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Studies of the electron transport chain of extremely halophilic bacteria. VIII. Respiration-dependent detergent dissolution of cell envelopes

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SUMMARY

The kinetics of the dissolution of the cell envelope of *Halobacterium cutirubrum*, in the presence of cetyl trimethylammonium bromide (CTAB) or Triton X-100 as perturbing agents, indicate that respiring cells are more resistant to disruption than those inhibited with cyanide, azide, 2-heptyl-4-hydroxyquinoline-*N*-oxide, and phenylmercuric acetate or uncoupled with carbonylcyanide *m*-chlorophenylhydrazone. The difference in the rate of dissolution between respiring and inhibited cells was 2.5-fold for CTAB and 30–40-fold for Triton X-100. Although the effectiveness of CTAB in dissolving the cell envelope is increased in the presence of KCN, such inhibition does not change the uptake of the detergent by the cells.

The cell envelope of the extremely halophilic bacterium, *Halobacterium cutirubrum*, disintegrates upon lowering the salt concentration[1-6]. As reported earlier[6], we found that as NaCl concentration was lowered from 3.4 M, the proteins released at high salt concentrations also showed increased release by hydrophobic bond breaking agents. Those membrane structures that required NaCl concentrations below 1 M for disruption, however, were little affected by such treatment. The present study concerns the effect of detergents on the cell envelope of halophilic bacteria. The disruption of membranes by detergents has been extensively studied[7-9]. The action of such amphipathic agents is thought to be due to the insertion of their hydrophobic portion into the lipid bilayer, thereby decreasing surface tension and cohesiveness. Bacterial cells are generally

Abbreviations: CTAB, cetyl trimethylammonium bromide; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

more resistant to detergents than expected on the basis of this mechanism because of the presence of a rigid carbohydrate cell wall[10,11]. *H. cutirubrum*, however, lacks such a structure[12] and it was expected that the response of the cell envelopes to detergents would reflect more closely the properties of the lipoprotein membrane.

The effect of Triton X-100 on respiring and nonrespiring cells of *H. cutirubrum* was tested by adding the detergent, to 0.15% final concentration, to a late logarithmic phase culture[13] and transferring the flasks to 22 °C for further incubation. Turbidity was followed with a Klett photometer, utilizing a side arm in the flasks. Fig. 1 shows the changes in turbidity following the addition of the detergent in (1) shaking (aerobic) cultures, (2) standing (semianaerobic) cultures, and (3) KCN-inhibited (nonrespiring) cultures. In the absence of detergent the turbidity increased slightly in 4 h of incubation under all the above conditions. In those cultures where Triton X-100 was added, turbidity decreased dramatically in KCN-inhibited and standing cultures, while the shaking (respiring) culture appeared to be unaffected. When the air in another culture (not shown) was replaced with nitrogen and, after adding Triton X-100, the flask was stoppered and shaking was resumed, the decrease in turbidity was similar to that observed with the standing culture (Fig. 1). It appears from these experiments that lysis of the cells by the detergent is dependent on the state of respiration, fully respiring cells not being susceptible to

