Membrane Perturbation by Cerulenin Modulates Glucosyltransferase Secretion and Acetate Uptake by *Streptococcus salivarius*

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Cerulenin and dodecanoic acid prevented the synthesis and secretion of glucosyltransferase in non-proliferating cell suspensions of *Streptococcus salivarius* ATCC 25975 under conditions that also inhibited the incorporation of radioactively labelled acetate into the cell. In the presence of Tween 80, acetate incorporation was not markedly affected by cerulenin despite the fact that glucosyltransferase secretion was still inhibited. Cerulenin and dodecanoic acid were found to prevent the incorporation of radioactively labelled acetate by affecting the uptake of acetate by the cell. In the case of cerulenin, the inhibition of uptake of acetate by the cell was partially relieved by the addition of Tween 80. These and other observations strongly suggested that cerulenin inhibited glucosyltransferase secretion and acetate incorporation by perturbing the membrane, rather than by directly inhibiting lipid synthesis.

INTRODUCTION

The glucosyltransferase(s) of oral streptococci are secreted as a mixture of at least two enzymes which act in a concerted manner *in vivo*. When separated *in vitro* these enzymes react with sucrose to synthesize either a soluble glucan with a characteristic branched α -(1→6) structure or a water-insoluble glucan with a relatively linear α -(1→3) structure (Hare *et al.*, 1978).

Despite the apparent degree of characterization of the pattern of action of these enzymes, the mechanism of secretion of glucosyltransferase still remains to be elucidated. Growth conditions appear to be important in this process since the amount of glucosyltransferase secreted by oral streptococci varies markedly depending upon the constituents of the growth medium employed (Carlsson & Elander, 1973; Schachtele *et al.*, 1976; Janda & Kuramitsu, 1976; Wittenberger *et al.*, 1978) and upon the growth rate and the carbohydrate available to the organism in continuous culture (Carlsson & Elander, 1973; Hamilton *et al.*, 1979; Hardy *et al.*, 1981). In particular, the enhancement of glucosyltransferase synthesis and secretion by the non-ionic surfactants Emanon 4115 (polyethylene glycol monooleate) and Tween 80 (polyoxyethylene sorbitan monooleate) (Umesaki *et al.*, 1977; Wittenberger *et al.*, 1978) has implicated lipid synthesis in controlling the expression of the enzyme. The antibiotic, cerulenin, has been used to support this view. Cerulenin [(3R)-2,3-epoxy-4-oxo-7,10-dodecadienoyl amide] specifically inhibits the condensation reactions between acyl- and malonyl-thioesters by acting as a non-competitive inhibitor of the enzyme of fatty acid chain elongation (D'Agnolo *et al.*, 1973; Omura, 1976; Vance *et al.*, 1972). The antibiotic also prevents the synthesis and secretion of glucosyltransferase by oral streptococci (Kuramitsu & Wondrack, 1980; Leung *et al.*, 1980) as well as other extracellular proteins by bacilli (Caufield *et al.*, 1979; Paton *et al.*, 1980).

However, the direct involvement of lipid synthesis in the secretion of glucosyltransferase has to be questioned since other Tween detergents do not promote the secretion of the enzyme in a similar manner to Tween 80 (Wittenberger *et al.*, 1978). Furthermore the interpretation of the mode of action of cerulenin in suppressing secretion by inhibiting lipid synthesis is in doubt

(Petit-Glatron & Chambert, 1981). As cerulenin is an amphiphilic molecule it has been argued that it can penetrate and interact with the lipid bilayer. This hypothesis has been supported by studies with dodecanoic acid, a fatty acid with a hydrocarbon backbone the same length as that of cerulenin. Dodecanoic acid has been reported to prevent levansucrase synthesis and secretion in *Bacillus subtilis* without affecting acetate incorporation into lipids. To account for this and other observations it has been concluded that cerulenin inhibits exoenzyme synthesis and secretion by *B. subtilis* as a result of its physicochemical interaction with the membrane (Petit-Glatron & Chambert, 1981).

In view of these recent findings with cerulenin, the role of the antibiotic in modulating the synthesis and secretion of glucosyltransferase by *Streptococcus salivarius* has been reappraised. Whereas dodecanoic acid did not directly distinguish between a role for lipid synthesis or a perturbation of the cytoplasmic membrane as a mechanism for modulating exoenzyme secretion in *S. salivarius* in the same way as reported for *B. subtilis* (Petit-Glatron & Chambert, 1981), the use of Tween 80 allowed this distinction to be made.

