Biochemical Pharmacology, Vol. 24, pp. 2163–2166. Pergamon Press, 1975. Printed in Great Britain.

THE EFFECT OF CHLORHEXIDINE ON THE ANAEROBIC FERMENTATION OF *SACCHAROMYCES CEREVISIAE*

JORN ERIK JENSEN

The Royal Dental College, Department of Biochemistry, Vennelyst Boulevard, DK—8000 Århus C, Denmark

(*Received* 23 *October* 1974; *accepted* 12 *May* 1975)

Abstract—The effect of chlorhexidine (Chx) on the anaerobic fermentation of baker's yeast has been studied. A concentration-dependent inhibition of glucose consumption and CO_2 production was observed. The concentration for 50% inhibition of glucose consumption was about 2 nmoles Chx per mg yeast while total inhibition was obtained at concentrations above 4 nmoles Chx per mg yeast. The concentration giving 50% inhibition of the production of CO_2 with glucose as a substrate was 7 nmoles Chx per mg yeast while maximal inhibition was observed at concentrations above 35 nmoles Chx per mg yeast. Chx conferred an ability on the cells to decarboxylate exogenous pyruvate, the concentration giving 50% of maximal stimulation being 10 nmoles Chx per mg yeast while maximal stimulation was obtained above 37 nmoles Chx per mg yeast. The decarboxylase activity was not released from the cells by Chx, nor were the cells enabled to produce CO_2 from exogenous glucose-6-phosphate and glycerate-3-phosphate. Cations exerted a concentration-dependent protection of the cells against the effect of chlorhexidine. The relative efficacy of protection was in the order $Na^+ < K^+ < Mg^{2+} < Ca^{2+} < Zn^{2+} < Ba^{2+} < Mn^{2+}$. The binding of Chx to whole cells was reduced by Ca^{2+}. The mode of action of Chx is discussed in the light of the present results.

Chlorhexidine (Hibitane®), 1,1′-hexamethylene bis(5-(4-chlorophenyl))biguanide exerts concentration-dependent effects on the cell membranes of microbial cells causing leakage of cytoplasmic constituents and loss of viability [1–4]. It inhibits the formation of acid [5, 6] and the stimulation of glycolysis associated with K^+ uptake but apparently does not affect the formation of ATP from glycolysis [7]. Chlorhexidine thus seems to interfere with essential parts of the glycolysis and in order to obtain insight into the mechanism of the action of chlorhexidine, it was found of interest to examine further the effect of chlorhexidine on some parameters of the anaerobic fermentation of yeast. The present paper reports findings concerning the effect of chlorhexidine on the production of CO_2, consumption of glucose, decarboxylation of exogenous pyruvate and the protective action of certain cations.

MATERIALS AND METHODS

Yeast. All experiments were performed on non-growing suspensions of commercial baker's yeast (De Danske Spritfabrikker, Copenhagen). The yeast was suspended in deaerated distilled water 0·5 hr prior to the experiments. The weight of yeast used in the experiments is given as wet weight.

Determination of CO_2 production. The production of CO_2 was determined manometrically by Warburg constant volume respirometers by the conventional technique [8]. The deaerated incubation medium consisted of 3·3 mM KH_2PO_4, 95·8 mM glucose, 0–690 μM chlorhexidine and 17·2 mM sodium acetate buffer pH 5·0. The amount of yeast used was 4·0 mg in 2·9 ml incubation medium. The respirometers were flushed for 10 min and filled with oxygen-free N_2. The experiments were performed at 30°, and the reaction initiated by the addition of glucose. Chlorhexidine was added concomitantly with the glucose and all determinations performed in duplicate. Cations, when used, were added before chlorhexidine. The corresponding concentrations of cations and chlorhexidine giving half-maximal inhibition of the control CO_2 production were determined as the mean from two dose–response curves. Each dose–response curve was obtained from a series of experiments with constant amounts of cations present and varying concentrations of chlorhexidine. Experiments with glucose-6-phosphate and glycerate-3-phosphate were performed by addition of these substrates to final concentrations of 5 mM instead of glucose.