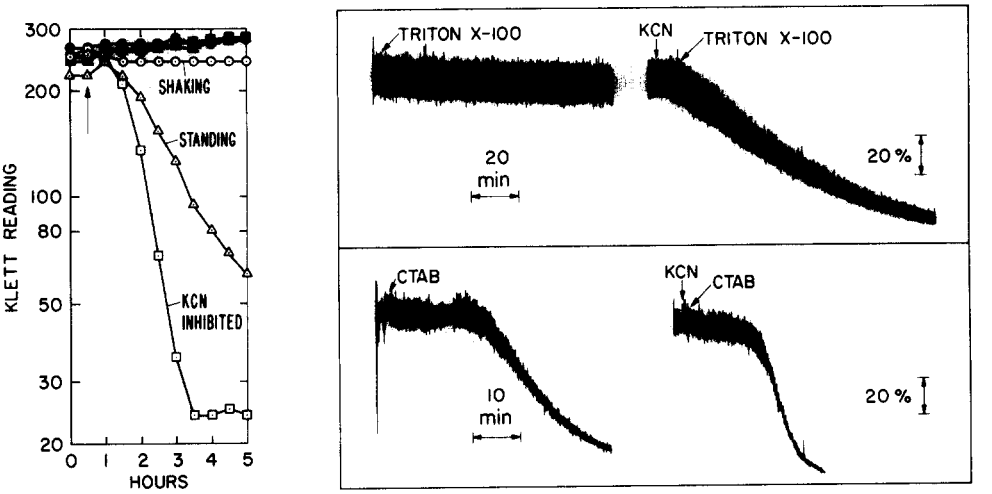

Fig. 1. Turbidity changes in *H. cutirubrum* cultures after addition of Triton X-100. Experimental details described in the text. The detergent was added (to 0.15% final concn) at the time indicated with an arrow. Open symbols, detergent experiments; closed symbols, controls, to which no detergent was added. ⊙ and ●, shaking cultures; △ and ▲, standing cultures; ⊡ and ■, KCN-inhibited cultures (final concn, 10 mM, added together with the detergent).

Fig. 2. Recorder traces of light-scattering changes occurring on adding detergents alone and detergents *plus* KCN to *H. cutirubrum* cells. The cells were diluted 40-fold into buffer containing 3.4 M NaCl and basal salts, light-scattering intensity was followed as described in the text. Final concentrations: Triton X-100, 0.15%; CTAB, 0.00025%; KCN, 10 mM. Ordinate, percent light-scattering intensity; abscissa, time.

disruption. To test the possibility that the resistance of respiring cells to Triton X-100 might be due to the metabolic destruction of the detergent at a rate faster than its adsorption to the cells, a culture was shaken with detergent, as in Fig. 1, for 2 h, showing no decrease in turbidity. KCN was then added. The decrease in turbidity followed the same course as shown for the cyanide-inhibited cells in Fig. 1, without previous aerobic incubation. Since the rate of lysis was proportional to the detergent concentration, it seems very unlikely that the cells metabolize the detergent at an appreciable rate. The lysis of the cells is accompanied by disintegration of the cell envelopes, to an extent as yet unknown, since only a small amount of sedimentable material was obtained at speeds which are sufficient to recover envelope vesicles[6].

The dissolution of the cells with detergents was further studied in a better defined system. 0.5 ml of bacterial culture was diluted into 19.5 ml buffer, containing 3.4 M NaCl and basal salts[6] and light scattering at 45° forward angle was followed as described before[6]. Since it had been shown that at this angle light scattering by bacterial cells is primarily due to the cell envelopes[14] rather than the contents of the cells, it was reasonable to expect that the time-course of light scattering changes would reflect the disintegration of the peripheral structures of the cells. Recorder traces of light scattering intensity changes after addition of Triton X-100 or cetyl trimethylammonium bromide (CTAB) are shown in Fig. 2. The traces show that (1) the light scattering decrease follows simple kinetics, approximately first order in light scattering intensity. (It was possible therefore to use these traces to calculate first-order rates of dissolution by dividing the slopes by the initial light scattering intensities.) (2) Addition of KCN causes a large increase in the rate of dissolution of cell envelopes by either Triton X-100 or CTAB, the experiments with the latter detergent exhibiting less increase than the former. The rate of respiration of cells (see below) was rather low and allowed the contents of the light-scattering cuvette to remain aerobic. The residual light scattering approached 10–15% of the initial intensity, a value similar to the residual light scattering observed during the salt-dependent disintegration of membrane vesicles from the same organism[6]. The pH dependence of the action of Triton X-100 on cells was investigated in 3.4 M NaCl buffer, containing basal salts, between pH 5.5 and 8.5. Below pH 5.5 the cells showed extensive aggregation, independently of the detergent. Above this pH, the rate of disintegration in the presence of 10 mM KCN and 0.15% Triton X-100 was somewhat dependent on pH, showing 3-fold increase over 3 pH units. The rate of dissolution in the absence of cyanide showed a similar increase, but the rate-enhancing effect of KCN-inhibition persisted over the entire pH range studied. The detergents Triton X-114, X-100, X-102, and X-165 contain *tert*-octylphenol as their hydrophobic moiety and ethoxy groups of 7–8, 9–10, 12–13, and 16 repeating units, respectively[15]. Despite the differences in the size of their hydrophilic portions there was no variation in the rate of dissolution of *H. cutirubrum* cells in the presence of any of these detergents. Neither Tween 80 (polyoxethylene monooleate sorbitan) nor BRIJ 35 (polyoxyethylene lauryl ether) caused disintegration of these cells, even at concentrations up to 1%.

