

Electron Microscopic and Microcalorimetric Investigations of the Possible Mechanism of the Antibacterial Action of a Defined Propolis Provenance

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Abstract

Microcalorimetric and electron microscopic studies on the mode of the antibacterial action of propolis were performed on *Streptococcus agalactiae*. It was shown that propolis inhibits bacterial growth by preventing cell division, thus resulting in the formation of pseudo-multicellular streptococci. In addition, propolis disorganized the cytoplasm, the cytoplasmic membrane, and the cell wall, caused a partial bacteriolysis, and inhibited protein synthesis. It was evident that the mechanism of action of propolis on bacterial cells is complex and a simple analogy cannot be made to the mode of action of any classic antibiotics.

Key words

Propolis, antibacterial action, bacteriolysis, electron microscopic studies, microcalorimetric method, inhibition of protein biosynthesis.

Introduction

Propolis, a natural resinous substance produced by honey bees, is employed for the treatment of various infectious diseases in otorhinolaryngology, gynecology, stomatology, and dermatology. Many medical properties, including antibacterial, antimycotic, antiprotozoan, antiviral, anti-inflammatory, and immunostimulatory activities, have been ascribed to propolis.

The antimicrobial and anti-inflammatory properties of propolis have been attributed to the flavonoids pinocembrin, galangin, pinobanskin, pinobanskin-3-acetate as well as to the *p*-coumaric acid benzyl ester and caffeic acid ester derivatives (1–4), while local anesthetic activity was due to pinocembrin, pinostrobin, and a mixture of caffeic acid esters (5). The stimulating effect of propolis on cellular immunity, by increasing the number of plaque-forming cells in the spleen (6), and on mammalian tissue regeneration was ascribed to the presence of free amino acids such as arginine, which stimulates mitosis and

enhances protein biosynthesis, and proline, which promotes the build-up of collagen and elastin (7). In addition, allergic contact dermatitis induced by propolis was due to cinnamic acid ester (8) and caffeic acid esters (9).

In a previous study, Simuth et al. (10) demonstrated that several UV-absorbing components from propolis inhibited the DNA-dependent RNA polymerases of *Escherichia coli* and *Streptomyces aureofaciens*. Therefore, the mechanism of propolis action on microorganisms seems to be complex with respect to those components which are presently known. This fact led us to study the mode of antibacterial action of propolis on *Streptococcus agalactiae* by means of electron microscopy and microcalorimetry.

Materials and Methods

Propolis extract

Propolis was collected in Mainburg/Oberbayern in 1991 (70% conifers: *Abies alba*, *Larix decidua*, and *Picea abies*, 30% mixed woodland: *Alnus glutinosa*, *Betula pendula*, *Corylus avellana*, *Fagus sylvatica*, *Populus nigra*, *Quercus robur*, and *Salix alba*) and a voucher is available on request. We made an ethanolic solution (25% v/v ethanol) from the raw propolis. The ethanolic extract of propolis (EEP) contained: 0.82% caffeic acid, 1.24% *p*-coumaric acid, 0.62% ferulic acid, 0.4% isoferulic acid, 0.84% benzoic acid, and 0.35% 3,4-dimethoxycinnamic acid, further flavonoids and sugars. The flavonoid total content was 1.8%. Chrysin, fisetin, galangin, luteolin-7-glucoside, and quercetin were qualitatively determined.

Bacterial growth and experimental conditions

Streptococcus agalactiae, strain NCTC 8181 (type IIb) from the Central Public Laboratory (London) and strain 58/59 (type Ia), kindly provided by Dr. J. Rotta (Institute of Hygiene and Epidemiology, Prague), were grown in Trypticase peptone-yeast extract broth supplemented with maltose at 37°C under constant shaking (120 rpm) as described earlier (11). Growth was monitored each hour at 660 nm in a PMQ3 Zeiss-Photometer, samples being diluted with medium if necessary.

Exponential growing cultures at A₆₆₀ = 0.5 were divided into 10 ml portions for incubation with ethanolic extract of propolis (EEP). This was the starting point for the EEP treatment. EEP was added to each portion of the culture suspension at concentrations up to 0.25, 0.5, and 1.25 µg/ml, and the incubation was carried out at 37°C for 2 h with shaking. One portion of the bacterial suspension was incubated with 100 µl of a

25% ethanol solution to serve as control. At the end of the incubation period, 5 μ l of each culture were applied on propolis-free blood agar plates and incubated at 37 °C under 5% CO₂ atmosphere for 48 h in order to test the ability of bacterial regeneration after treatment with different concentrations of EEP.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Intracellular proteins and proteins secreted by *S. agalactiae* treated with different concentrations of EEP were analyzed using SDS-PAGE according to the Laemmli method (12).