Determination of pyruvate decarboxylation. The formation of CO_2 was estimated as described above. The deaerated incubation medium consisted of 3·3 mM KH_2PO_4, 5·0 mM sodium pyruvate, 0–690 μM chlorhexidine and 17·2 mM sodium acetate buffer pH 5·0. The amount of yeast used was 8·0 mg in 2·9 ml incubation medium. The reaction was initiated by the addition of pyruvate. Chlorhexidine was added concomitantly with the pyruvate. The experiments were performed at 30°. Cations, when used, were added before chlorhexidine. All experiments were performed in duplicate.

Determination of the solubilizing effect of chlorhexidine on the pyruvate decarboxylase activity. Yeast (8 mg) were incubated for 15 min in 2·9 ml deaerated medium consisting of 3·3 mM KH_2PO_4, 0–690 μM chlorhexidine and 17·2 mM sodium acetate buffer pH 5·0. The mixture was then centrifuged at 5000 g for 10 min. The supernatant was collected and the

pellet resuspended in a medium equal to that removed. Supernatant and suspended pellet were placed into respirometers and the reactions initiated by the addition of sodium pyruvate to a final concentration of 5·0 mM. The formation of CO_2 was estimated as described previously.

Determination of glucose consumption. The experiments were performed in Thunberg tubes. The deaerated incubation medium consisted of 3·3 mM KH_2PO_4, 9·3 mM glucose, 0–690 μM chlorhexidine and 16·7 mM sodium acetate buffer pH 5·0. The amount of yeast used was 15·0 mg in 3·0 ml incubation medium. The tubes were filled with oxygen-free N_2 and kept at 30° with shaking. The reaction was initiated by the addition of the yeast. Glucose was analysed by a colorimetric glucose oxidase method (GOD-Perid), Boehringer kit No. 15756. All experiments were performed in duplicate.

Binding of chlorhexidine to yeast cells. Yeast (20 mg) were incubated for 30 min at 30° in 3·0 ml medium consisting of 0–3000 μg chlorhexidine digluconate, 1·4 μg (0·1 μCi) of ^{14}C-labelled chlorhexidine digluconate, 3·3 mM KH_2PO_4 and 16·6 mM sodium acetate buffer pH 5·0. Following incubation the mixture was centrifuged at 1000 g for 10 min and the radioactivity of the supernatant (0·25 ml) was determined as described in the following section. For each concentration of chlorhexidine a control sample prepared as described above but without yeast was run in parallel with the test sample. The determinations were performed in duplicate.

Measurement of radioactivity. As a rule 250 μl of the radioactive solution was used and added to 6·75 ml scintillator fluid. This consisted of 80 g naphthalene, 5 g PPO and 0·05 g POPOP in a mixture of 333 g dioxane, 333 g xylene and 333 g ethanol. The radioactive material was placed in counting vials (5·5 × 2·5 cm) and measured in an IDL liquid scintillation counter.

Chemicals. Chlorhexidine digluconate labelled with ^{14}C was generously supplied by I.C.I., Macclesfield, England. Unlabelled chlorhexidine was obtained from I.C.I., Macclesfield, England. Glycerate-3-phosphate and glucose-6-phosphate was obtained from C. F. Boehringer & Soehne, Mannheim, GFR. Other chemicals were of analytical grade.

RESULTS

Effect of chlorhexidine on the production of CO_2 by yeast. The production of CO_2 was directly proportional to the amount of yeast present in the controls. From Fig. 1a it is evident that chlorhexidine exerts a concentration-dependent inhibitory effect on the anaerobic CO_2 production by yeast. Fifty per cent of the control activity was obtained at a concentration of chlorhexidine about 10 μM (7 nmoles chlorhexidine per mg yeast). At concentrations above 50 μM chlorhexidine the activity was depressed to a constant level of 5–10% of the control activity, and was independent of the presence of glucose. It might therefore be suggested that this basic activity is due to decarboxylation of endogenous substrates.

