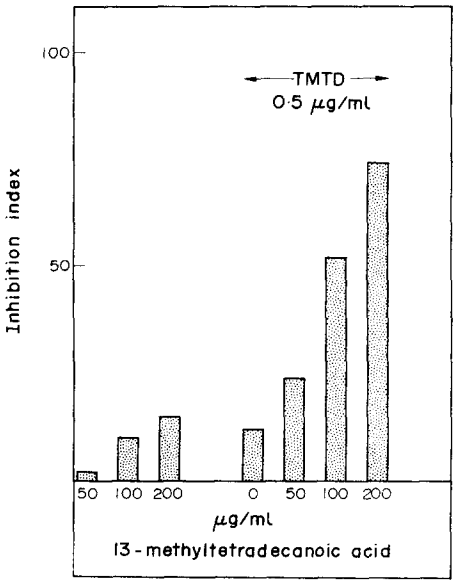

FIG. 2. Inhibition of the germination of conidia of *Fusarium roseum*, incubated for 4 hr at 25° in F-solution supplemented with TMTD, 0·5 μg/ml, and various amounts of 13-methyltetradecanoic acid. Relative number of germinating conidia in F-solution (control) = 100.

the activity was measured of the lipid component alone as well as in combination with TMTD. It has therefore been possible to separate the independent effect of the lipid component alone from the synergetic effect (enhancement of the inhibition effect). The synergetic relations are summarized in Table 2.

The results given for nonanoic acid are representative for the *normal* fatty acids of chain lengths C_4–C_{12}. The *iso*-branched fatty acids in the C_4–C_{11} range behaved in the same way. No enhancement effect is obtained, although the lipid component added to the system results in inhibition of the germination.

A quite different behaviour was observed in 13-methylpentadecanoic acid, 2-methyloctadecanoic acid, 17-methyloctadecanoic acid, 20-methylheneicosanoic acid and to a somewhat less extent tetradecanoic acid. When used alone they hardly affected the germination of the conidia. Even at an incubation of only 3 hr their inhibition index was smaller than 4. In spite of that, their effect in combination with TMTD was considerable. Thus, after 3 hr of incubation, the inhibition indices of these lipids component + TMTD were about 80. Most of these great effects disappeared however, when the incubation was prolonged.

As is obvious from Table 1 the strongest enhancement of antimicrobial effect was obtained from 12-methyltridecanoic acid and 13-methyltetradecanoic acid. 13-Methyltetradecanoic acid has also been tested in combination with other fungicides. The activity measurements have involved captafol (*N*-tetrachloroethylthiotetrahydrophthalimide), captan (*N*-trichloromethylthio-4-cyclohexene-1,2-dicarboximide), dinocap (2-1′-methyl-4,6-dinitrophenyl-crotonate), zineb (zincethylenebisdithiocarbamate) and a 20:3 mixture of hexachlorobenzene and 2-(2-furyl)-benzimidazole. In combination with all these fungicides 13-methyltetradecanoic acid acted in a synergetic manner,

TABLE 1. INHIBITION OF THE GERMINATION OF CONIDIA OF *Fusarium roseum*, INCUBATED FOR 4 hr AT 25° IN F-SOLUTION SUPPLEMENTED WITH TMTD, 0·5 μg/ml AND VARIOUS LIPID COMPONENTS

Lipid components added to F-solution	Amount (μg/ml)	Inhibition index for		
		Lipid components alone	TMTD (0·5 μg/ml)	Fatty acid + TMTD (0·5 μg/ml)
m-Nonanoic acid	35	5	14	21
	47	24	14	38
	70	48	14	66
m-Tetradecanoic acid	50	2	11	15
	100	1	11	25
12-Methyltridecanoic acid	50	2	13	27
	100	6	13	56
	200	8	11	80
13-Methyltetradecanoic acid	50	2	12	24
	100	10	12	52
	200	15	12	74
13-DL-Methylpentadecanoic acid	100	3	15	9
	200	8	15	46
13-Ethylpentadecanoic acid	100	0	15	3
	200	2	15	20
2-DL-Methyloctadecanoic acid	130	4	16	20
	260	4	15	31
9-DL-Methyloctadecanoic acid	130	3	16	15
	260	4	15	20
17-Methyloctadecanoic acid	130	6	16	20
	260	8	15	35
8-Oxooctadecanoic acid	130	0	15	10
	260	4	15	35
20-Methylheneicosanoic acid	150	7	16	34
1-Monoglyceride of *iso*-tetradecanoic acid	133	45	16	68
1,3-Dimyristin	210	0	15	18
	420	0	15	36

