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Surface-Active Lipids from *Nocardia erythropolis* Grown on Hydrocarbons

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Nocardia erythropolis (ATCC 4277) was grown in a 28-liter fermentor on mineral salts medium and 4% hydrocarbon. Extraction of the neutral lipids with pentane removed approximately 90% of the surface activity of the culture medium. The residual surface activity of the culture medium was attributed to the polar lipid fraction which was not extracted with pentane. Analysis of the pentane extracts with thin-layer chromatography showed the presence of four major compounds. A fatty alcohol reached a maximum concentration in the early log phase of growth and then decreased to the end of the fermentation. A monoglyceride, an ester, and a fatty acid appeared during the log phase of growth and continued to increase until the end of the fermentation. The fatty acids isolated from the culture grown on hexadecane had a carbon skeleton with the same length as the substrate, with 70% of the component as the saturated acid and 30% as a monounsaturated homolog. When isolated from a kerosene culture, the fatty acids consisted of a number of homologs from C_{18} to C_{20}, including branched-chain and unsaturated acids, reflecting the distribution of the branched-chain isomers in the substrate.

Because of increased demands for biodegradable surfactants and for a wider range of surfactant properties, considerable interest has arisen concerning the use of biosurfactants produced by microbes (1, 2).

A preliminary study of *Nocardia erythropolis* (ATCC 4277) showed that this organism produces biosurfactants when grown on a hydrocarbon (8). *N. erythropolis* reduced the surface tension to 29 mN/m and the interfacial tension against hexadecane to less than 3 mN/m from 65 and 35 mN/m, respectively, in mineral salts medium before inoculation. The surface tension value is good when compared with other surfactants, since only a limited number reduce this value to less than 30 mN/m (1). However, the interfacial tension of 3 mN/m is exceptionally low, and very few biosurfactants have been reported to be this effective (1). Further work is now presented on the characterization of these potent biosurfactants and the times of production of these metabolites during the growth cycle of the organism.

Some data have been published concerning the effect of substrate on the lipids of *Nocardia* species (4, 11) which indicate that a strong correlation exists between the substrate and the structure of the lipids. Also, the surfactant properties of glycolipids from other *Nocardia* species have been studied (5; P. Rapp and F. Wagner, Abstr. 5th Int. Ferment. Symp., no. 7.03, p. 113, 1976). This paper characterizes the major neutral lipids found in the surface-active whole broth of *N. erythropolis* and presents the surfactant properties of these and the polar lipids.

MATERIALS AND METHODS

Fermentation by *N. erythropolis*. *N. erythropolis* (ATCC 4277) was grown in a 28-liter New Brunswick fermentor (working volume, 20 liters) on mineral salts medium and 4% hexadecane or Imperial No. 9 kerosene. The agitation rate was 250 rpm, and the temperature was 25°C. The fermentation was monitored by aseptically removing samples approximately every 4 h for the duration of the experiment. Analyses for biomass, surface tension, interfacial tension, and surfactant concentration were conducted on each of the samples. Surfactant concentration measurement was repeated on the samples after extraction with pentane to ensure that the procedure had been effective.

Biomass was determined by passing a portion of broth through a prewashed, preweighed filter paper (0.22 μm). The paper was then dried for 6 to 8 h at 105°C before reweighing. Surface and interfacial tensions were measured with a Fisher Autotensiomat (model 215). All interfacial tension measurements were made against hexadecane.

Surfactant concentration was determined by adding equal portions of culture medium to distilled water until the critical micelle concentration (CMC) of the solution was attained. The CMC is the concentration where the surfactant molecules saturate the solution and form micelles upon further addition of surfactant (1, 2). All surfactant concentrations reported here are in terms of the dilution factor required to reach the CMC.

The surface tensions of the individual fractions and isolated compounds were determined by dissolving the compounds in pentane and adding equal portions of this solution to a known quantity of water. The pentane was then removed by passing air over the sample, and the surface tension was measured. This process was repeated until the CMC was reached. Hexadecane was then layered on the water phase, and the interfacial tension was measured.