From Fig. 1a it can also be seen that Ca^{2+} suppresses the inhibitory effect of chlorhexidine, as more chlorhexidine is necessary to provoke the same effect

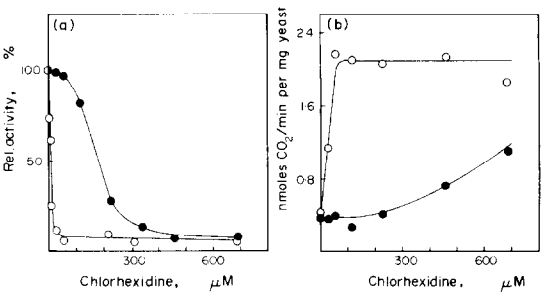

Fig. 1. The abscissae give total concentrations of chlorhexidine. Experiments with and without Ca^{2+} are represented by —●— and —○— respectively. (a) The effect of chlorhexidine on anaerobic CO_2 production in bakers yeast. The ordinate shows the activity relative to the control value without chlorhexidine. Fermentation with glucose and no chlorhexidine produced 4·1 ± 0·2 (mean ± S.E.M.) (n = 4) nmoles CO_2/min per mg yeast and the same in the presence of 21 mM Ca^{2+} was 3·9 ± 0·2 (mean ± S.E.M.) (n = 4) nmoles CO_2/min per mg of yeast. (b) Effect of chlorhexidine on anaerobic decarboxylation of pyruvate. The ordinate shows the total amount of CO_2 developed. The experiments with Ca^{2+} were performed at a concentration of 21 mM of the ion. For further details see Materials and Methods.

in the presence of this ion. In the selected experiments with 21 mM Ca^{2+}, 50% of the control activity was obtained at a concentration of chlorhexidine about 180 μM (130 nmoles chlorhexidine per mg yeast). Above 500 μM chlorhexidine the activity was depressed to the basic level.

Chlorhexidine did not confer on the cells an ability to produce CO_2 from exogenous glucose-6-phosphate or glycerate-3-phosphate at any of the concentrations investigated.

Effect of chlorhexidine on pyruvate decarboxylase activity. Chlorhexidine stimulates the ability of the cells to decarboxylate exogenous pyruvate as shown in Fig. 1b. Half-maximal stimulation was obtained at about 27 μM chlorhexidine (10 nmoles chlorhexidine per mg yeast). The stimulation reached a maximal level at about 100 μM chlorhexidine, and retained its value even in the presence of chlorhexidine up to 690 μM, indicating that the decarboxylase is not inhibited by chlorhexidine.

From Fig. 1b it can also be seen that Ca^{2+} inhibits the stimulatory effect of chlorhexidine on pyruvate decarboxylase activity as more chlorhexidine is necessary to provoke the same effect as that obtained in the abscence of Ca^{2+}. In selected experiments with 21 mM Ca^{2+}, the half-maximal stimulation was observed at about 690 μM chlorhexidine. Controls with pyruvate and without chlorhexidine showed the same activity as samples without pyruvate and chlorhexidine. As intact cells are assumed not to be able to decarboxylate exogenous pyruvate the basic activity might be explained by decarboxylation of endogenous substrates. Chlorhexidine did not release the decarboxylase activity from the cells as all the activity was confined to the pellet after centrifugation of the chlorhexidine treated cells.

Effect of chlorhexidine on glucose consumption. Chlorhexidine exerts a concentration-dependent inhibitory effect on the glucose consumption of yeast

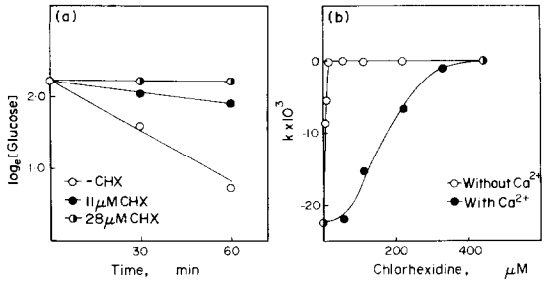

Fig. 2. The effect of chlorhexidine on the anaerobic glucose consumption of baker's yeast. (a) Semilog plot showing typical alterations in the concentration of glucose with time in the presence of selected concentrations of chlorhexidine. The ordinate gives the logarithm of the glucose concentration in mM. (b) The ordinate shows the rate constant k calculated from the rate expression for a first-order reaction. The unit of k is min^{-1}. The graph with Ca^{2+} was obtained in the presence of 41 mM of the ion. For further details consult Materials and Methods.