METHODS

Organism and medium. *Streptococcus salivarius* ATCC 25975 was used throughout these studies. Stock cultures were stored at 4 °C in semi-defined medium containing an excess of CaCO₃ (20 mg ml⁻¹). Transfers were made to fresh medium every month. The semi-defined medium was a modification of the strictly defined medium described by Wittenberger *et al.* (1978) in which all amino acids except cysteine were replaced with casein hydrolysate (5 g l⁻¹) (Oxoid) and no further NaCl was added. The amount of each amino acid in the casein hydrolysate was known from the manufacturer's analysis. Glucose (25 mM) served as the fermentable carbon source.

Chemicals. [U-glucosyl-¹⁴C]Sucrose, [1,2-¹⁴C]acetate, 2-[G-³H]deoxy-D-glucose, and the liquid scintillation cocktail Aquasol-2, were purchased from New England Nuclear. Chloramphenicol and cerulenin were purchased from Calbiochem-Behring Australia, Sydney, Australia; dodecanoic (lauric) acid from Ajax Chemicals, Sydney, Australia and Tween 80 from BDH. Stock solutions of cerulenin and dodecanoic acid were prepared in ethanol. As ethanol can perturb the membranes of bacteria (Buttke & Ingram, 1980; Rigomier *et al.*, 1980) including the membrane of *S. salivarius* (unpublished observations), all traces of ethanol were removed under a stream of nitrogen before use.

Growth measurements. *Streptococcus salivarius* was grown at 37 °C in screw-cap bottles containing 50 to 100 ml of the semi-defined medium. Growth was monitored in 14 mm i.d. test tubes with the aid of a Corning Colorimeter model 252 using a 600 nm filter (Evans Electroselenium, Halstead, Essex, U.K.). The dry weight of bacteria growing exponentially was linearly related to optical density. An optical density of 1.0 in this system was equivalent to 0.82 mg dry wt cells ml⁻¹.

Non-proliferating cell suspensions. Non-proliferating cell suspensions were used to measure the rate of secretion of glucosyltransferase and acetate incorporation by *S. salivarius*. This was necessary since the use of batch cultures presented a number of problems. For instance, plotting the rate of exoenzyme secretion in the presence of cerulenin either against time or increasing cell number during a period in which the doubling time of the cells altered exaggerated the effects of the antibiotic (cf. Caufield *et al.*, 1979; Kuramitsu & Wondrack, 1980; Leung *et al.*, 1980). Consequently, by using non-proliferating cell suspensions accurate changes in the rate of exoenzyme secretion with time could be determined.

Cells incubated in non-proliferating suspension medium for 120 min underwent an approximate twofold increase in optical density. The use of mutants unable to synthesize intracellular polysaccharide confirmed that this density change was due to the production of the storage polymer. No measurable increase in cell number was observed when cells present in serial dilutions of the suspension mixture were counted under the microscope (unpublished observations).

The essential requirements for the production of glucosyltransferase in non-proliferating cell suspensions of *S. salivarius* under defined conditions have been referred to previously (Wittenberger & Wolf, 1979). Cells from 100 ml culture were harvested at mid-exponential phase by centrifugation (8000 g, 4 °C, 10 min), washed and resuspended to 6 ml in 10 mM-potassium phosphate buffer pH 6.5 containing 10 mM-MgSO₄. Cells were stored on ice until used (within 10 min of harvesting) and then equilibrated to 37 °C for 3 min prior to addition to pre-equilibrated suspension medium. Cells were generally resuspended to a concentration of 0.50 ± 0.1 mg dry wt ml⁻¹ in 5 ml volumes of suspension medium containing 200 mM-potassium phosphate buffer (pH 6.5), 7.6 mM-(NH₄)₂SO₄, 0.8 mM-MgSO₄, 50 mM-glucose and hydrolysed casein (5 g l⁻¹) as a source of amino acids. Although these components were sufficient for the production of glucosyltransferase by non-proliferating cell suspensions,