Extraction of lipids. Neutral lipids were extracted from the culture medium with pentane (1 ml of pentane/5 ml of culture medium), followed by centrifugation for 20 min at 5,000 rpm and pipetting off the pentane phase. Two samples were extracted repeatedly with pentane, with surface tension measurements being conducted between each extraction.

Lipids were removed from 4.5 g of lyophilized surface-active product by a standard method (6). The material was combined with 45 ml of eluting solvent (chloroform-methanol-water, 25:25:4) and stirred overnight. An equal volume of water was added, and the solution was stirred vigorously for 1 h and then centrifuged for 20 min at 5,000 rpm. The aqueous and organic phases were then separated, and the respective solvents were removed. The organic phase was extracted twice with acetone, leaving the polar lipids as a white solid. Surface tension measurements were then conducted on the aqueous phase, the acetone-soluble (neutral lipid) fraction, and the acetone-insoluble (polar lipid) fraction.

TLC. All preparative and analytical thin-layer chromatography (TLC) studies were conducted with commercially prepared plates with Silica Gel G and a $CaSO_4$ binder (Fisher Rediplate analytical and Analtech preparative plates). The solvent systems used for preparative and analytical TLC were: (i) hexane-isopropyl ether-methanol (70:30:15), (ii) hexane-isopropyl ether-acetic acid (15:10:1), (iii) chloroform-acetone-methanol-acetic acid-water (7:8:2:2:1), (iv) chloroform-methanol-water (65:25:4), and (v) chloroform-methanol-acetic acid-water (25:15:4:2). The first two solvent systems were used for the neutral lipids which were extracted by pentane; the others were used for the polar lipids.

In the analytical TLC studies, the compounds were visualized by spraying the plates with concentrated H_2SO_4 and charring at 150°C. Alternatively, the sprays ninhydrin, phospray, α-naphthol with concentrated H_2SO_4, and 2,4-dinitrophenylhydrazine were used to indicate the presence of specific functional groups (6). After these sprays were used, the plates were charred to determine the total number of components present.

Two-dimensional TLC studies were conducted on both the neutral lipid and polar lipid fractions. In the study of neutral lipids, where solvents i and ii were used, solvent system i was developed first. With the polar lipid fraction (solvents iii, iv, and v), solvent system iv was developed before either of the remaining two solvents.

The formation of specific compounds throughout a fermentation was studied by spotting 100 μl of unlyophilized residue from the pentane extract of whole broth samples over two analytical plates, in the same sequence that the samples were removed from the fermentation. The two plates were then developed together, and the components were charred for visualization.

Isolation and identification of compounds. All components were isolated with preparative TLC (6). The compounds were eluted from the silica gel with chloroform-ether-methanol (1:1:1) or the Bligh-Dyer solvent, chloroform-water-methanol (1:0.8:2). The solvents were removed by rotary evaporation, lyophilization, or both. Before the silica gel was removed from the plates, compounds were visualized by using ultraviolet light. Under ultraviolet light the compounds appeared as light and dark bands without the use of fluorescing dyes.

Fatty acids were esterified to the methyl esters, using anhydrous hydrochloric acid in methanol (6). The monoglyceride was converted to the trimethylsilyl ether by using the commercial agent TRI-SIL"Z" (Pierce Chemical Co., Rockford, Ill.).