The rates of dissolution of cells at various concentrations of CTAB were investigated in more detail. The results, expressed in first-order rates as functions of detergent concentration, are shown in Fig. 3. Graphs a and b were obtained with buffers containing 3.4 M NaCl and 3.4 M NaCl *plus* basal salts, respectively. These data indicate that (1) the rate of light-scattering decrease observed, due to the dissolution of cells, shows a threshold at a low detergent concentration, and above this value it is proportional to the amount of detergent added; (2) the threshold concentration is not affected by the presence of KCN but the rates of dissolution are increased 2–3-fold; and (3) the absence of basal salts, consisting primarily of $MgCl_2$ and $CaCl_2$, drastically increases the sensitivity of cells to CTAB 3–5-fold without changing the threshold concentration of the detergent or the KCN enhancement of the dissolution. Once the detergent concentration was raised above the threshold value the decrease in light-scattering intensity proceeded to completion, as in Fig. 2. In Fig. 3c the concentration dependence of the rate of light-scattering decrease was determined with different amounts of cells added. The threshold concentration of CTAB was found to be directly proportional to the number of cells added to the light-scattering cuvette.

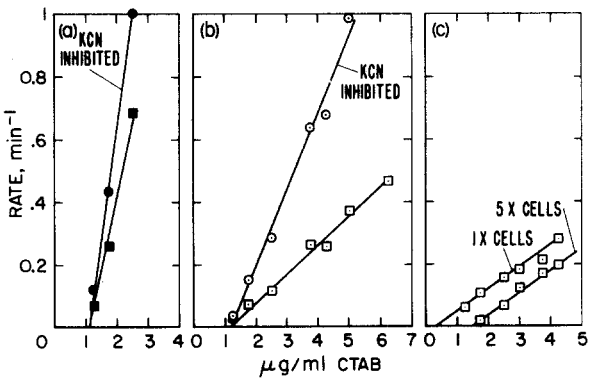

Fig. 3. Dependence of the rate of dissolution of cells on CTAB concentration. The rate of light-scattering decrease, after addition of the detergent, was determined for respiring cells (□ and ■) and for KCN-inhibited cells (final concn, 10 mM; added 3–4 min before adding the detergent) (◎ and ●). Graphs a and b show the results obtained in buffers containing 3.4 M NaCl and 3.4 M NaCl *plus* basal salts, respectively. Graph c shows an experiment where different amounts of cells were used in buffer containing 3.4 M NaCl *plus* basal salts but not KCN.

Kinetic studies were carried out also with Triton X-100. The results, shown in Fig. 4 as dissolution rates *vs* Triton X-100 concentration, are similar to those obtained with CTAB. As seen in Fig. 4a, with Triton X-100 KCN inhibition increases the rate of dissolution 30–40-fold. A comparison between Figs 4a and 4b reveals that in the absence of added divalent cations (final concentration: Mg^{2+} approx. 2.5 mM, Ca^{2+} approx. 0.04 mM) the rate of dissolution is increased by a factor of approximately 300. Thus, in a buffer resembling the salt composition of the growth medium, containing 3.4 M NaCl and basal salts, respiring cells show unusually high resistance to Triton X-100. In contrast, KCN-inhibited cells at the same ionic strength but in the absence of divalent cations are

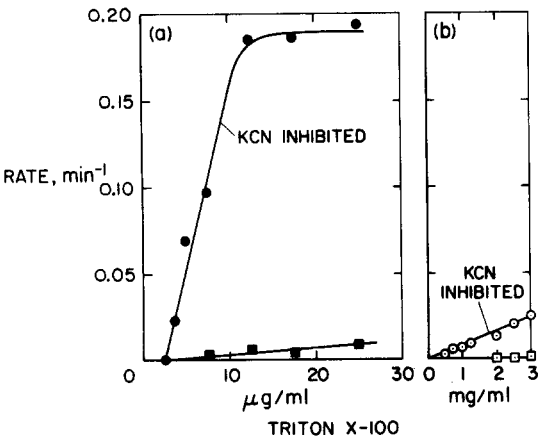

Fig. 4. Dependence of the rate of dissolution of cells on Triton X-100 concentration. Experimental procedure and symbols are explained under Fig. 3. Buffer contained 3.4 M NaCl, (a) without basal salts, (b) with basal salts.

very sensitive to the detergent. The increase in the effect of Triton X-100, brought about by changing both KCN inhibition and divalent cations, amounts to 4 orders of magnitude.