sodium acetate was added to a concentration of 200 μM in order that all experiments were comparable with those where [^{14}C]acetate uptake and/or incorporation were studied. After centrifugation (8000 g, 4 $^{\circ}\text{C}$, 10 min), the supernatant was used as the source of secreted enzyme. In order to assay cell-associated glucosyltransferase activity, cells from the non-proliferating cell suspensions obtained by centrifugation were washed and resuspended to 5 ml volumes in phosphate buffer as described above. In order to obtain cell-free extracts these cells were disrupted by sonication (Branson Sonic Power Company, Conn., U.S.A.: Model S75) at 4 $^{\circ}\text{C}$ and 10 A, 10×30 s with 30 s cooling periods, and then reassayed for glucosyltransferase activity. All experiments reported were repeated at least once and all normalized data are accurate within a s.e. of $\pm 3\%$ of the values shown.

Assay of glucosyltransferase activity. Glucosyltransferase activity was assayed by a modification (Wittenberger *et al.*, 1978; Jacques & Wittenberger, 1981) of the procedure described by Robrish *et al.* (1972). The standard radioactive assay contained 100 mM-potassium phosphate buffer pH 6.0, 10 mM-NaF, 0.1 mM-histidine, 0.5 μl Tween 80 ml^{-1} , enzyme source (20 to 500 μl) and 10 mM-[U-glucosyl- ^{14}C]sucrose (1.39 mCi mol^{-1} ; 51.43 MBq mol^{-1}) in a total volume of 2.0 ml. The tubes were incubated at 37 $^{\circ}\text{C}$ for 60 min, after which time the radioactively labelled polysaccharide was precipitated with ethanol, filtered and washed (Jacques & Wittenberger, 1981). Radioactivity was measured in a Beckman liquid scintillation counter (model LS9000). One unit of enzyme activity (U) was defined as the amount of glucosyltransferase that catalysed the incorporation of 1 μmol of the glucose moiety of sucrose into 75% (v/v) ethanol-insoluble polysaccharide min^{-1} . In all cases, glucosyltransferase activity was expressed as U (mg dry wt of cells) $^{-1}$, as a means of indicating the amount of enzyme secreted by a given cell mass.

When *S. salivarius* was grown in batch culture in semi-defined medium, the average level of glucosyltransferase produced at stationary phase was threefold greater than the average value of 50 mU (mg dry wt) $^{-1}$ produced by cells grown in the strictly defined medium of Wittenberger *et al.* (1978). Irrespective of the medium used, the amount of glucosyltransferase produced in non-proliferating cell suspensions after 120 min of incubation was generally twice that produced by the corresponding batch culture. The average levels of glucosyltransferase observed in non-proliferating cell suspensions containing components of the semi-defined medium were therefore about 300 mU (mg dry wt) $^{-1}$.

Acetate incorporation as a measure of lipid synthesis. The rate of lipid synthesis was measured by the rate of incorporation of acetate into TCA-precipitable material (Kuramitsu & Wondrack, 1980; Leung *et al.*, 1980). Cell suspensions were incubated in the presence of [1,2- ^{14}C]acetate (200 μM ; 1 Ci mol^{-1} ; 37 GBq mol^{-1}). Aliquots (200 μl) were taken at the indicated times and added to 200 μl volumes of 10% (w/v) TCA on ice. After all samples had been collected, a 2 ml volume of 10% TCA at 4 $^{\circ}\text{C}$ was added to each tube and the contents filtered through 2.5 cm Whatman GF/B glass fibre pads under vacuum (Whatman). The sample tubes and filter pads containing the trapped precipitated material were each washed a further three times with 2 ml volumes of 10% TCA at 4 $^{\circ}\text{C}$. After drying at 90 $^{\circ}\text{C}$ the discs were counted in a liquid scintillation counter as described above.

Uptake of acetate and 2-deoxyglucose by the cell. Transport and incorporation of acetate across the cell membrane of *S. salivarius* was determined by measuring the rate of uptake of [^{14}C]acetate over a period of 10 min. At zero time, 100 μl 10 mM-[^{14}C]acetate (2 Ci mol^{-1} ; 74 GBq mol^{-1}) was added to 4.9 ml of a non-proliferating cell suspension and 500 μl volumes were removed at 2 min intervals. The 500 μl volumes were filtered under vacuum on 2.5 cm Whatman GF/B glass fibre discs and washed immediately with six 2 ml volumes of ice-cold 10 mM-potassium phosphate buffer pH 6.5 containing 10 mM-MgSO₄. This treatment was followed by a further two washes with 2 ml volumes of 75% ethanol. After completion of drying at 90 $^{\circ}\text{C}$, the discs were counted in a liquid scintillation counter as described above.