Instrumentation and chemicals. Infrared (IR) spectra were determined on a Beckman IR-20 spectrometer, using chloroform as the solvent. The gas chromatograph used in the fatty acid analysis was a Varian Aerograph series 1800 with a column packed with 3% SE on 60/80 Chromosorb W AW (Supelco, Bellefonte, Pa.). All chromatographs were conducted isothermally at 190°C. Standard mixtures of the methyl esters of saturated and unsaturated even-chain fatty acids from C_{12} to C_{20} (Serdary Research, London, Canada) were compared with isolated fatty acids. Mass spectral analysis was conducted on a Varian spectrometer (model MAT 311A). Mono-, di-, and triglyceride standard lipids were obtained from Serdary Research; aldehyde and ketone standards were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Fermentation by *N. erythropolis*. The surface tension, interfacial tension, biosurfactant concentration (dilution to the CMC), and biomass are shown in Fig. 1 for a typical fermentation. Minimum surface tension and interfacial tension were 33 and 2 mN/m, respectively. The maximum surfactant concentration was 160 × CMC of the biosurfactant formed. Maximum biomass concentration was 2.5 g/liter. The culture medium during this fermentation was a pale, milklike liquid which settled into two distinct layers, an upper fraction containing the hexadecane with cellular material and a lower aqueous phase.

The effect of repetitive pentane extractions on the surface-active components of the samples taken at 42 and 94 h is shown in Fig. 2. In both samples, the surfactant concentration of the culture medium was reduced to a minimal value with four washings, after which some residual surface-active material remained. The concentrations were reduced from maxima of 67 and 91 × CMC to minima of 4.5 and 9 × CMC for the 42- and 94-h samples, respectively. The surface tension remained between 34 and 37 mN/m for

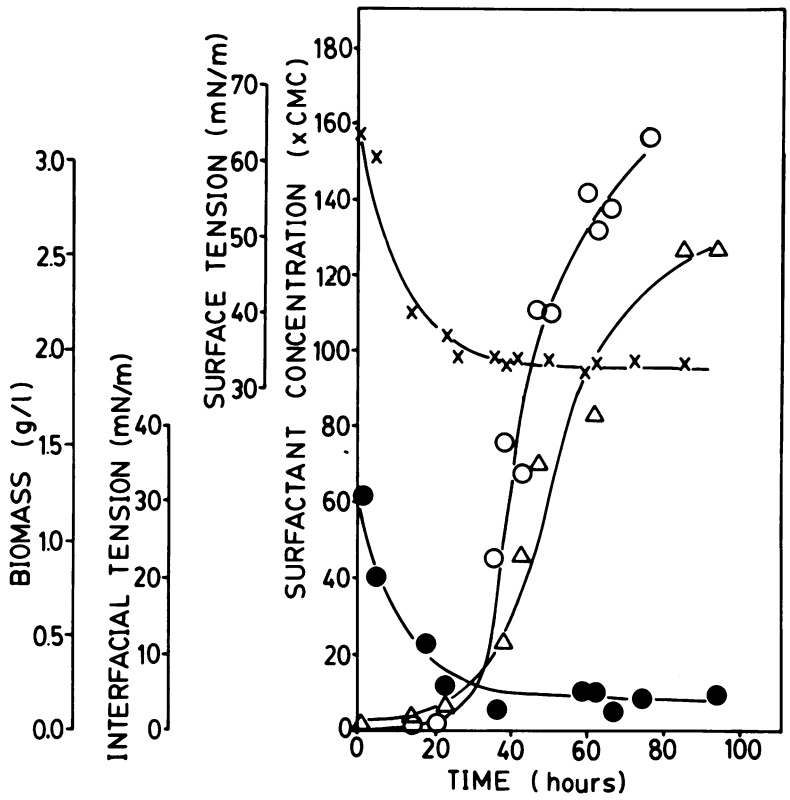

FIG. 1. *Data from a fermentation of N. erythropolis grown on mineral salts medium containing 4% hexadecane, as plotted against fermentation time. Symbols: ○, surfactant concentration; ×, surface tension; ●, interfacial tension; △, biomass concentration.*

both samples during the extraction procedure. Interfacial tension remained constant at 2 mN/m.

TLC of pentane-extractable lipids. TLC data on the pentane extracts of the whole broth gave four major compounds which occurred at different times during the fermentation. Two-dimensional TLC showed the presence of four minor components which were obscured by the major components. None of the components reacted positively with the indicator sprays for phospholipids, lipopeptide, and glycolipids.