The threshold detergent concentration is presumably related to the equilibrium between free and bound detergent and the minimum number of detergent molecules that can be inserted into the cell envelope without causing disintegration. For CTAB the threshold amount (Fig. 3) is $8.6 \cdot 10^{-15}$ g/cell or $3.0 \cdot 10^{-17}$ mole/cell. For Triton X-100 the corresponding values, obtained from Fig. 4a, are $2.0 \cdot 10^{-14}$ g/cell or $3.3 \cdot 10^{-17}$ mole/cell.

It is evident from Figs 3 and 4 that the basal salts of the growth medium have a stabilizing effect on the *H. cutirubrum* cell envelopes. The sensitivity of the cells to detergents could be lowered by adding either $MgCl_2$ (up to 100 mM) or $CaCl_2$ (up to 3–4 mM) to buffers containing 3.4 M NaCl, but the low rate of dissolution observed in the complete salt buffer could be achieved only in the presence of both of these divalent cations.

The respiratory chain of *H. cutirubrum* has been described before and the sites of the action of some inhibitors have been identified[16,17]. Oxygen uptake by whole cells was determined in the presence of various inhibitors and the uncoupling agent, carbonyl-cyanide *m*-chlorophenylhydrazone (CCCP) with a vibrating Pt electrode. The results, shown in Table I, indicate that KCN, NaN_3, and 2-heptyl-4-hydroxyquinoline-*N*-oxide are inhibitory to respiration at concentrations usually employed in bacterial systems. Of the two mercurials tested, phenylmercuric acetate and *p*-chloromercuribenzoate, only the former showed inhibition. This finding is consistent with our previous suggestion[17] that negatively charged agents do not interact well with halophilic membranes. The enhancing effect of CCCP on respiration indicates that electron transport in these cells is tightly coupled and is subject to respiratory control.

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TABLE I

RELATIONSHIP OF RESPIRATION STATE AND DETERGENT DISSOLUTION IN *H. CUTIRUBRUM* CELLS

Inhibitor	*Concn (M)*	*O_2 uptake* (nmoles O_2/min)*	*Rate of light-scattering decrease (min^{-1})*		
			*CTAB***	*Triton X-100*	
			Saline buffer + basal salts	*Saline buffer****	*Saline buffer § + basal salts*
None	–	5.00	0.138	0.019	⩽0.0007
	$5 \cdot 10^{-4}$	0	0.211	0.054	0.0146
KCN	$1 \cdot 10^{-3}$	–	0.272	0.099	0.0173
	$2 \cdot 10^{-3}$	–	0.287	0.176	0.0182
NaN_3	$5 \cdot 10^{-3}$	0.60	0.257	0.110	0.0172
	$1 \cdot 10^{-2}$	0.48	0.314	0.125	0.0242
2-Heptyl-4-hydroxyquinoline-	$2.5 \cdot 10^{-6}$	1.30	0.245	0.172	0
N-oxide	$5 \cdot 10^{-6}$	0.71	0.271	0.192	0.0012
Phenylmercuric acetate	$1 \cdot 10^{-5}$	1.88	0.220	0.125	0.0207
p-Chloromercuribenzoate	$1 \cdot 10^{-5}$	4.94	0.114	0.017	0
CCCP	$3.75 \cdot 10^{-7}$	8.46	0.202	0.024	0
	$7.5 \cdot 10^{-7}$	10.59	0.218	0.044	0

* Oxygen uptake was determined with 10 times the cells used in light scattering experiments; the values obtained were divided by 10.

** 2.5 μg CTAB/ml.

***12.5 μg Triton X-100/ml.

§4 mg Triton X-100/ml, initial rates.

The rate of dissolution of cells by CTAB and Triton X-100 was determined in the presence of the agents described above (Table I). The rate of dissolution by CTAB in the complete salt buffer is seen to be increased 2–2.5-fold by those agents that inhibit respiration and by CCCP. Most striking are the results obtained with the mercurials, where *p*-chloromercuribenzoate is inactive; the influence of these compounds on the dissolution of cells by CTAB is seen to parallel their inhibitory effect on oxygen uptake. The increase in the effectiveness of CTAB on adding the above agents does not appear to be due to a generalized destabilization of the cell envelope. Rather, the sensitivity of the cells to the detergent varies between two values, those of the respiring system and the inhibited or uncoupled system. Variations in the nature of the inhibitors and in their concentrations above that sufficient for inhibition have little further effect on the response of the cells to the detergent. The transition in the response of the cells, from the resistant to the more sensitive state, is thus seen to be brought about by blocking the respiratory chain at any point, from NADH dehydrogenase to cytochrome oxidase, or by uncoupling oxidative phosphorylation. The increased resistance of respiring cells to detergents is therefore not associated with the redox state of any single respiratory component, but rather, depends on electron flow coupled to energy conversion.