The uptake of 2-deoxyglucose in the presence of glucose was determined in a similar manner to that for the uptake and incorporation of acetate. A non-proliferating cell suspension (5 ml) with or without cerulenin and/or Tween 80 was harvested (8000 g, 4 $^{\circ}\text{C}$, 10 min) after 45 min incubation at 37 $^{\circ}\text{C}$ and resuspended and equilibrated at 37 $^{\circ}\text{C}$ in 4.9 ml of an equivalent suspension mixture devoid of glucose. At zero time (2 min after equilibration), 100 μl 10 mM-glucose containing 4 μM -2-[^3H]deoxyglucose (5000 Ci mol^{-1} ; 185 TBq mol^{-1}) was added. Volumes of 500 μl were filtered and processed at 1 min intervals for 5 min as described above and the rate of uptake of 2-deoxyglucose was calculated.

Preparation and analysis of fatty acids. Samples of cells for fatty acid analysis (10 to 50 ml) were harvested by centrifugation (10000 g, 4 $^{\circ}\text{C}$, 10 min), washed and resuspended in distilled water (2.5 ml). The membrane-bound lipids were extracted according to the method of Bligh & Dyer (1959), and methyl esters were prepared as reported previously (Jacques, 1982). The fatty acid methyl esters were analysed on a Hewlett-Packard GLC (7600 A Chromatograph System). Samples (1 μl) were injected at an initial temperature of 150 $^{\circ}\text{C}$, the temperature being programmed to rise at 6 $^{\circ}\text{C min}^{-1}$ after 1 min to a final temperature of 240 $^{\circ}\text{C}$. The column (2.5 mm $\times$ 170 cm) was filled with a commercially prepared packing of 10% (w/w) SP-2330 on 100/120 Chromosorb (Supelco, Bellefonte, Pa., U.S.A.). Identification of saturated and unsaturated fatty acids was determined by co-chromatography with known standards. The area under each peak was determined in duplicate by the use of a Summagraphics area digitizer (Summagraphics, Fairfield, Conn., U.S.A.). The accuracy of this procedure was confirmed using fatty acid mixtures of known composition.

RESULTS

Effect of cerulenin and dodecanoic acid on the synthesis and secretion of glucosyltransferase

Cerulenin and dodecanoic acid were added in increasing concentrations to non-proliferating cell suspensions. After incubation at 37 °C for 120 min, the glucosyltransferase activity was measured. Cerulenin (3 µg ml⁻¹), inhibited glucosyltransferase synthesis and secretion by 79%, whereas 20 µg dodecanoic acid ml⁻¹ was required to obtain a similar degree of inhibition (83%) (Table 1). During the course of all incubations, the optical density of the suspensions was monitored at 15 min intervals to determine possible cell lysis. None was apparent.

Neither cerulenin nor dodecanoic acid in the range of concentrations used had any effect on the activity of glucosyltransferase in the *in vitro* assay system, nor was there any increase in the cell-associated activity or the intracellular activity of disrupted cells after prior incubation for 120 min in non-proliferating suspension medium. The results indicated that a range of concentrations for cerulenin and dodecanoic acid existed which gave similar inhibitory patterns for the secretion of glucosyltransferase and supported the view that the synthesis of glucosyltransferase in *S. salivarius* was linked to secretion (Wittenberger *et al.*, 1978).

Effect of Tween 80 on glucosyltransferase secretion

When cells were incubated in non-proliferating suspension medium for 120 min with Tween 80 at the relatively low concentration of 0.05 µl ml⁻¹ (Wittenberger *et al.*, 1978), the synthesis and secretion of glucosyltransferase was stimulated. The amount of glucosyltransferase secreted increased from 300 ± 50 mU (mg dry wt)⁻¹ in the absence of the detergent, to 650 ± 50 mU (mg dry wt)⁻¹ in its presence.