TLC studies on the pentane extracts derived from the repetitive washing of the 42- and 94-h samples showed that, for each of the major compounds, most of the compound was removed from the culture medium in the first two pentane extractions. This observation correlated well with the loss of surface activity shown in Fig. 2. These results indicated that two pentane extractions of each sample of culture medium were sufficient to remove most of the biosurfactant.

The changes of the individual components with time throughout the fermentation were studied by extracting each sample twice with pentane. The pentane was removed, the oil was dissolved in hexane, and 100 μl of each extract was spotted on the TLC plate in the sequence that they were removed from the fermentation. Figure 3 illustrates the rapid increase in concentration of one compound (the alcohol) to a maximum at 23 to 26 h and a subsequent decrease until the end of the fermentation. The other major compounds were first observed in the samples taken at 18 to 20 h and continued to increase until the end of the fermentation.

It is important to note that the hexadecane, which was extracted from the culture medium with the neutral lipids, suppressed the R_f value of the upper components (alcohol, etc.) because of its high concentration in the region of R_f values of 0.6 to 1.0 (Fig. 3). Consequently, all samples were lyophilized to remove most of the hexadecane to obtain the R_f values shown in Table 1. This lyophilization step was also important in the preparative TLC, where the hexadecane would saturate the silica gel and be removed with the lipid components. The samples used for the analytical comparison plates, however (e.g., Fig. 3), were not lyophilized. Al-

though this affected the R_f values of the upper components, it ensured no loss of volatile components.

TLC of total lipid extract. TLC was used to differentiate the components in the acetone-soluble (neutral lipids) and acetone-insoluble (polar lipid) fractions extracted from floating product which had been lyophilized to remove hexadecane. The polar lipid fraction contained at least five components which reacted positively to phospray (phospholipids) and also one glycolipid (Table 2).

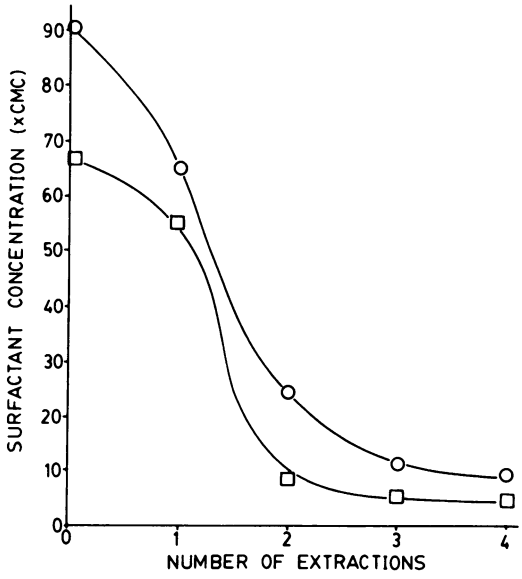

FIG. 2. *Removal of surface-active neutral lipids from two samples of culture medium by extraction with pentane (1 ml of pentane/5 ml of culture medium). The samples were taken at 42 (□) and 94 (○) h.*

There were a number of neutral lipids present in the acetone-soluble phase, including the four major components isolated from the pentane extracts (Table 1). There was also one glycolipid found in the acetone-soluble fraction (Table 2). No lipopeptides were observed in either the acetone-soluble or acetone-insoluble fractions.

Characterization of major neutral lipids. The component that arose early in the fermentation and then decreased in concentration behaved as an alcohol when compared with a standard (stearic alcohol) in solvents i and ii.

A second compound with high R_f values appeared in the late stages of the fermentation, but was obscured by the hexadecane, particularly if the extracts were not lyophilized. The R_f values (Table 1) compared favorably with those of the ester palmityl stearate. Subsequent IR spectra of the isolated compound showed a strong $\nu C = 0$ peak at 1,720 cm^{-1}. It was concluded from this that the compound was a long-chain ester.