Relative number of germinating conidia in F-solution (control) = 100.

enhancing their inhibitory effect strongly. In combination with mercury compounds, such as methoxy-ethyl mercury salts, on the other hand, the fatty acid gave no visible effect. 12-Methyltridecanoic acid and 13-methyltetradecanoic acid have also been studied with respect to their effect in combination with penicillin and streptomycin. The results indicate that the lipid components give a strong enhancement of the antibiotic effect on Gram-positive bacteria, whereas the Gram-negative species are less

TABLE 2. SUMMARY OF OBSERVATIONS ON SYNERGETIC EFFECT OF LIPID COMPONENTS ON FUNGICIDES

Lipid component	Synergetic effect
Nonanoic acid	
Tetradecanoic acid	×
12-Methyltridecanoic acid	××
13-Methyltetradecanoic acid	××
13-Methylpentadecanoic acid	×
13-Ethylpentadecanoic acid	
2-Methyloctadecanoic acid	×
9-Methyloctadecanoic acid	
17-Methyloctadecanoic acid	×
8-Oxooctadecanoic acid	×
20-Methylheneicosanoic acid	×
1-Monoglyceride of *iso*-tetradecanoic acid	
1,3-Dimyristin	×

Enhancement of the total biological effect in relation to the sum of the individual components is indicated by crosses (double crosses indicate strong enhancement).

susceptible. Thus, in the presence of 10 μg of either of the two *iso*-branched fatty acids per millilitre medium, the minimum concentration of streptomycin to produce an inhibitory effect on *Micrococcus aureus* was reduced from 10 μg/ml to less than 0·5 μg/ml. In the case of *Escherichia coli* the minimum concentration of streptomycin and penicillin was reduced to 50 per cent by an addition of 400 μg/ml of the lipid component.

In order to obtain information on the membrane function, which is believed to be involved in the synergetic effect of these lipids on antibiotic action, the interaction with cholesterol has been examined. The medium for the conidia was supplemented with 12-methyltridecanoic acid, TMTD and various amounts of cholesterol. The results, presented in Table 3, showed that in the presence of increasing amounts of cholesterol the inhibitory effect decreased successively, i.e. cholesterol reduces the enhancement effect obtained by the *iso*-branched fatty acid.

TABLE 3. EFFECT OF CHOLESTEROL ON THE INHIBITION OF THE GERMINATION OF *Fusarium* CONIDIA OF 12-METHYLTRIDECANOIC ACID AND TMTD

Amount of cholesterol added to F-solution (μg/ml)	Inhibition index for		
	iso-acid 200 μg/ml	TMTD 0·5 μg/ml	*iso*-acid, 200 μg/ml TMTD, 0·5 μg/ml
0	10	7	73
50	—	6	65
100	—	6	53
200	—	7	15

Incubation for 4 hr at 25°. Relative number of germinating conidia in F-solution (control) = 100.

Surface film behaviour

Iso-*branched fatty acids.* The π–A isotherms of *iso*-pentadecanoic acid and *iso*-docosanoic acid are shown in Fig. 3. *Iso*-docosanoic acid shows a very remarkable surface film behaviour. At compression a solid condensed phase with high compressibility is formed, and it collapses at 26·5 Å^2/molecule (Å^2/M). When compression is continued beyond the collapse point there is a considerable reduction in pressure, and if the movement of the booms is stopped until equilibrium is obtained, the pressure approaches zero. One such stop is shown in Fig. 3. If the compression rate is extremely slow and also if the temperature is lowered to about 10° at the usual compression rate of 6 Å^2/M/min the curve obtained shows the same low pressure.