Microcalorimetry

Microcalorimetric investigations of the effect of propolis on the growth and metabolic activity of *S. agalactiae* were performed as previously reported (11).

Electron microscopy

An exponentially growing culture was incubated for 4 h with EEP at a concentration of 2.5 μ g/ml. Thereafter, cells were harvested by centrifugation at 12,000 \times g (Sorvall SS34 rotor) and 4 °C for 10 min, and washed with 0.1 M cacodylate buffer, pH 7.2. Streptococci were prepared for electron microscopy by fixation in a 2.5% glutaraldehyde solution (Serva, Heidelberg, FRG), post-fixed with 1.5% osmium tetroxide plus 1.65% potassium bichromate in cacodylate buffer for 1 h at room temperature (13) and stained with 1% uranyl acetate. After dehydration with an increasing alcohol series, the bacterial samples were embedded in LR White. Thin sections were cut with glass knives on a Reichert OMU 3 ultramicrotome, post-stained with lead citrate (Reynolds, 1963), and examined in a Philips EM 400 electron microscope at 80 kV.

Results

Viability of streptococcal cells after treatment with propolis extract

Exponentially growing cells of *S. agalactiae*, strains 8181 (type IIb) and 58/59 (type Ia), were incubated with 0.25, 0.5, and 1.25 μ g EEP/ml medium. A significant decrease in the optical density of the bacterial culture suspension was obtained in the presence of EEP concentration of 0.5 μ g/ml. Figure 1 shows the capability of streptococcal cells for regeneration on blood agar plates after treatment with different concentrations of EEP. In comparison to the control without propolis, the ability of propolis-treated cells to regenerate on drug-free blood agar decreased and was completely inhibited by higher concentrations of EEP (1.25 μ g/ml) for strain 58/59. These observations were in accord with the decrease of the optical density of propolis-treated cell cultures. It was found that strain 58/59 was more sensitive to propolis than strain 8181.

SDS-PAGE analysis of extracellular proteins from streptococcal cells after 2 h incubation with different concentrations of EEP showed a marked reduction in the density of protein bands as compared to the control (Fig. 2). The intensity of the inhibition of synthesis and secretion of proteins correlated with the concentrations of EEP used for treatment. Similar results have been obtained by analysis of intracellular proteins of propolis-treated cells.