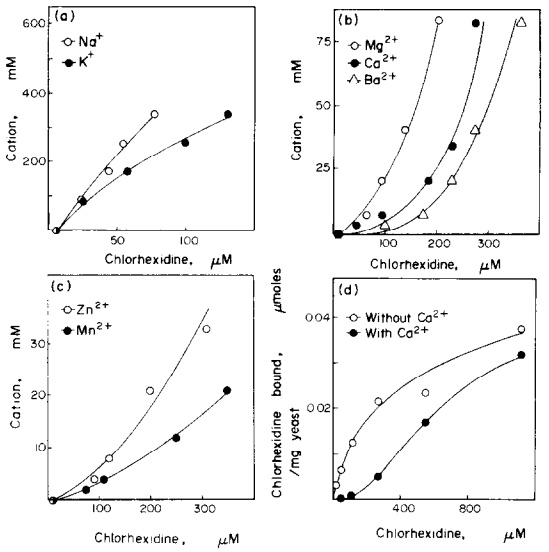

Fig. 3. The abscissae give total concentrations of chlorhexidine. (a) Protective effect of Na^+ and K^+ against the inhibitory action of chlorhexidine on anaerobic fermentation. The ordinate gives the concentrations of the cation added. The graphs show the corresponding concentrations of chlorhexidine and cation bringing about 50% of the control formation of CO_2 without chlorhexidine. The production of CO_2 in the controls with Na^+ and K^+ amounted to 4·3 ± 0·1 (mean ± S.E.M.) (n = 8) and 4·2 ± 0·1 (mean ± S.E.M.) (n = 8) nmoles/min per mg of yeast respectively. (b) Protective effect of Mg^{2+}, Ca^{2+} and Ba^{2+} against the inhibitory action of chlorhexidine on anaerobic fermentation. The ordinate gives the concentration of cation added. The graphs show the corresponding concentrations of chlorhexidine and cation giving 50% of the control production of CO_2 without chlorhexidine. The formation of CO_2 of the controls with Mg^{2+} was 4·0 ± 0·3 (mean ± S.E.M.) (n = 10) nmoles/min per mg of yeast, with Ca^{2+} 3·9 ± 0·3 (mean ± S.E.M.) (n = 10) and with Ba^{2+} 4·2 ± 0·3 (mean ± S.E.M.) (n = 10). (c) Protective effects of Zn^{2+} and Mn^{2+} against the inhibitory action of chlorhexidine on anaerobic fermentation. The ordinate gives the concentration of cation added. The graphs show the corresponding concentrations of chlorhexidine and cation giving 50% of the CO_2 production in the controls without chlorhexidine. The CO_2 formation of the controls with Zn^{2+} was 3·3 ± 0·2 (mean ± S.E.M.) (n = 8) nmoles/min per mg of yeast and with Ba^{2+} 3·2 ± 0·2 (mean ± S.E.M.) (n = 8). (d) The graphs show the amount of chlorhexidine bound to whole yeast cells without Ca^{2+} and in the presence of 40 mM Ca^{2+}. For further details consult Materials and Methods.

as shown in Fig. 2b. Controls showed a direct proportionality between the rate constant of the glucose consumption and the amount of yeast present. The rate constant of the consumption was reduced to one-half of that of the control at a concentration of chlorhexidine about 8 μM (2 nmoles chlorhexidine per mg yeast), while total inhibition was observed at about 30 μM. From Fig. 2b it can also be seen that Ca^{2+} suppresses the inhibitory effect of chlorhexidine. In the selected experiments with 41 mM Ca^{2+}, 50% of the control consumption without chlorhexidine was obtained at a concentration of chlorhexidine about 160 μM (32 nmoles chlorhexidine per mg yeast) while maximal inhibition was acheived above 400 μM chlorhexidine.

Effect of cations on the inhibition of CO_2 production. Cations suppress the inhibitory effect of chlorhexidine on the production of CO_2. This is shown in Fig. 3a–c from which it can be seen that the order of protective effect of the cations investigated is $Na^+ < K^+ < Ca^{2+} < Zn^{2+} < Ba^{2+} < Mn^{2+}$.

