BBA 75743

ULTRASTRUCTURAL AND CHEMICAL ALTERATIONS INDUCED BY DICUMAROL IN *STREPTOCOCCUS FAECALIS*

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(Received May 4th, 1971)

SUMMARY

1. The effect of dicumarol on the ultrastructure, total RNA and phospholipid content of *Streptococcus faecalis* incubated in phosphate buffer with glucose was studied.

2. Electron microscopy showed a change in the geometry of the cytoplasmic membrane resulting in a symmetric profile and a progressive depletion in ribosomes; signs of cell lysis appeared at a later stage.

3. When compared with cells incubated without dicumarol, the levels of total RNA and of lipid phosphorus per cell were consistently lower, the phospholipid composition showing a reduction in the proportion of aminoacyl derivatives of phosphatidylglycerol and an increase in the proportion of cardiolipin.

INTRODUCTION

It was previously reported[1] that dicumarol increases the rate and degree of metabolic swelling[2], eventually leading to lysis, of protoplasts from *Streptococcus faecalis*. Later it was found[3] that in the presence of dicumarol the total amount of ATP produced during metabolization of glucose by whole cells of *S. faecalis* exceeds the theoretical value expected with this bacterium[4]. Recent data[5] showed that after such a treatment most of the cells became unable to grow when reinoculated in fresh culture medium. Thus, it was considered of interest to study the ultrastructure of this bacterium during incubation with dicumarol *plus* glucose. The results obtained prompted us to look also for alterations in the phospholipid and RNA content of the cells.

MATERIALS AND METHODS

Cells of *S. faecalis* ATCC 9790, grown, harvested, and washed as described previously[3], were suspended in buffer (0.075 M K_2HPO_4–HCl, pH 7.2) in a final concentration of 2.5 mg dry weight of cells per ml. To one aliquot of this suspension dicumarol (Sigma Chemical Co.) was added (final concentration 0.01, 0.1 or 0.5 mM)

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followed, 15 min thereafter, by glucose (11 mM) (time zero). In another aliquot (control) dicumarol was omitted. The aliquots were incubated at 37° under aerobic conditions, with shaking. Samples taken at various times during the incubation were centrifuged and the harvested cells studied as follows.

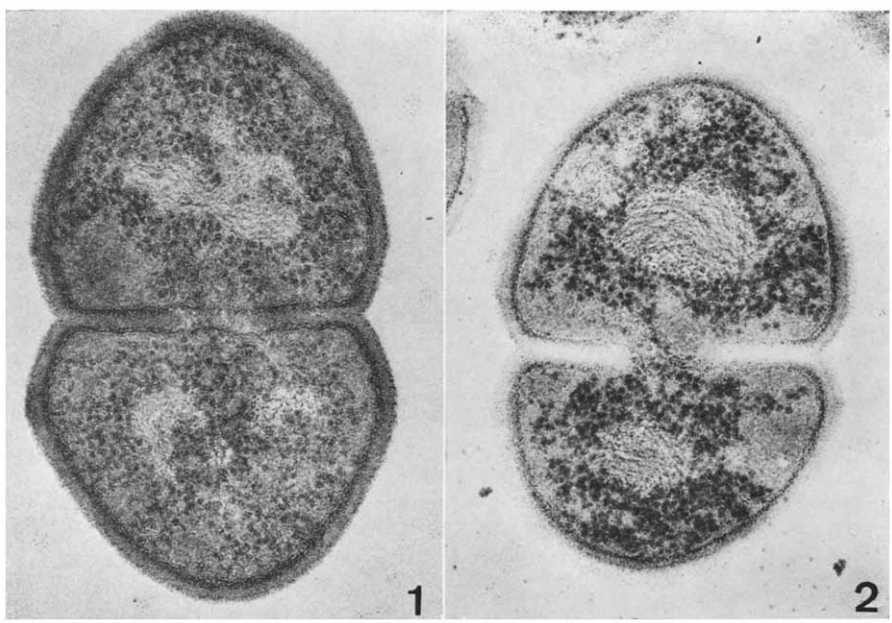
Electron microscopy

The cells were fixed for 6–16 h at room temperature by the standard fixative procedure of RYTER AND KELLENBERGER[6], including the postfixation with uranyl acetate. The prefixative step was omitted because of the presence of phosphate in the buffer[7]. In some experiments parallel samples were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h, and embedded without any postfixation. Further processing was carried out as previously described[8].