The effect of Tween 80 on modulating the effects of cerulenin and dodecanoic acid on glucosyltransferase secretion was examined. Non-proliferating cell suspensions were incubated for 30 min in the presence and absence of 2 µg cerulenin ml⁻¹. After 30 min, the cell suspension containing the antibiotic was divided into two and 0.05 µl Tween 80 ml⁻¹ added to one half. All three suspensions were incubated at 37 °C for a further 90 min. Samples were taken at 15 min intervals throughout the 120 min incubation period and chloramphenicol (50 µg ml⁻¹) was added to prevent any further synthesis of glucosyltransferase (Wittenberger *et al.*, 1978). Cerulenin at 2 µg ml⁻¹ markedly reduced the level of glucosyltransferase secreted during the 120 min incubation. In contrast, cell suspensions containing 2 µg cerulenin ml⁻¹ to which 0.05 µl Tween 80 ml⁻¹ had been added secreted glucosyltransferase at a higher rate. The increased rate of secretion was such that 90 min after adding the detergent, the amount of glucosyltransferase secreted was the same as in control suspensions devoid of both antibiotic and detergent (Fig. 1*b*). Similar results were obtained when 2 µg cerulenin ml⁻¹ was replaced by 20 µg dodecanoic acid ml⁻¹ (Fig. 1*a*), or when cells were harvested, washed and resuspended in fresh medium devoid of cerulenin. The latter result of reversing the inhibitory effects of

Table 1. *Effect of cerulenin and dodecanoic acid on synthesis and secretion of glucosyltransferase in non-proliferating cell suspensions*

100% activity for cerulenin treated cell suspension represented 311 mU (mg dry wt)⁻¹ and for dodecanoic acid 295 mU (mg dry wt)⁻¹. The dry weight of cells in both cases was 0.52 mg ml⁻¹.

Cerulenin		Dodecanoic acid	
Concentration (µg ml ⁻¹)	Glucosyltransferase activity (%)	Concentration (µg ml ⁻¹)	Glucosyltransferase activity (%)
0.0	100	0	100
1.0	82	10	85
2.0	43	20	17
3.0	21	30	8
4.0	9	40	3
5.0	7	50	1

Fig. 1. Effect of Tween 80 on reversing the effect of (a) 20 μg dodecanoic acid ml^{-1} and (b) 2 μg cerulenin ml^{-1} on glucosyltransferase secretion. Samples (2 ml) were removed from 35 ml or 60 ml cell suspensions at the indicated times and cooled on ice in the presence of chloramphenicol (50 μg ml^{-1}). After centrifugation, glucosyltransferase activity was assayed. The arrow indicates the point at which the experimental culture was divided into two and 0.05 μl Tween 80 ml^{-1} added to one half. ●, No inhibitor; □, inhibitor; ■, inhibitor plus 0.05 μl Tween 80 ml^{-1} .

Table 2. Fatty acid profile of *S. salivarius* grown in the presence or absence of Tween 80

Cells were grown to mid-exponential phase in the strictly defined medium of Wittenberger *et al.* (1978) as described in the text. The results are expressed as the percentage of the total fatty acids present in the cell lipids.

Fatty acid	Amount of fatty acid (%)	
	No Tween 80	Tween 80 (0.5 μl ml^{-1})
Tetradecanoic ($\text{C}_{14:0}$)	4.6	3.5
Hexadecanoic ($\text{C}_{16:0}$)	37.7	28.6
Hexadecenoic ($\text{C}_{16:1}$)	10.3	11.8
Unknown	0.0	1.0
Octadecanoic ($\text{C}_{18:0}$)	6.3	3.8
Octadecenoic ($\text{C}_{18:1}$)	31.4	42.7
Eicosanoic ($\text{C}_{20:0}$)	0.0	1.0
Eicosenoic ($\text{C}_{20:1}$)	9.8	7.7
Unsaturated/saturated fatty acid ratio	1.06	1.65

cerulenin by removing the antibiotic has been well established (Kuramitsu & Wondrack, 1980; Petit-Glatron & Chambert, 1981).