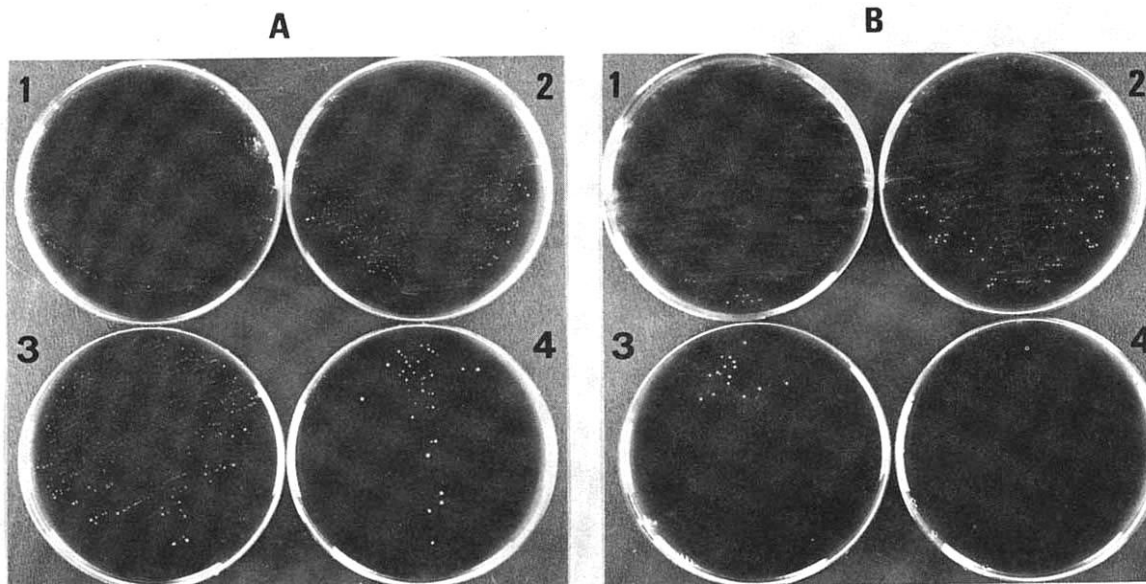


Fig. 1 Recovery of *S. agalactiae*, strains 8181 (A) and 58/59 (B), on drug-free blood agar plates after 2 h treatment with ethanolic extract of propolis (EEP) during growth in Trypticase peptone-yeast extract broth. 5 μ l of each culture were applied on each plate. 1: Control streptococcal cells. 2: Streptococcal cells incubated with 0.25 μ g/ml EEP. 3: Streptococcal cells incubated with 0.50 μ g/ml EEP. 4: Streptococcal cells incubated with 1.25 μ g/ml EEP. Blood agar plates were incubated for 48 h at 37 °C under 5% CO₂ atmosphere.

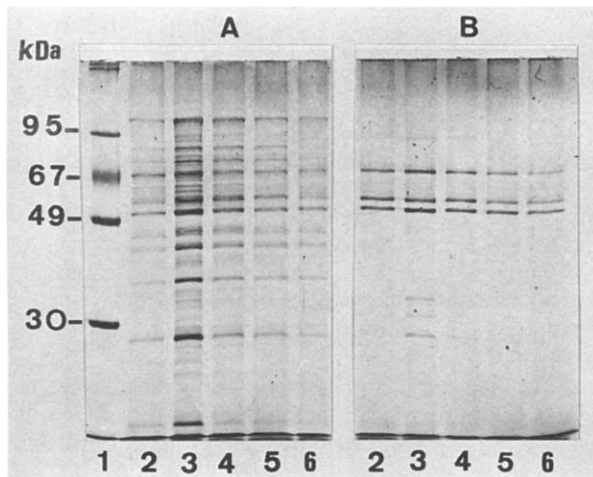
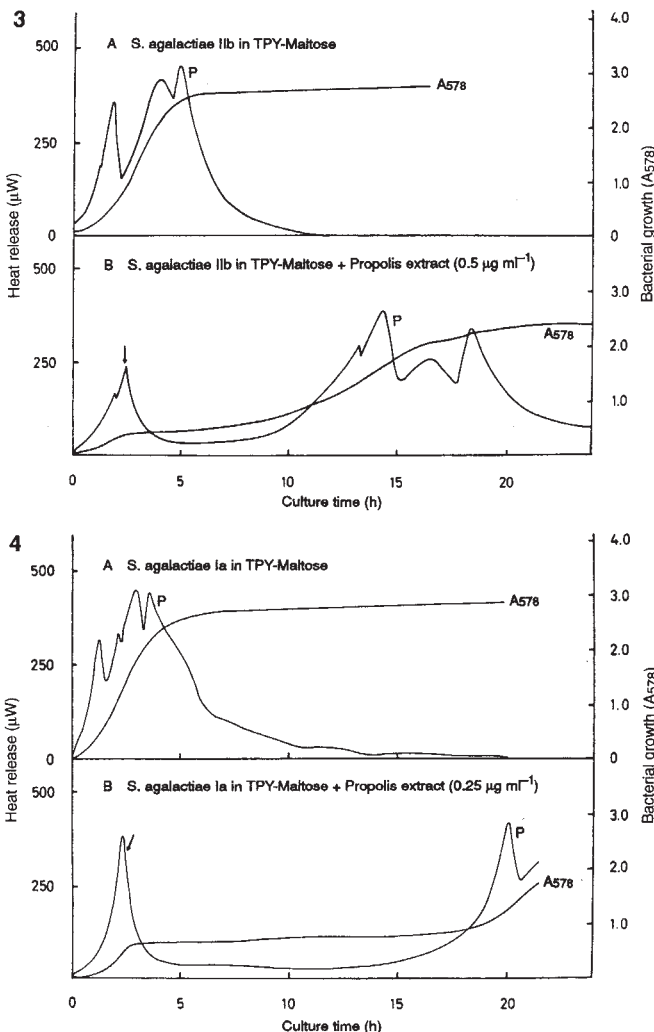


Fig. 2 Analytical SDS-PAGE of extracellular proteins from growing *S. agalactiae*, strains 8181 (A) and 58/59 (B), after 2 h incubation with ethanolic extract of propolis (EEP) in Trypticase peptone-yeast extract broth. Proteins from 500 μ l of each culture were precipitated with 2 volumes of cold ethanol (95 % v/v at -20°C for 30 min. **1:** Protein markers. **2:** Culture prior to propolis addition (T_0). **3:** Control culture without propolis (T_2). **4:** Culture after treatment with 0.25 $\mu\text{g/ml}$ EEP. **5:** Culture after treatment with 0.50 $\mu\text{g/ml}$ EEP. **6:** Culture after treatment with 1.25 $\mu\text{g/ml}$ EEP. Protein bands were stained with 0.3% Coomassie brilliant blue R-250.