A third compound which showed R_f values identical to those of standard fatty acids was isolated from the pentane extracts. The IR spectrum for the compound showed $\nu C = 0$ peaks at 1,560 and 1,700 cm^{-1}, νC–0 stretch at 1,200 cm^{-1}, and a weak $\nu 0$–H at about 3,200 cm^{-1}. After derivatization to the methyl ester, the $\nu C = 0$ peak moved to 1,720 cm^{-1} with the νC–0 stretch in the region of 1,160 cm^{-1}. The $\nu 0$–H band was not present after esterification. Both the IR

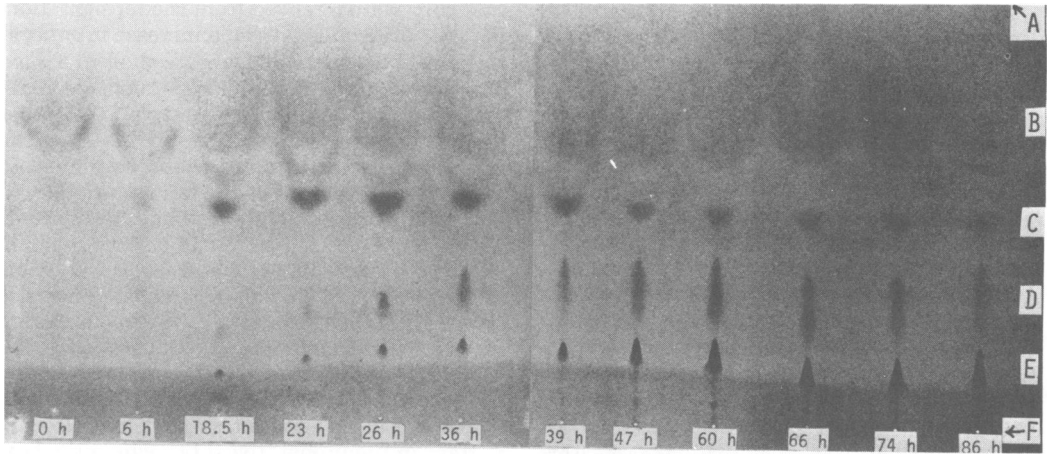

FIG. 3. *TLC of unlyophilized pentane extracts of culture medium taken throughout a fermentation of N. erythropolis grown on 4% hexadecane. One hundred microliters of redissolved extract was spotted at each position on the plate. Solvent system: hexane-isopropyl ether-methanol (70:30:15, vol/vol). (A) Solvent front; (B) hexadecane; (C) alcohol; (D) fatty acid; (E) monoglyceride; (F) origin.*

TABLE 1. *R_f values of neutral lipid components and standard compounds in solvent systems i and ii*

Substance	Solvent system	
	i	ii
Compound[a]		
Fatty acid	0.30–0.55[b]	0.51
Monoglyceride	0.14	0.01
Alcohol	0.61	0.24
Ester	0.90	0.76
Standard		
Palmitic acid	0.30–0.60[b]	0.60
Tetradecanol	0.70	0.54
10-Nonadecanone	0.83	0.74
Palmityl stearate	0.82	0.77
Stearyl alcohol	0.61	0.29
1-Monopalmitin	0.10	0.03

[a] Tentative assignments based on R_f values, IR data, and mass spectral data.

[b] Fatty acid components are very diffuse in solvent system i.

TABLE 2. *R_f values of compounds removed during extraction of total lipids*

Component	Solvent system		
	iii	iv	v
Phospholipid			
A	0.01	0.13	0.42
B	0.15	0.20	0.47
C	0.41	0.53	0.69
D	0.50	0.38	0.80
E	0.81	0.90	0.97
Glycolipid			
A	0.14	0.36	0.61
B[a]	0.30	0.41	

[a] Extracted in the acetone-soluble portion of the total lipid extract.

spectra and the R_f values were characteristic of carboxylic acids, their anions, and their methyl esters, respectively.