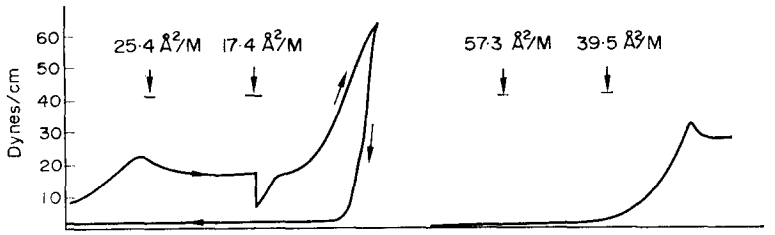

FIG. 3. π–A isotherms of *iso*-docosanoic acid at 21·5° (to the left) and *iso*-pentadecanoic acid at 21° (to the right).

When the area has been reduced to that corresponding to a triple layer there is a steep rise in pressure. At an area of 7·6 Å^2/M, near collapse of the triple layer, the film was expanded. The small pressure obtained after expansion to an area too large for a triple layer indicates that the molecules are transformed directly into a gaseous state.

It seems natural to regard the triple layer as a crystal. It is in fact the thinnest possible fatty acid crystal in the air–water interface. The crystal unit layer consists of a bimolecular layer of hydrogen-bonded dimers, and an extra layer is needed in order to obtain a hydrophilic surface in contact with water. The crystal structure of an *iso*-branched fatty acid has been determined by Abrahamsson,[22] and the area of a triple layer calculated from the angle of tilt of the molecules is in good agreement with the observed area of the surface film.

A certain molecular concentration on the surface must of course be reached before crystallization can start, and the upper part of the peak corresponding to the monomolecular film on the pressure–area isotherm can be regarded as the supersaturation needed for crystallization. After crystallization has started there is thus a fall in pressure. We have found that this hump on the curve is of great importance, as it can be correlated with biological activity.

The isotherm obtained for *iso*-pentadecanoic acid (Fig. 3) shows that a liquid-expanded phase is formed at condensation of the film, and a similar hump is observed at collapse of the film (at 25·7 Å^2/M). *Iso*-tetradecanoic acid, which also possesses very high biological effect, gives the same type of surface film with a liquid-expanded phase showing a hump at the collapse point. Shorter *iso*-acids are discussed below in connection with *normal* fatty acids.

Normal fatty acids. Heikkila *et al.*[23] have recently reported an accurate study of fatty acid surface films, which proved that the condensed phases *LS* and *S* are metastable and collapse above their equilibrium spreading pressure. The crystallization process, with initial formation of a triple layer was, however, not revealed. Tetradecanoic acid shows a hump at collapse of the solid condensed phase, and it exhibits about the same synergetic effect on fungicides as *iso*-docosanoic acid (cf. Table 2). The hump is less pronounced at longer chain lengths. Dodecanoic acid and shorter homologues do not give any condensed surface film phases, and the corresponding shorter members of the *iso*-acids behave in the same way. As mentioned earlier they give no enhancement of the effect of fungicides, although they have an independent fungistatic effect.

Various branched fatty acids. Three methyl-branched octadecanoic acids were studied in order to compare the effect of branches in different positions. They were selected in order to represent the various occurring structure types according to their known crystal structures.[22] The isotherm observed for the *iso*-branched member is given in Fig. 4. A solid condensed phase is formed which collapses at 22·9 Å^2/M, where the pressure decreases in the same way as described earlier, and the expected synergetic activity is also observed (cf. Table 2).