Figs. 3 and 4 Microcalorimetric measurement of the effect of ethanolic extract of propolis (EEP) on *S. agalactiae*, strains 8181 (Fig. 3) and 58/59 (Fig. 4), growing in maltose-containing Trypticase peptone-yeast extract broth. **A:** Control culture. **B:** Culture in the presence of EEP. **P:** Power-time curve (heat production). **A₅₇₈:** Bacterial growth curve (optical density at 578 nm). The arrows show the time at which EEP was added to each streptococcal culture.

Microcalorimetric measurement of anti-bacterial activity of propolis extract

Figures 3 and 4 show recordings of the heat evolution and optical density during growth of *S. agalactiae*, strains 8181 and 58/59, in the absence (A) or presence of propolis (B), respectively. The addition of EEP (0.25 or 0.5 $\mu\text{g/ml}$) caused an immediate inhibition of both heat production and bacterial growth as compared to untreated control cultures. The growth and power-time curves returned to minimal levels, and remained in a long plateau-phase (up to 15 h) before they recovered their normal output. Strain 8181 (type IIb) showed less sensitivity to propolis than strain 58/59 (type Ia). The minimal inhibitory concentration of EEP on *S. agalactiae* was found to be between 0.5 and 1.5 $\mu\text{g/ml}$.

In addition, electron micrographs of EEP-treated cells revealed the formation of pseudo-multicellular (polycaryotic) streptococci due to the inhibition of cell separation processes (Fig. 5 D). EEP also caused a disturbance of the cytoplasm characterized by the presence of empty spaces (Fig. 6 A) or fibrous-like structures in the cytoplasm (Fig. 6 B).

Electron microscopy of streptococcal cells treated with propolis extract

Electron microscopic studies on the anti-microbial activity of propolis have, to our knowledge, not been performed to date. Figures 5 and 6 shows cells of *S. agalactiae* (strain 8181) after 2 h of incubation with EEP (2.5 $\mu\text{g/ml}$) at 37°C . An untreated streptococcal cell, which was in the division process, is shown in Figure 5 A. Intact cytoplasmic membrane and cell wall can still be seen. Figures 5 B–D as well as Figures 6 A and B depict structural variations induced in streptococci by propolis. In this way, propolis inhibited the cell division, causing the non-separation of daughter cells (Fig. 5 B and C). Furthermore, alterations of the cytoplasmic membrane as well as defects in the cell wall structure could be observed (Fig. 5 C and D and 6 B).