Mass spectral analysis of the esterified compound was characteristic of a normal-chain ester with the molecular ion (M) at 270 mass units, corresponding to a fatty acid of 16 carbon atoms, with a small peak corresponding to the monounsaturated acid. The mass spectra contained the fragments typical of the methyl ester of a fatty acid (9). These included the acylium ion (M-30) at *m/e* 239, a large peak at *m/e* 74 due to the McLafferty rearrangement, and other typical fragments at M-43 (*m/e* 239) and M-29 (*m/e* 241). The presence of the monounsaturated acid was confirmed by gas chromatography, which showed a strong peak in the $C_{16:0}$ position and a weaker peak close to the $C_{16:1}$ position (*cis*-9-hexadecanoate standard). The proportion of saturated to unsaturated fatty acid was approximately 7:3.

Fatty acids were also isolated from a fermentation of *N. erythropolis* grown on 4% kerosene as a carbon source instead of hexadecane. The IR spectra were identical for the fatty acids, regardless of the carbon source. The mass spectral analysis of the fatty acid esters derived from the kerosene culture showed parent ions at 298, 312, and 326 mass units, corresponding to acids $C_{18:0}$, $C_{19:0}$, and $C_{20:0}$, with the dominant homolog being $C_{19:0}$. Gas chromatography of this mixture showed at least six peaks between standard $C_{18:0}$ and $C_{19:0}$ acids, suggesting the presence of branched-chain isomers as well as unsaturated fatty acids. The presence of unsaturated fatty acids was supported by minor peaks in the mass spectrum at 2 mass units lower than the parent ions.

The fourth component was tentatively identified as a monoglyceride from R_f values as well as the IR spectra of the compound and its trimethylsilyl ether. The R_f value of the compound in solvent system i was low (0.10), but in solvent system ii the compound was almost immobile (R_f = 0.03). This compared favorably with the standard 1-monopalmitin. The IR spectrum showed peaks at 1,720 and 1,190 cm^{-1} as well as a broad υ0–H peak at about 3,320 cm^{-1}. After derivatization to the trimethylsilyl ether, the spectrum included peaks at 1,725 and 1,200 cm^{-1} as well as a strong υC–Si peak at 840 cm^{-1}, and no υ0–H peak was observed.

Surface activity of the isolated components. The minimum surface tension produced by the fatty acid (33 mN/m) was identical to the minimum surface tension of the whole broth before extraction with pentane (Table 3). The lowest interfacial tension of water against hexadecane (4.0 mN/m) was produced by the ester and corresponded to the minimum interfacial tension of the whole broth. The monoglyceride also showed strong surfactant properties, with surface tension and interfacial tension of 37 and 7 mN/m, respectively.

The production of these compounds began in approximately the 20th hour of the fermentation (Fig. 3), which compared favorably with the

TABLE 3. *Surface activity of neutral lipids extracted with pentane from the culture medium of N. erythropolis*

Component	Minimum surface tension (mN/m)	Interfacial tension (mN/m)[a]
Ester	37	4.0
Monoglyceride	37	7.0
Fatty acid	33	8.0
Whole broth	33	2.0

[a] Measured against hexadecane.

minimum surface and interfacial tensions of the fermentation (Fig. 1). The increase in the concentration of surface-active materials (Fig. 1) also reflected the increase of the individual components removed by pentane (Fig. 3).

Surface activity of fractions from total lipid extract. The surface activity of the three fractions derived by a chloroform-methanol-water extraction of floating product is shown in Table 4. The aqueous portion was an opaque yellowish color, suggesting the presence of solid material, yet the surface activity was poor.

The acetone-soluble fraction contained a number of neutral lipids, including the four major components removed in the pentane extraction process. It caused a low surface tension and a low interfacial tension (<1.0 mN/m) when added to distilled water. This low interfacial tension could be the result of the mixture of neutral lipids, the glycolipid, or some unidentified compound. The acetone-insoluble (polar lipid) fraction also produced a very low interfacial tension.