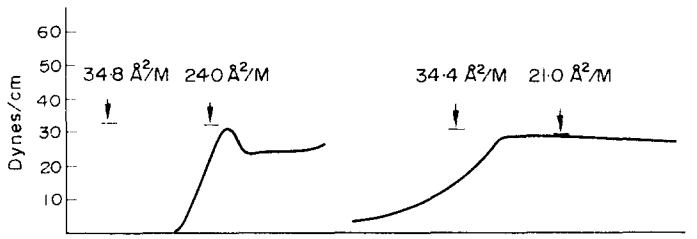

FIG. 4. π–A isotherms of 17-methyloctadecanoic acid at 20° (to the left) and 9-DL-methyloctadecanoic acid at 21° (to the right).

9-DL-Methyloctadecanoic, on the other hand, shows only a liquid expanded phase with no hump after the collapse at 28·2 Å^2/M (Fig. 4), and neither does it enhance the effect of fungicides after four hours of incubation. It is not surprising that no crystallization is observed for this member as the molecules in the solid state are so much tilted[22] that the structure is not of usual layer type.

The surface film behaviour of 2-DL-methyloctadecanoic acid is shown in Fig. 5. It is very similar to that of *iso*-docosanoic acid with a well-defined triple layer formed

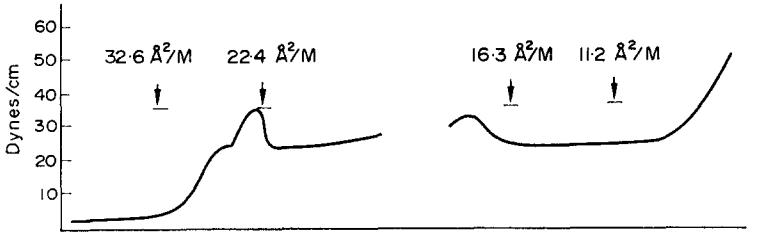

FIG. 5. π–A isotherms of 2-DL-methyloctadecanoic acid at 20°.

after crystallization. The solid monolayer collapses at 19·5 Å²/M indicating that the molecules are vertical, whereas the corresponding area observed for the triple layer (7·9 Å²/M) indicates that the molecules are strongly tilted as in the solid state.[22] The biological activity is, as expected, similar to that of the *iso*-acid.

13-DL-Methylpentadecanoic acid (*anteiso*-branched) was studied for comparison with 13-methylpentadecanoic acid (*iso*-branched). The isotherm is shown in Fig. 6. At collapse (25·4 Å²/M) there is a very small hump, and as can be seen in Table 2 it also exhibits synergetic action.

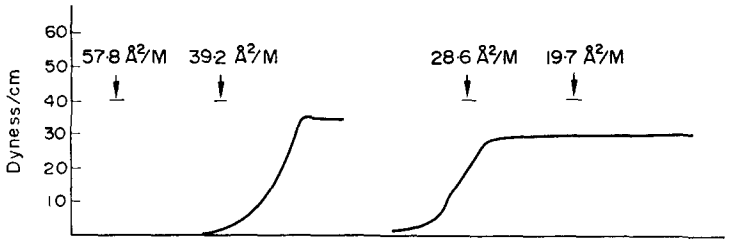

FIG. 6. π–A isotherms of 13-DL-methylpentadecanoic acid and 13-ethylpentadecanoic acid (to the left and to the right respectively) at 21°.

13-Ethylpentadecanoic acid was investigated in order to see the effect of a larger branch. As can be seen from the isotherm in Fig. 6 there is no decrease in pressure after collapse (at 27·1 Å²/M), and in accordance with the earlier observations no enhancement of the biological effect was obtained.

A keto-fatty acid, 8-oxooctadecanoic acid, was studied in order to see the effect of a polar substituent. The isotherm given in Fig. 7 shows clearly the formation of a triple layer in the same way as discussed earlier. The observed areas of the condensed monolayer (17·7 Å²/M at collapse) and that of the triple layer (6·0 Å²/M at the same