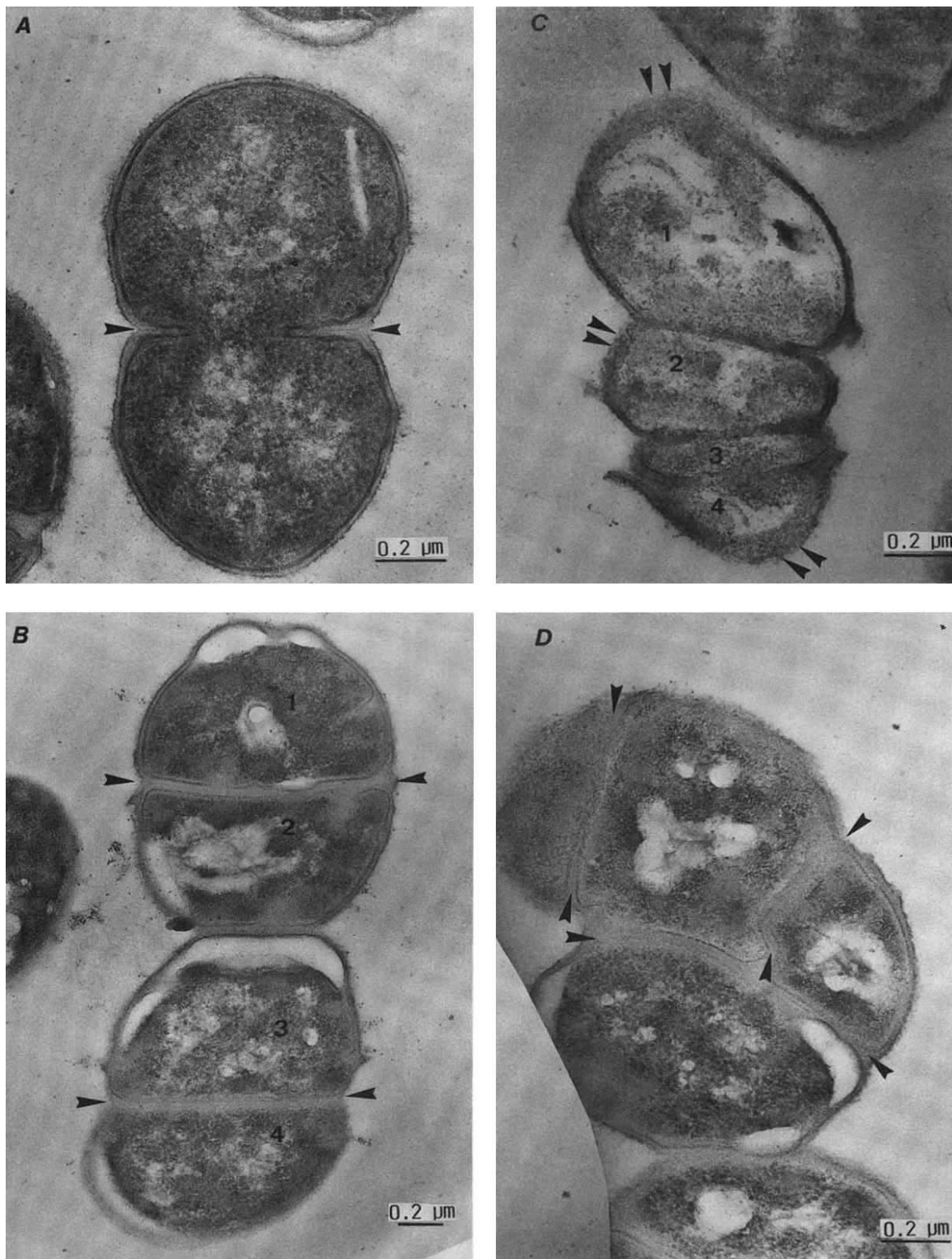


Fig. 5 Electron microscopy of thin sections of *S. agalactiae* NCTC 8181 after a 2 h treatment with ethanolic extract of propolis, EEP, ($2.5 \mu\text{g}/\text{ml}$) during growth in maltose-containing Trypticase peptone-yeast extract broth at 37°C . **A**: Control: streptococcal cell in the process of division; note the presence of cross wall initiation (arrows). **B**, **C**, and **D**: Thin sections of streptococci treated with EEP. **B**: Blockage of cell division by propolis: non-separation of cross wall (arrows) of daughter cells (1, 2, 3, 4). **C**: Inhibition of separation of daughter cells (1, 2, 3, 4) after division; note the defective structure of the cell wall (two arrows) and the material disturbance in the cytoplasm. **D**: Evidence of disturbance of cell division from the appearance of different division planes (arrows): formation of a pseudo-multicellular bacterium as a result of inhibition of cross wall separation by EEP. Bars = $0.2 \mu\text{m}$.