Fatty acid composition of S. salivarius grown in the presence and absence of Tween 80

Cells grown in the absence of Tween 80 were used as a 5% (v/v) inoculum for two 100 ml volumes of medium, one of which contained 0.5 μl Tween 80 ml^{-1} . The fatty acid profile of cells sampled at various stages throughout the growth cycles of both cultures was similar to those given in Table 2 for mid-exponential phase cells. Cells grown in the presence of 0.5 μl Tween 80

Fig. 2. Effect of cerulenin on (a) glucosyltransferase synthesis and secretion and (b) acetate incorporation. Samples (2 ml) were removed for assay of glucosyltransferase activity as described in Fig. 1. In separate 5 ml cell suspensions containing [¹⁴C]acetate, 200 μ l samples were taken at the indicated times and mixed with 200 μ l TCA (10%) on ice. Acetate incorporation was then determined. ●, Control suspensions, no cerulenin added; ■, cerulenin (2 μ g ml⁻¹); ▲, cerulenin (5 μ g ml⁻¹).

ml⁻¹ were enriched with the unsaturated fatty acid, octadecenoic acid, in the membrane lipids, mainly at the expense of the saturated fatty acids hexadecanoic acid and octadecanoic acid. The incorporation of octadecenoic acid resulted in an increase in the unsaturated/saturated fatty acid ratio from 1.06:1.00 for cells grown in the absence of Tween 80 to 1.65:1.00 for cells grown in its presence (Table 2).

Effect of cerulenin on cells enriched with octadecenoic acid

Non-proliferating cell suspensions were prepared from bacteria grown in the presence and absence of 0.5 μ l Tween 80 ml⁻¹, and incubated with or without 2 μ g cerulenin ml⁻¹ for 120 min. Measurement of glucosyltransferase activity indicated that 2 μ g cerulenin ml⁻¹ caused a 15% further decrease in secretion of the enzyme in those bacteria pre-grown in the presence of Tween 80 compared with those grown in its absence. The result indicated that an increase in the membrane fatty acid, octadecenoic acid, by prior growth in the presence of Tween 80 did not prevent cerulenin inhibition of the secretion of glucosyltransferase.

Cellular incorporation of acetate in the presence of cerulenin and dodecanoic acid

The incorporation of radioactively labelled acetate and the secretion of glucosyltransferase in the presence of 0, 2 and 5 μ g cerulenin ml⁻¹ were examined at 15 min intervals. At each concentration of cerulenin the decrease in the amount of acetate incorporated after 120 min of incubation correlated well with the decrease in the secretion of glucosyltransferase. Furthermore, the time course for the incorporation of acetate possessed similar kinetics to that for glucosyltransferase secretion for each concentration of cerulenin used. For instance, with cerulenin at 5 μ g ml⁻¹, incorporation of acetate appeared to cease after 30 min. The secretion of glucosyltransferase followed a similar pattern (Fig. 2).

Increasing the concentration of dodecanoic acid also resulted in a decrease in the incorporation of acetate. In the presence of 10, 20, 30, 40 and 50 μ g dodecanoic acid ml⁻¹, incorporation of acetate appeared to be linear with time over a 120 min incubation period (results not shown). This observation differed from that with cerulenin where the amount of acetate incorporated reached a plateau by 120 min.

Fig. 3. Degree of inhibition of glucosyltransferase secretion and acetate incorporation by (a) dodecanoic acid and (b) cerulenin in the presence and absence of Tween 80. Cell suspensions (5 ml) were incubated for 120 min, centrifuged and glucosyltransferase activity determined. Acetate incorporation was measured after 120 min in similar cell suspensions containing [^{14}C]acetate. $\square$, Glucosyltransferase activity (no Tween 80); 100% activity in (a) was 295 mU (mg dry wt) $^{-1}$ and in (b) 304 mU (mg dry wt) $^{-1}$; $\blacksquare$, glucosyltransferase activity (0.05 μl Tween 80 ml $^{-1}$); 100% activity in (a) was 1179 mU (mg dry wt) $^{-1}$ and in (b) 774 mU (mg dry wt) $^{-1}$; $\circ$, acetate incorporated (no Tween 80); 100% incorporation in (a) was 116 nmol (mg dry wt) $^{-1}$ and in (b) 86 nmol (mg dry wt) $^{-1}$; $\bullet$, acetate incorporated (0.05 μl Tween 80 ml $^{-1}$); 100% incorporation in (a) was 88 nmol (mg dry wt) $^{-1}$ and in (b) 85 nmol (mg dry wt) $^{-1}$.

Effect of Tween 80 on the incorporation of acetate in the presence of cerulenin and dodecanoic acid

The relationship between acetate incorporation and glucosyltransferase secretion by cell suspensions was studied in the presence of 0.05 μl Tween 80 ml $^{-1}$. At this concentration, Tween 80 had no significant effect on either the rate or the degree of incorporation of acetate into the cell during a 120 min incubation period.