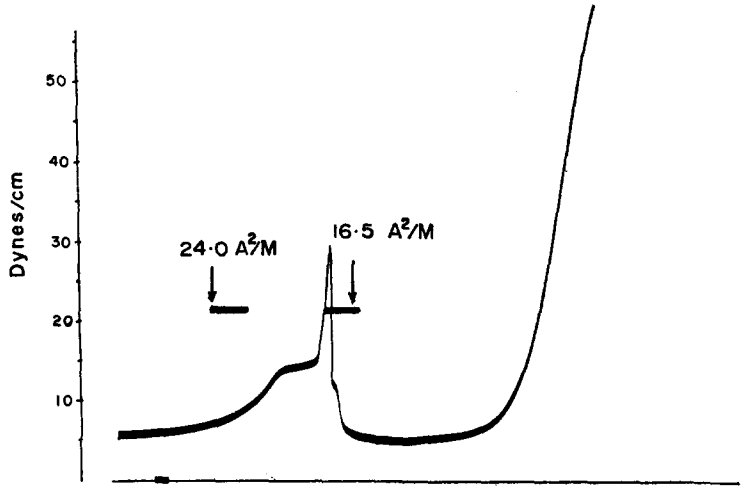

FIG. 7. π–A isotherm of 8-oxooctadecanoic acid at 19°.

pressure) indicate that the molecules are packed vertically according to the orthorhombic chain packing as in the solid state.[17] The triple layer appears to be very stable with a linear pressure–area relation between 22 and 54 dynes/cm. The same behaviour at expansion of the triple layer as shown for *iso*-docosanoic acid was found. The biological activity was also similar to that of the *iso*-branched acid.

Some more complex lipids. As the *iso*-configuration seemed to give the largest biological effect with respect to synergetic behaviour in combination with fungicides, another lipid with an *iso*-branched chain was studied. The racemic 1-monoglyceride of *iso*-tetradecanoic acid was used in order to see the effect of a larger polar group. However, as shown in Table 2, no enhancement effect in the biological system was obtained. The pressure–area curve shown in Fig. 8 obeys the relation found for the others, giving no decrease in pressure after collapse of the liquid expanded phase at 26·1 Å²/M.

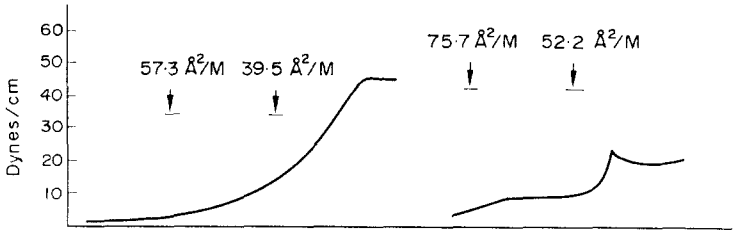

FIG. 8. π–A isotherms of the racemic 1-monoglyceride of *iso*-tetradecanoic acid at 20° (to the left) and 1,3-dimyristin at 20° (to the right).

The effect of addition of cholesterol to the system is given in Table 3. No enhancement was obtained, and neither is there any hump at collapse of the monomolecular surface film.

Finally some more complex lipids were studied. The surface film behaviour at room temperature of monoacid, di- and triglycerides of different chain lengths was examined with regard to collapse properties. The only one which showed this hump in the isotherm was dimyristin. The pressure–area relations are shown in Fig. 8. A liquid expanded phase is first formed, which transforms into a solid condensed phase at 67·5 Å²/M. After collapse of the solid phase at 44·0 Å²/M there is a reduction in pressure. If this is correlated with the biological activity (Tables 1 and 2) it can be seen that the relation observed for all the other lipids is fulfilled.

Interaction with cholesterol. There are numerous reports on the effect of cholesterol on phospholipid monomolecular layers (cf. Ref. 24). The results indicate that cholesterol has a condensing effect on the surface film, and a similar mechanism is believed to be operational in membranes. If *iso*-branched fatty acids act by expanding the cell membrane, as suggested in this paper, cholesterol should be expected to counteract the *iso*-acid, and consequently inhibit the enhancement of the antimicrobial effect. As evident from the activity results shown in Table 3 such a reduction on the effect from *iso*-pentadecanoic acid was in fact obtained. No effect of cholesterol alone on TMTD was observed.