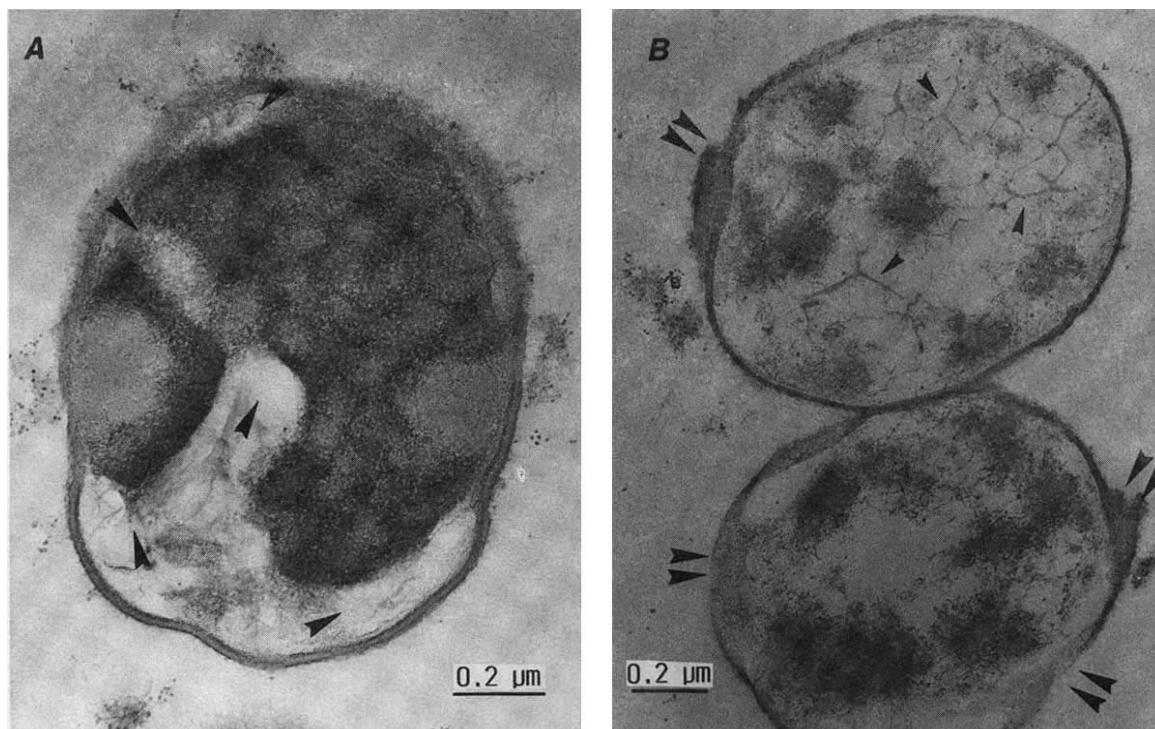


Fig. 6 Thin sections of growing *S. agalactiae* NCTC 8181 after 2 h incubation with ethanolic extract of propolis, EEP, (2.5 µg/ml). **A:** Beginning of cytoplasmic disorganization by EEP (arrows). **B:** Evidence of a total disturbance of the cytoplasm which was emptied of its content. Note the presence of fibrous-like structures (arrows) in the cytoplasm, and of defective sites on the cell wall (two arrows). Bars = 0.2 µm.

Discussion

The treatment of growing cells of *Streptococcus agalactiae* with ethanolic extract of propolis (EEP) from Provenance Mainburg/1991 resulted in the reduction or loss of the ability to regenerate on a propolis-free culture medium (Fig. 1), proving in this way the antibacterial property of this natural remedy. The alterations of the characteristic power-time curves obtained by microcalorimetry during streptococcal growth provided evidence for a reduction or inhibition of the bacterial metabolic activity by EEP. The MIC of propolis observed by microcalorimetric investigations was found to be between 0.5 and 1.5 µg propolis/ml culture medium.

The action of the *S. agalactiae* seemed to be complex as demonstrated by electron microscopic studies of propolis-treated streptococcal cells. The inhibition of cell division and of cross wall separation of daughter cells by EEP led to the formation of pseudo-multicellular streptococci. This effect could be due to the blockage of the so-called splitting system of the cross wall as was demonstrated by *S. aureus* during treatment with trimethoprim (14). The inhibition of cell division observed in the presence of EEP suggested that this natural drug would act like nalidixic acid which is known to inhibit DNA replication and, indirectly, cell division.

The effect on the cell wall caused by EEP could explain the synergism in the effect of propolis with

benzylpenicillin observed in propolis-resistant strains of *S. aureus* (15). One could further extrapolate that the antifungal activity of propolis preparations (16, 17) is believed to be like that of amphotericin B, which forms tight complexes with sterols (ergosterol) of the fungal membranes. Treatment of bacterial cells with EEP led to the disturbance of the cytoplasm (Fig. 6) and sometimes to a partial bacteriolysis. In addition, SDS-PAGE analysis of cellular and secreted proteins from propolis-treated cells (Fig. 2) indicated that propolis inhibited the synthesis and secretion of proteins from the bacterial cells. The fact that the treatment of streptococcal cells with EEP did not lead to the formation of thick cell walls suggested that the mechanism of propolis action was different from that of tetracyclines, chloramphenicol, or macrolides (18–20). Simuth et al. (10) reported that the effect of several UV-absorbing components of propolis upon transcription (*in vitro*) resembles that of rifamycin, which is known to inhibit the bacterial DNA-dependent RNA polymerases.

As demonstrated above, the mechanism of action of propolis appears to be complex and, therefore, a simple analogy cannot be made to the mode of action of the classic antibiotics.

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