Treatment with ribonuclease (L. Light and Co.; 0.1 % solution in water, pH 6.8, for 4 h at 37°), was carried out on sections of cells which had been fixed with glutaraldehyde or by the procedure of RYTER AND KELLENBERGER[6]; in the latter case, the sections were previously oxidized with H_2O_2 (ref. 8).

Estimation of lipid phosphorus

Lipid material was extracted and fractionated by thin-layer chromatography as described before[9]. Quantitative estimation of the phospholipids was done by assaying phosphorus[10].



Figs. 1–11. Cells of *S. faecalis* incubated in phosphate buffer *plus* glucose, with or without dicumarol, and fixed by the procedure of RYTER AND KELLENBERGER[6], without prefixation. Sections contrasted with lead citrate.

Fig. 1. Control cell (no dicumarol) incubated for 2 h. Notice the asymmetric cytoplasmic membrane and the numerous ribosomes in the cytoplasm. × 62400.

Fig. 2. As in Fig. 1, but with oxidation of the sections with H_2O_2 before staining, in order to enhance the contrast of the ribosomes. × 55000.

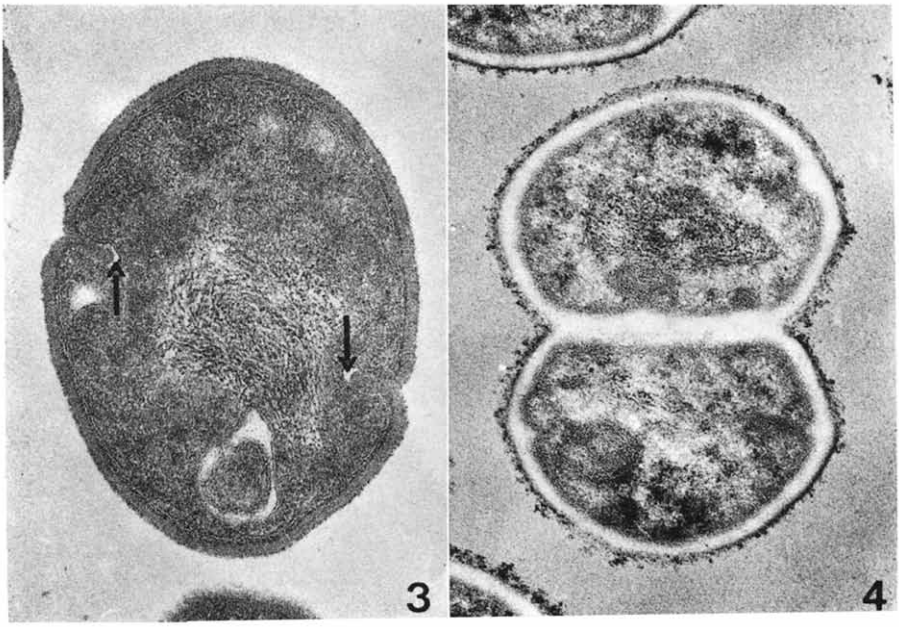


Fig. 3. Cell treated with 0.1 mM dicumarol for 2 h. Notice the symmetric profile of the cytoplasmic membrane, the absence of ribosomes and the first signs of lytic activity at the site of septa formation (arrows). × 58000.

Fig. 4. Cell treated with 0.1 mM dicumarol for 2 h. Section prepared as in Fig. 2. Notice the absence of ribosomes and the presence of dence granular material. × 51000.

Estimation of total RNA

The cells were treated with ice-cold 15% trichloroacetic acid. Lipid material was removed from the precipitate with acetone and ether. From the lipid-free remainder RNA was extracted according to the method of SHORTMAN AND FUKUHARA[11]. The RNA content was determined both from the absorbance at 260 nm and by the orcinol reaction[12] after hydrolysis in 1 M KOH.

RESULTS

Ultrastructural data

Fig. 1 represents a control cell, harvested after 2-h incubation, showing clusters of ribosomes in the cytoplasm, an asymmetric cytoplasmic membrane, and an intact cell wall. Fig. 2 shows a control cell preferentially contrasted for ribosomes. A high magnification of the cytoplasmic membrane of a control cell, showing the asymmetry of its profile, is shown in Fig. 6.