As the concentration of dodecanoic acid was increased, acetate incorporation was inhibited to the same extent irrespective of the presence or absence of the detergent (Fig. 3a). Although the pattern of inhibition of glucosyltransferase was similar in the presence of Tween 80, inhibition was not as pronounced for a given concentration of dodecanoic acid. This gave rise to a shift to the right for the inhibition curve for glucosyltransferase secretion despite the same degree of inhibition of acetate incorporation (Fig. 3a).

When similar experiments were performed using cerulenin instead of dodecanoic acid, only a minor shift in the inhibition curve for glucosyltransferase synthesis and secretion was observed in the presence of Tween 80. However, the inhibition of acetate incorporation by cerulenin was significantly reduced in the presence of the detergent (Fig. 3b). The amount of acetate incorporated in the presence of 2 μg cerulenin ml $^{-1}$ was in fact the same as that in the presence of 5 μg cerulenin ml $^{-1}$ plus 0.05 μl Tween 80 ml $^{-1}$.

Uptake of acetate by S. salivarius

The incorporation of radioactively labelled acetate into the lipids of *S. salivarius* is dependent upon two factors. First, the added acetate has to be transported into the cell, and secondly, it has to be incorporated into the lipids. If cerulenin and dodecanoic acid were altering membrane processes directly, then incorporation of radioactively labelled acetate into the cell might be modulated by an effect of these compounds on the transport of acetate by the cell. This possibility was investigated by measuring the rate of uptake of acetate by cell suspensions after

Fig. 4. Effects of (a) cerulenin and (b) dodecanoic acid on the uptake of acetate in the presence or absence of Tween 80. Cell suspensions (4.9 ml) were incubated in the presence or absence of inhibitor for 40 min after which time 100 µl 10 mM-[¹⁴C]acetate was added. Samples (500 µl) were removed at the indicated times, filtered and washed to determine the amount of acetate transported into the cell. Filled symbols, no Tween 80; open symbols, 0.05 µl Tween 80 ml⁻¹. ●, ○, No inhibitor; ■, □, in (a) cerulenin (2 µg ml⁻¹) and in (b) dodecanoic acid (20 µg ml⁻¹); ▲, ▽, in (a) cerulenin (5 µg ml⁻¹) and in (b) dodecanoic acid (50 µg ml⁻¹).

an initial 40 min incubation in the presence of a given concentration of cerulenin or dodecanoic acid. In the presence of 2 or 5 µg cerulenin ml⁻¹, the rate of uptake of acetate was reduced by 70% and 97%, respectively. However, a significant reversal of the inhibition of acetate uptake by the antibiotic was observed when 0.05 µl Tween 80 ml⁻¹ was added (Fig. 4a). Interestingly, the rate of uptake of acetate in the presence of 2 µg cerulenin ml⁻¹ was the same as that in the presence of 5 µg cerulenin ml⁻¹ plus 0.05 µl Tween 80 ml⁻¹ (Fig. 4a).

Uptake of acetate was also dependent upon the concentration of dodecanoic acid, but independent of the presence or absence of 0.05 µl Tween 80 ml⁻¹ (Fig. 4b).

Uptake of 2-deoxyglucose by S. salivarius

Streptococcus salivarius ATCC 25975 possesses two phosphoenolpyruvate:glucose phosphotransferase systems for the uptake of glucose, one of which is specific for the uptake of the non-metabolizable substrate 2-deoxyglucose (Vadeboncoeur & Trahan, 1982). It was of interest therefore to determine whether the rate of transport of the non-metabolizable substrate 2-deoxyglucose could also be altered by cerulenin. In the presence of 5 µg cerulenin ml⁻¹, the rate of uptake of 2-deoxyglucose was reduced by 48% compared with control cell suspensions devoid of the antibiotic, where the rate of uptake was equivalent to 187 pmol (mg dry wt)⁻¹ h⁻¹. Tween 80 (0.05 µl ml⁻¹) totally reversed this effect. Interestingly, in the absence of added cerulenin, 0.05 µl Tween 80 ml⁻¹ increased the rate of uptake of 2-deoxyglucose by 42% compared with control cultures devoid of the detergent, further indicating that low concentrations of Tween 80 could influence membrane properties.