Interaction with membrane-like surface films. A glycosphingolipid and a diglyceride have been used in order to represent a model membrane. Both have the same molecular

geometry as most membrane lipids (two hydrocarbon chains attached to a polar end group), and whereas the glycosphingolipid has a very large polar group, the diglyceride is weakly polar. It is obvious that the glycosphingolipid is more representative of membrane lipids. The diglyceride was chosen in order to detect effects in the hydrophobic interaction with higher sensitivity.

The glycosphingolipid used was sodium sulphatide, the surface film behaviour of which is described in Ref. 1. The diglyceride used was 1,3-dicaprin. This short chain length was chosen as the surface film at room temperature then is in a liquid expanded state, i.e. the hydrocarbon chains are in a liquid state as in membranes.

Binary mixtures of the membrane-like lipid and the very active compound—*iso*-pentadecanoic acid—were spread, and the pressure–area isotherms were recorded.

A plot of the average molecular area at two pressures for the system sulfatide-*iso*-pentadecanoic acid is shown in Fig. 9. At the lower pressure in the beginning of the film condensation as well as at the higher pressure near collapse of the film there is an expansion effect over the whole composition range. It can also be seen that addition of only small amounts of the *iso*-acid results in a pronounced expansion of the film.

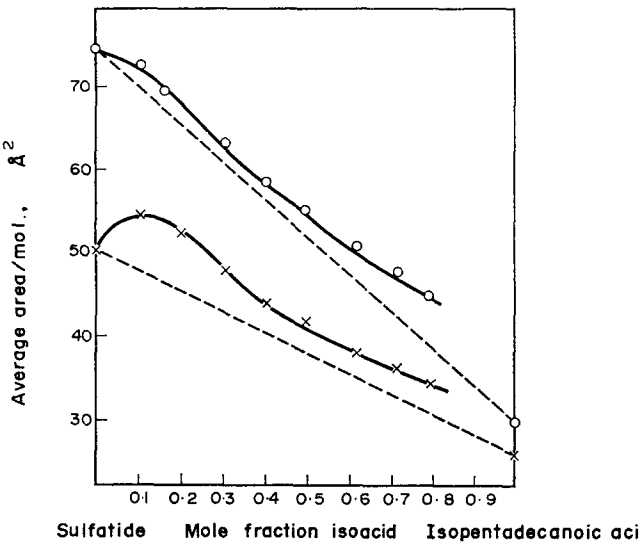

FIG. 9. A plot of the mean molecular area vs. composition in mixed monolayers of sulfatide-*iso*-pentadecanoic acid at 21°. The pressure 15 dynes/cm is indicated by crosses, and that of 30 dynes/cm by open circles.

The average molecular area at the collapse point versus composition of binary films of dicaprin-*iso*-pentadecanoic acid is indicated in Fig. 10. All compositions show only one condensed phase, the liquid expanded state, and as the collapse pressure is almost constant the area at collapse is given. Also in this case it can be seen that the *iso*-branched acid expands the film, and the effect is even stronger than in the sulfatide film. This difference is not surprising as the diglyceride film should be more sensitive for disturbances in the hydrocarbon chain interaction introduced by the *iso*-configuration than the sulfatide film, in which the independent interaction between the polar end groups gives a larger contribution to the film stability.