Incubation with dicumarol resulted in some ultrastructural alterations: (1) A progressive cellular depletion of ribosomes was already detected with a dicumarol concentration of 0.01 mM, although only in occasional cells and after an incubation of 2 h. With 0.1 mM dicumarol the depletion was noticeable in some cells after an incubation of 15 min; by 2 h most of them appeared completely depleted of ribosomes (Figs. 3 and 4). In some specimens of these depleted cells, a granular material was ob-

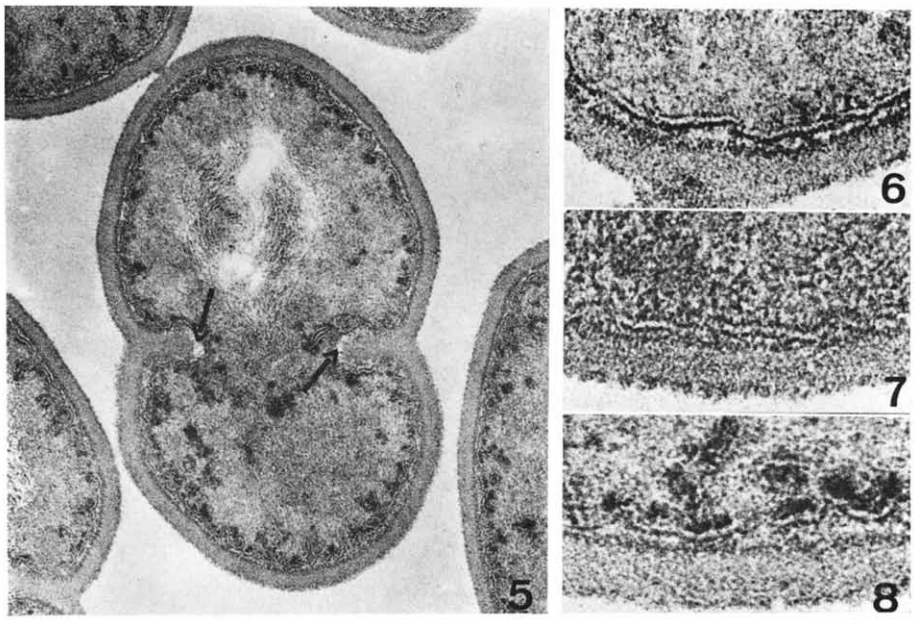


Fig. 5. As in Fig. 3, but additionally showing the granular material contacting the membranes. × 60000.

Figs. 6–8. Higher magnifications (× 200000) of the profile of the cytoplasmic membrane.

Fig. 6. Control cell showing an asymmetric profile.

Fig. 7. Cell treated with 0.1 mM dicumarol for 2 h, showing a symmetric profile.

Fig. 8. As in Fig. 7, but showing the granular material contacting the membrane.

served (Fig. 4) predominating at the cell periphery and often in contact with the membranes (Figs. 5 and 8); this material disappeared as incubation proceeded. It was the most densely contrasted one in sections of glutaraldehyde-fixed cells stained with lead, a result which points to the presence of RNA[13]. Accordingly, this material was completely removed after treatment with ribonuclease. In all experiments a few cells retained their ribosomes even after 6 h of incubation and with concentrations of dicumarol up to 0.5 mM. (2) The cytoplasmic membrane showed a change in its geometry so that the membrane profile appeared symmetric (Figs. 3 and 7). With 0.1 mM dicumarol this change was detected in some cells after an incubation of only 15 min, becoming a rather general pattern with either longer incubation periods (2 h) or higher concentrations of dicumarol (0.5 mM). It could not be found in cells treated with 0.1 or 0.5 mM dicumarol for only 5 min; this result precludes the possibility of the alteration in the membrane profile being a consequence of an interference of dicumarol with the fixation process. (3) Alterations described for other bacteria as signs of lysis[14] were also found in most cells treated with 0.1 mM dicumarol for 6 h or more, or with 0.5 mM dicumarol for 2 h or more. These signs included: abnormal membrane systems consisting in arrays of parallel or concentric membranes with a symmetric profile as described above for the cytoplasmic membrane (Figs. 9 and 11); dispersed DNA areas with signs of increased hydration (Fig. 9); and cell wall damage