DISCUSSION

Cerulenin and dodecanoic acid affected exoenzyme secretion and acetate incorporation by *S. salivarius* in two ways that were significantly different from those reported for *B. subtilis* (Petit-

Glatron & Chambert, 1981). First, cerulenin affected secretion at lower concentrations than dodecanoic acid and, secondly, both cerulenin and dodecanoic acid inhibited acetate incorporation throughout the concentration range in which they affected the secretion of glucosyltransferase. Consequently, dodecanoic acid could not be used to distinguish between lipid synthesis or membrane perturbation as the primary site of modulation of exoenzyme secretion in *S. salivarius*, as was the case with *B. subtilis* (Petit-Glatron & Chambert, 1981).

The correlation between cerulenin and dodecanoic acid inhibition of glucosyltransferase secretion and acetate incorporation appeared at first sight to support a role for lipid synthesis in the secretion of glucosyltransferase by oral streptococci (Kuramitsu & Wondrack, 1980; Leung *et al.*, 1980). However, while there was no known effect of dodecanoic acid as an inhibitor of lipid synthesis, the fatty acid had been reported to modify the phase transition profile of a bilayer of dipalmitoylglycerophosphocholine by penetrating the lipid phase (Jain & Wu, 1977), as well as to associate reversibly with the cytoplasmic membrane of a Gram-positive organism (Petit-Glatron & Chambert, 1981).

The non-ionic detergent Tween 80 was found to reverse the inhibitory effect of 2 µg cerulenin ml⁻¹ on glucosyltransferase secretion (cf. Fig. 1). If cerulenin were in a state of equilibrium with the cell and its external milieu, Tween 80 might then bind the external antibiotic and so alter its equilibrium with the cell. If this were the case, reversal of the inhibition of the secretion of glucosyltransferase by the detergent could be easily explained in a similar manner to washing cells free of the antibiotic (Kuramitsu & Wondrack, 1980; Petit-Glatron & Chambert, 1981; and this paper). However, the degree of inhibition of glucosyltransferase secretion with increasing concentrations of cerulenin was essentially the same whether or not Tween 80 was present (cf. Fig. 3a), while the absolute amount of glucosyltransferase secreted in the presence of Tween 80 was always two- to threefold greater, irrespective of the concentration of the antibiotic used in this study. The apparent reversal by Tween 80 of the inhibition of glucosyltransferase secretion observed in the presence of 2 µg cerulenin ml⁻¹ was therefore due to the stimulatory property of the detergent. This stimulatory effect required the presence of the detergent since the synthesis and secretion of glucosyltransferase was more susceptible to cerulenin in cells enriched with octadecenoic acid by prior growth in the presence of the detergent.

The use of Tween 80, however, enabled a distinction to be made between the role of cerulenin as an inhibitor of lipid synthesis or as a membrane modulating agent. Any apparent correlation between the degree of acetate incorporation into the cell and the inhibition of glucosyltransferase secretion no longer pertained in the presence of 0.05 µl Tween 80 ml⁻¹. Acetate incorporation, over the range of cerulenin concentrations tested, occurred at elevated rates in the presence of Tween 80, albeit at slightly reduced rates compared with cells suspended in the absence of the antibiotic. These observations suggested that cerulenin could be affecting the transport of acetate across the cell membrane. Studies on the uptake of acetate by the cell supported this view. Of particular interest was the fact that the rate of uptake of acetate in the presence of 2 µg cerulenin ml⁻¹ was the same as in the presence of 5 µg cerulenin ml⁻¹ plus 0.05 µl Tween 80 ml⁻¹. This gave rise to an equivalent degree of incorporation of acetate into the cell for these two conditions. Furthermore, cerulenin decreased the rate of uptake of the non-metabolizable substrate 2-deoxyglucose, whilst the presence of 0.05 µl Tween 80 ml⁻¹ reversed the effect. The primary role of cerulenin as an inhibitor of glucosyltransferase secretion and acetate incorporation in *S. salivarius* therefore appeared to be due to its ability to perturb membrane structure rather than to inhibit lipid synthesis directly. Such perturbations might influence one or several events required for the initiation of glucosyltransferase secretion, including the direct interaction between polysomes and membranes or the mechanism of translocation of the protein across the cytoplasmic membrane (Smith *et al.*, 1979; Wickner, 1979). Examination of these processes in more detail should enable some of these questions to be answered.

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