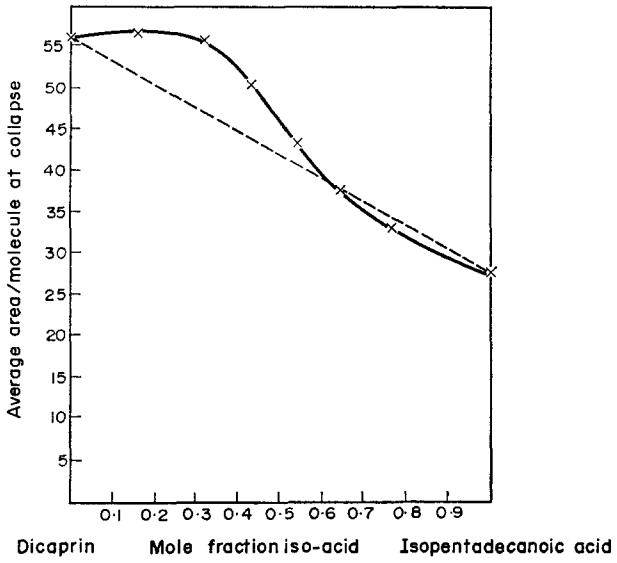

FIG. 10. A plot of the mean molecular area at the collapse point vs. composition of mixed monolayers of dicaprin *iso*-pentadecanoic acid at 21°.

DISCUSSION

The agreement between the synergetic effect of lipids like *iso*-pentadecanoic acid on antibiotic agents and their surface film behaviour observed in this study indicates that the surface film technique can be used in order to provide guidance in the search of membrane-active substances with optimal pharmacologic effect. Synergetic effect should thus be expected if there is a pronounced reduction in pressure after the collapse point, provided that there still is a coherent surface film. The highest effect should be obtained if the monomolecular layer exists in a liquid-expanded state only. Furthermore, it is suggested that the magnitude of the reduction in pressure at collapse of the liquid expanded monolayer is interpreted as a measure of the strength of the biological effect.

A probable mechanism that can explain the enhancement on antibiotic effect obtained from these lipids is that they make the cell membrane more permeable for the active agents. Evidence for this is given in Fig. 11, in which swelling of hyphae of *Fusarium roseum* is obtained by addition of *iso*-fatty acids. The synergetic effect was observed on the fungicides studied except for the mercury compounds. A possible explanation is that this type of compound denaturates the cell proteins in a non-specific way.

The possibility that surface-active types of antibiotics act by increasing membrane permeability to certain substances was proposed long ago by Schulman and Armstrong[25] from observations on the decolorization of methyl blue by yeast cells in the presence of biologically active alkyl succinic acid derivatives.

Extensive studies of the mechanism of action of different antibacterials affecting the cell membrane have been reported by Hamilton.[26] It has been found that cetyltrimethylammonium bromide destroys the semi-permeable character of the membrane in a non-specific way, whereas others, such as tetrachlorsalicylanilide and valinomycin,