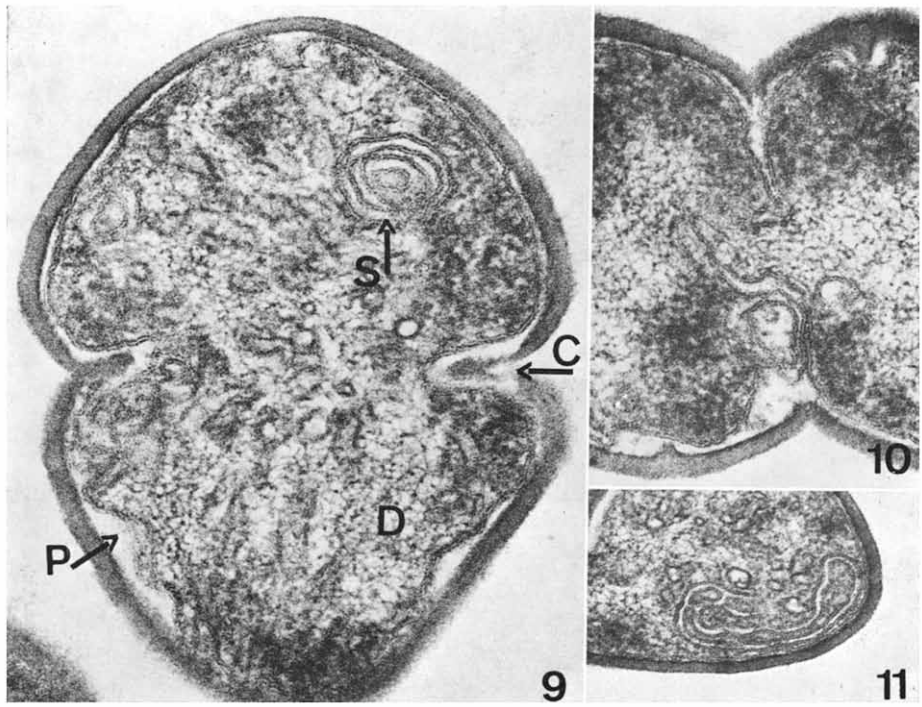


Fig. 9. Cell treated with 0.5 mM dicumarol for 2 h. Notice the symmetric profile of all the membranes, a system of concentric membranes (S), the hydrated DNA areas (D), the increased periplasmic space (P), and the signs of intense cell wall damage (C). × 80000.

Fig. 10. As in Fig. 9, showing intense signs of cell wall damage. × 80000.

Fig. 11. As in Fig. 9, showing a system of parallel symmetric membranes. × 62000.

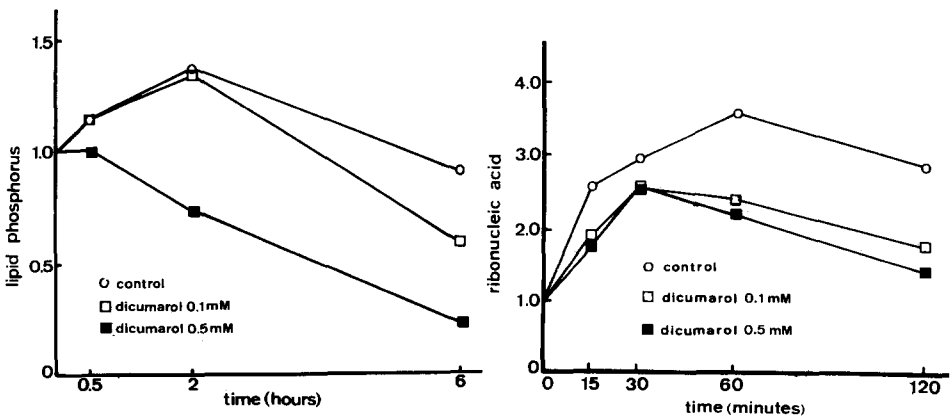


Fig. 12. Changes in the lipid phosphorus values per cell during incubation in phosphate buffer *plus* glucose, with or without added dicumarol. Values at time zero taken as unity.

Fig. 13. Changes in the total RNA values per cell during incubation in phosphate buffer *plus* glucose, with or without added dicumarol. Values at time zero taken as unity.

TABLE I

PHOSPHOLIPID COMPOSITION OF CELLS OF *S. faecalis* INCUBATED IN PHOSPHATE BUFFER FOR 2 h WITH GLUCOSE (CONTROL) OR WITH GLUCOSE *plus* DICUMAROL

DPG, diphosphatidylglycerol; PG, phosphatidylglycerol.

	Control	*Dicumarol-treated* 0.1 *mM*	0.5 *mM*
Total lipid phosphorus*	1.00	0.97	0.54
Percentage distribution of lipid phosphorus**			
DPG	10	19	33
PG	44	52	46
lysyl-PG + (?)	29	21	13
arginyl-PG***			
diglucosyl-PG	16	8	9
dilysyl-PG		traces	traces

* Value for control taken as unity.
** Identification based on previous work[9].
*** Presence of arginylphosphatidylglycerol was not confirmed.

(Figs. 9 and 10), preferentially at the sites of septa formation as in the case of the autolytic activity described by HIGGINS *et al.*[15]. Another finding was an increase in the periplasmic space (Fig. 9).