The results with Triton X-100 in buffer containing 3.4 M NaCl with and without divalent cations are similar to those obtained with CTAB. However, CCCP is ineffective in the absence of basal salts and both CCCP and 2-heptyl-4-hydroxyquinoline-*N*-oxide are inactive in the complete salt buffer. Since higher concentrations of Triton X-100 were needed for the rapid dissolution of the cells than CTAB, particularly in the presence of basal salts (Fig. 2), the inactivity of the above lipid-soluble agents may be due to their unfavorable partition between the cell envelope lipids and the Triton X-100 micelles. Indeed, in the presence of 0.4% Triton X-100 the effect of both CCCP and 2-heptyl-4-hydroxyquinoline-*N*-oxide on oxygen uptake by the cells was greatly diminished. The detergent itself inhibited respiration by about 20%.

Removal of CTAB by the cells from the buffer was studied by adding the detergent, as in the light scattering experiments, incubating at 22 °C, chilling the cell suspension, and removing the cells by centrifugation. The supernatant was assayed for CTAB, as indicated in Fig. 5a, and the decrease from control values, obtained without cells, was noted. The time-course of CTAB uptake is shown in Fig. 5b at $2.5 \cdot 10^{-6}$ g/ml detergent, where the respiration dependence of the dissolution is evident (Fig. 3b). No differences in CTAB uptake were observed, however, between respiring and KCN-inhibited cells for up to 3 min incubation. At longer times of exposure to the detergent the cell pellets, obtained by centrifugation, became smaller. Thus, the apparent release of previously bound CTAB, especially in the presence of KCN (Fig. 5b) is probably due to the solubilization of the cell envelope components. Uptake of CTAB was also investigated at various detergent concentrations in the presence and absence of KCN, as shown in Fig. 5c. In these experiments the cell suspensions were chilled and centrifuged after 2 min incubation. As in Fig. 5b, no significant differences were observed in CTAB uptake between respiring and KCN-inhibited cells. The shape of the uptake *vs* detergent concentra-

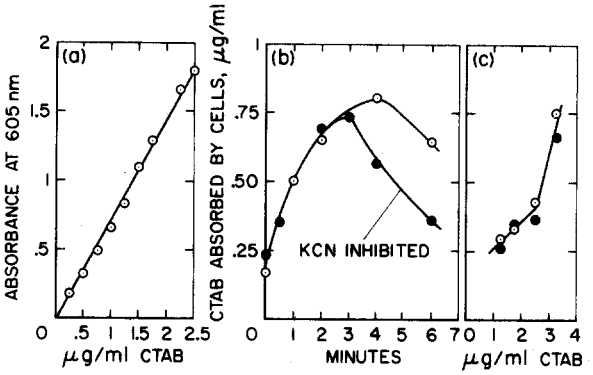

Fig. 5. CTAB uptake by *H. cutirubrum* cells. (a) Standard curve for the CTAB assay system, determined by shaking the given amount of detergent in salt buffer with ¼ vol. chloroform containing 0.04% tetrabromophenolphtalein ethyl ester. The blue dye–detergent complex was recovered in the organic phase and its absorption at 605 nm was measured. (b) Time-course of CTAB uptake, determined by incubating the cells, for the given time, with 2.5 μg/ml detergent, chilling and removing the cells by centrifuging. Subtraction of the CTAB concentration obtained in the supernatants from the total added gave the values shown for uptake. The buffer contained 3.4 NaCl and basal salts. ⊙, respiring cells; ●, KCN-inhibited cells (final concn, 10 mM). (c) Dependence of CTAB uptake on CTAB concentration. The cells were incubated with the detergent for 2 min. Experimental conditions and symbols are es explained under Graph b.

tion curve (Fig. 5c) is similar to the corresponding cell dissolution curves (Fig. 3b) where a threshold between $1 \cdot 10^{-6}$ and $2 \cdot 10^{-6}$ g/ml detergent was obtained. Since neither the threshold CTAB concentration nor the uptake of this detergent was changed upon addition of KCN it is concluded that the uptake of detergent molecules by the cells is unaffected by the state of respiration.

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