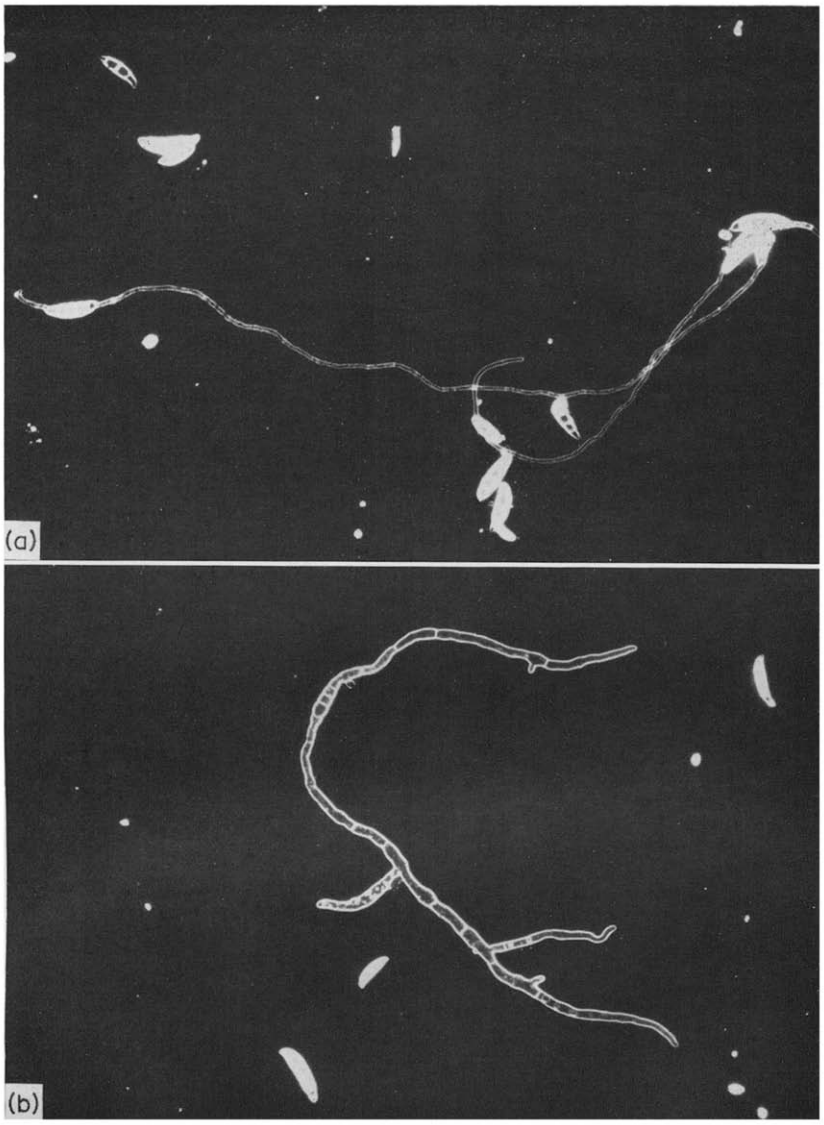

FIG. 11. Hyphae of *Fusarium roseum* grown in nutrient solution are shown above (a), and the effect of addition of *iso*-fatty acids (concentration 0·2 mg/ml) is shown below (b).

increase the permeability of certain ions. By measurements of lysis in different media the specificity of these antibiotic agents on permeability for different ions could be proved. Evidence was also given indicating that the cell wall is acting as barrier for these antibiotics in non-sensitive species.

A common feature of the antimicrobial agents reported by Hamilton[26] and the active lipids studied here is that they are active against Gram-positive organisms, whereas they have much less effect on Gram-negative species. This is probably due to the surface active nature of these compounds. The cell wall of a Gram-negative organism contains a large proportion of lipids in contrast to the Gram-positive ones, and any surface active agent must be expected to adsorb on the lipid surface in an aqueous medium.

For a complete understanding of the synergetic effect it is necessary to know more about the structure of biological membranes. In spite of intensive research in this field during the last years there are many fundamental features of their structure, which are still unknown. It is felt that the effects from influence on membrane transport mechanisms reported here give important information on membrane structure, and these aspects will briefly be discussed.

It has been suggested that the lipids of the cell membrane exist on the borderline of a phase transition.[27,28] Furthermore, there is much evidence indicating that the well-known bimolecular lipid layer constitutes one possible membrane structure type. Such bilayers are spontaneously formed by membrane lipids in the presence of water, and they exhibit many properties characteristic of biological membranes. The bimolecular layer conformation is a very efficient barrier against permeability, and any phase transition from this structure must result in increased permeability. If such a phase transition model is accepted it is obvious that the expansion effect on a membrane-lipid surface film reflects unfavourable interaction conditions between the lipid molecules. A lowering of the energy barrier, which must be overcome at a phase transition from the bimolecular layer conformation, should therefore be expected. Also the surface film behaviour of the synergetically active lipids alone, with the occurrence of metastable monolayers, indicates that the orientation of the lipid molecules into infinite layers in the membrane might be "labilized".

Finer *et al.*[29] have studied the effect of cyclic antibiotics on phospholipids, using NMR-technique, and found that they destroy the bilayer structure formed in aqueous systems by some sort of hydrophobic interaction. The effect of these agents should be expected to increase in the presence of such lipids as *iso*-pentadecanoic acid. The synergetic activity achieved by addition of these membrane-active lipids seems to represent a general mechanism, which might be utilized in the preparation of various drugs, where membrane permeability is involved in the pharmacological effect. Particular attention should, however, be given to the vehicle, as one requirement for synergy is that the active components reach their site of action simultaneously.

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