Binding of chlorhexidine to yeast cells. This is shown in Fig. 3d, from which it is apparent that the amount of chlorhexidine bound increases nearly linearly with the total concentration up to 100 μM. At higher concentrations the percentage bound decreases. The cells are not saturated with chlorhexidine at the concentrations investigated (below 1100 μM). From Fig. 3d it can also be seen that Ca^{2+} interferes with the binding of chlorhexidine to the cells, as Ca^{2+} reduces the amount of chlorhexidine bound. In the presence of 40 mM Ca^{2+} no chlorhexidine is bound at concentrations below 100 μM. At higher concentrations of chlorhexidine the percentage bound increases but a level of saturation is not achieved at the concentrations investigated.

DISCUSSION

In several papers it is described that chlorhexidine damages the membranes of microorganisms (e.g. bacteria [1–3] and yeast [9]) and the membranes of cellular organelles (e.g. mitochondria, lysosomes and peroxisomes [10, 11]). As a consequence, soluble and membrane-bound enzymes are affected [10–12] and some of the compounds from the membrane-enclosed spaces leak out [10, 11]. These effects are attended by loss of viability of all types of cells investigated [4, 9, 13]. The loss of viability indicates that essential metabolic events in the cells are inhibited. The present experiments with the anaerobic fermentation of yeast show that glucose consumption and CO_2 production are progressively inhibited by increasing concentrations of chlorhexidine. It is also shown that chlorhexidine concomitantly enables the cells to decarboxylate exogenous pyruvate. The inhibitory effect of chlorhexidine on glycolysis is not restricted to yeast as it has been observed in experiments with

Streptococcus mutans [6], *Streptococcus faecalis* [7] and rat intestinal mucosal cells [14]. The inhibition of the anaerobic glycolysis of yeast may be the result of the membrane-damaging effect, as yeast is known to lose K^+ in the presence of chlorhexidine [9]. It is therefore possible that the cells also lose necessary cofactors, coenzymes and/or enzymes needed in fermentation. However, alteration of the membrane does not necessarily cause release of the fermentation enzymes themselves from the cells [15]. In fact the pyruvate decarboxylation activity could be sedimented at 1000 *g* in the case of chlorhexidine-treated cells. The finding that pyruvate is decarboxylated when membrane permeability is affected is in agreement with previous studies [15–17]. In the present study, however, chlorhexidine did not confer an ability on the cells to produce CO_2 from exogenous glucose-6-phosphate or glycerate-3-phosphate. These phosphorylated intermediates seem not to penetrate the chlorhexidine-treated membrane and if they penetrate they presumably cannot reach the active sites of the enzymes, as the enzymes are assumed to be organized in a structure or matrix which is not directly affected by an increase in membrane permeability [15]. Another possibility is that one or more of the enzymes themselves are inhibited vigorously by chlorhexidine. If so it must be one or more of the enzymes of the glycolytic pathway prior to the decarboxylation of pyruvate, as pyruvate decarboxylating activity is stimulated and reaches a saturation level with increasing concentrations of chlorhexidine. The cause of the depression of glucose consumption could be that chlorhexidine, in addition to the possible effects mentioned above, inhibits glucose transport across the membrane and its access to the fermentation sites in the cell. The membrane-damaging effect of chlorhexidine is presumably dependent on the interaction between the cationic parts of the chlorhexidine molecule and the anionic groups of the cell membrane followed by a secondary interaction between their respective apolar parts, since an occupation of the anionic binding sites with inorganic cations protects the cells against chlorhexidine. In fact the presence of cations reduces the amount of chlorhexidine bound to whole cells (cf. Fig. 3d). Chlorhexidine thus behaves like basic dyes and quaternary ammonium detergents which bind to anionic groups on the cell surface and produce an effect from which protection is afforded by inorganic cations [9, 18, 19].

In conclusion, chlorhexidine exerts an inhibitory action on the anaerobic glucose consumption and the production of CO_2 and exerts concomitantly a stimulatory effect on the decarboxylation of exogenous pyruvate. Cations suppress these effects and reduce the binding of chlorhexidine to whole cells. From these findings it may be suggested that chlorhexidine interacts with the cell membrane, disturbs its integrity and interferes with its functions. However, the possibility remains that chlorhexidine furthermore penetrates the cell membrane and interferes with the function of the enzymes proper of the glycolytic pathway.

Acknowledgement—The author is indebted to Dr. Flemming Christensen for valuable suggestions and to Birte Esmann for skilful technical assistance.

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