DISCUSSION

During growth on hexadecane, *N. erythropolis* produces a mixture of biosurfactants. Most of this surfactant activity is due to neutral lipids which can be removed from the culture medium by extraction with pentane. Approximately 90% of the surface-active compounds are removed from the culture medium after four extractions (Fig. 2). The residual surface activity is due to a mixture of polar lipids and pentane-insoluble neutral lipids.

A good correlation exists between the increasing surfactant concentration throughout the fermentation and the quantity of pentane-extractable lipids present (Fig. 3). The monoglyceride and fatty acid first appear in the medium at about 20 h. This corresponds well with the time that the surface and interfacial tensions reach a minimum as the CMC is achieved. Beyond this time, the dilution necessary to reach the CMC increases as the amounts of the three major neutral lipids increase.

The only fatty acids found in the pentane extracts of the hexadecane culture were palmitic acid ($C_{16:0}$) and a monounsaturated homolog ($C_{16:1}$), which have the same carbon skeleton as the substrate. This type of correlation between hydrocarbon substrates and the fatty acids produced is well documented for other organisms (1–4, 7, 11). In the present study, when the substrate was kerosene, three major acids and their unsaturated homologs were found ($C_{18:0}$, $C_{19:0}$, $C_{20:0}$, $C_{18:1}$, $C_{19:1}$, and $C_{20:1}$). Furthermore, the gas chromatogram of the mixture showed that they were not the normal straight-chain acids but branched-chain isomers. The isolation of acids with even and odd carbon numbers and branched carbon skeletons is consistent with the mixture of alkanes present in the kerosene (H. Guignard, M.E.Sc. thesis, The University of Western Ontario, London, Ontario, Canada, 1976). However, the carboxylic acids, 18 to 20 carbon atoms long, are significantly longer than the kerosene alkanes, 10 to 15 carbon atoms long, with the majority of alkanes in lengths of 12 to 14 carbon atoms. This lengthening phenomenon has been reported elsewhere (3) and may indicate that the branched fatty acids do not adequately replace the *n*-isomers.

TABLE 4. *Surface activity of individual fractions derived from total lipid extraction of culture medium of N. erythropolis*

Fraction	Minimum surface tension (mN/m)	Interfacial tension (mN/m)
Aqueous	56	18.5
Acetone soluble	34	<1.0
Acetone insoluble	32	<1.0

Before the major neutral lipids appear in the fermentation medium, there is an early accumulation of fatty alcohol. This reaches a maximum concentration at about 26 h and then rapidly disappears. Suzuki and Ogawa (12) also noted an early maximum in the concentration of fatty alcohols during the growth of *Arthrobacter paraffineus* on hydrocarbons.

Alcohols have been suggested as intermediates in the bacterial oxidation of hydrocarbons (7). Thus, the alcohol in the *N. erythropolis* fermentation could be an intermediate in the formation of fatty acids which do not appear until after the alcohol has been synthesized.

The presence of polar lipids accounts for the residual surface activity after pentane extraction. They also account for the very low interfacial tension of 2 mN/m observed during the fermentation. This lowering of interfacial tension is in addition to the low interfacial tension produced by the compounds in the neutral lipid extract (i.e., 4.0 mN/m for the ester).

None of the surface-active phospholipids (Table 2) found in the polar lipid fraction have been assigned as known *Nocardia* lipids (10). Their R_f values did not compare to standards, including phosphatidyl serine, phosphatidyl glycerol, lysophosphatidyl choline, phosphatidyl inositol, and lecithin.

It can be concluded from this study that approximately 90% of the surface-active materials produced by *N. erythropolis* are pentane-extractable neutral lipids. The remainder of the activity is due to polar lipids. The carboxylic

acids are a major component of the biosurfactant mixture, and they reflect the nature of the alkane substrate.

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