Lipid phosphorus

The effect of the incubation with dicumarol on the lipid phosphorus values per cell is represented in Fig. 12. With 0.1 mM a decrease was evident only with long incubations. 0.5 mM dicumarol induced a much earlier decrease. The percentage distribution of the phospholipids is affected in both cases (Table I). The alteration concerns mainly an increase in the relative amount of cardiolipin and a decrease in the aminoacyl derivatives of phosphatidylglycerol[9].

Total RNA

Following the addition of glucose, there was an initial increase in the RNA values per cell of both control and dicumarol-treated samples (Fig. 13). As incubation proceeded the cellular level of RNA decreased. Dicumarol-treated cells were shown to have always a lower amount of RNA than control cells.

DISCUSSION

The ultrastructural results reported here show that most cells of *S. faecalis* when suspended in buffer and incubated with dicumarol *plus* glucose exhibit an early alteration in the profile of the cytoplasmic membrane and are progressively depleted of ribosomes; later clear signs of lysis do appear. It is not surprising that after such a treatment most of the cells are unable to grow when reinoculated in fresh medium[5]. Nevertheless, as described in RESULTS, a few cells persist with ribosomes. It is likely

that these cells are, by some reason still unclear, less damaged, or even unaffected bacteria, and thus would correspond to the minority which was shown previously[5] to remain viable.

Chemical analysis showed that the percentage distribution of phospholipids is significantly altered in conditions (*i.e.* 0.1 mM dicumarol up to 2 h) where the change in the geometry of the membrane is the only detectable membranous alteration. Although the possible relation between these findings is speculative, they were also observed to run parallel in other situations, namely when the cells were grown at different pH values (manuscript in preparation). A decrease in the cellular lipid phosphorus values was only observed with either longer incubation periods or higher concentrations of dicumarol. Since it is known[16] that in this bacterium almost all the phospholipid material is located in the membranes, this result might be related to the signs of lysis observed by electron microscopy and represent a loss of membrane substance.

The increase in the cellular level of RNA in control cells might be related to the availability of a carbon source (glucose) to cells which were starving after harvesting from the culture medium[17]. The increase was followed by a decrease but at the last sampling (2 h) the cellular level of RNA was still above the original one. This was also found for cells incubated with dicumarol, in spite of the observation that towards the end of that period most of them were depleted of ribosomes. Thus, the RNA assayed in these dicumarol-treated cells is not present in the form of ribosomes. Part of it is located in the dense granular material which appears in these cells. One possibility to consider is that this material represents a product of the disassembly of ribosomes.

The lower RNA content of dicumarol-treated cells obtained in comparison with the controls might represent an inhibited production and/or an enhanced degradation of RNA. In the latter case the additionally degraded RNA could represent the source of the "extra energy" reported before as produced during metabolization of glucose by *S. faecalis* in the presence of dicumarol[3]. In *Aerobacter aerogenes* it was reported[18] that degradation of RNA might supply easily metabolizable sources of carbon (ribose) and nitrogen (ammonia).

The early change in the geometry of the membrane was similar to that shown to occur in several bacteria under different conditions leading to lysis[14,19,20]. It cannot be decided whether this change is, in our case, the direct result of the action of dicumarol or represents the membranous counterpart of an injury produced by dicumarol somewhere in the cell. However, compounds that uncouple or inhibit oxidative phosphorylation, as is the case with dicumarol, have been suggested to have direct effects on cellular membranes in general[21,22]. Since *S. faecalis* lacks cytochromes and relies upon glycolysis for the generation of ATP[4,23], it is possible that a direct effect does exist in our case. Nor is it clear if this early change in the cell membrane and the ribosomal depletion are correlated in some way or if they represent independent alterations. In most situations in which bacterial ribosomes were reported to be degraded, damage of the cell membrane seems to be primarily involved, and at least in some of these situations[24–27] membrane damage has been suggested to affect the ribosomes through the action of membrane-bound degradative enzymes.

The growth of several bacteria besides *S. faecalis* was reported[28,29] to be affected by the presence of dicumarol. Studies on the structure and metabolism of these

bacteria during incubation with dicumarol seem necessary before the pertinence of this work to bacterial membrane morphology and metabolism in general can be ascertained.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. J. A. F. Op den Kamp for his suggestions for the manuscript, and to Dr. Maria-Laura R. Sousa, Miss Maria-Luísa Abreu and Mrs. Maria-Conceição Teles for technical assistance.

This work was supported in part by grants from Fundação Calouste Gulbenkian and Instituto de Alta Cultura.

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