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Influence of Electron Transport on the Interaction between Membrane Lipids and Triton X-100 in *Halobacterium cutirubrum*†

Janos K. Lanyi

ABSTRACT: Earlier results (Lanyi, J. K. (1972), *Biochim. Biophys. Acta 282*, 439) showed that respiring *Halobacterium cutirubrum* cells were resistant to Triton X-100 treatment but respiration-inhibited cells could be lysed readily. In this study, the kinetics of (1) turbidity decrease, (2) the appearance of menadione reductase activity, an enzyme that marks the interior surface of the cell membrane, (3) the release of various intracellular constituents, and (4) the shift in the bacteriorubberin absorption spectrum, which indicates the association of this pigment and presumably other membrane lipids with Triton X-100, were followed after adding the detergent to potassium cyanate inhibited and respiring cells. The results indicate that respiring cells lose the integrity of their cell envelope, but, unlike the inhibited cells, resist the penetration of Triton into the membrane lipid phase. The maintenance of respiring cell envelopes in the presence of the detergent is apparently a consequence of this resistance to perturbation.

Numerous enzyme systems have been described which show increased resistance to denaturation in the presence of substrates (Sulkowski and Laskowski, 1968; Reshef and Heller, 1969; Linn *et al.*, 1969; Sudi, 1970), presumably because of structural changes on binding. In biological membranes, which are complex lipoprotein entities, the metabolic state of respiratory enzymes has been found in some cases to influence the behavior of the lipid phase as well. Thus the solubilization of complex III and succinic dehydrogenase in mitochondria by hydrophobic bond-breaking agents is diminished when substrates are present (Rieske *et al.*, 1967; Baginsky and Hatefi, 1969). Respiratory inhibitors, which prevent the resolution of membrane proteins by chaotropes (which increase the lipophilicity of water), also inhibit spontaneous lipid oxidation (Hatefi and Hanstein, 1970). In submitochondrial particles, hydrolysis of lipids by cobra venom phospholipase was found to be less extensive during active electron transport (Luzikov and Romashina, 1972). Another example is the observation that the extraction of lipoproteins from *Escherichia coli* cells by sodium dodecyl sulfate and lysis is dramatically enhanced when the respiratory system is inhibited with potassium cyanide (Bolle and Kellenberger, 1958; Woldringh and van Iterson, 1972). Recently, we found that the *Halobacterium cutirubrum* cell envelope, which contains the electron transport mechanism (Cheah, 1969; Lanyi, 1971), was nearly completely resistant to the nonionic detergent Triton X-100, but when any one of a number of respiratory inhibitors was added the cells rapidly disintegrated (Lanyi, 1972a). Because the halophilic cells, unlike *E. coli*, do not possess a rigid carbohydrate cell wall (Kushner *et al.*, 1964), we expected that the dissolution of the cell envelope in these cells was primarily dependent on disrupting protein–lipid interactions. In the present study, we have investigated the mechanism of the dispersing effect of Triton X-100 on the cell envelope and the membrane lipids, and the possibility that the metabolic state of membrane enzymes has an influence on these processes. While halophilic membranes exhibit some unique characteristics, most notably disintegration upon removal of salt (Kushner, 1964; Stoeckenius and Rowen, 1967; Lanyi, 1971), the results presented in this paper have little obvious relation to such properties. On the contrary, it is our hope that the conclusions drawn may be of general interest in the study of the structure of bacterial membranes.

Materials and Methods

H. cutirubrum cells were grown as described by Hochstein and Dalton (1968), but the medium also contained 0.05 M Tris adjusted to pH 7.0 using a Corning triple-purpose glass electrode (with low Na error). As before (Lanyi, 1972a), late logarithmic phase cells were used, at 3–6 × 10^9 cells/ml, de-

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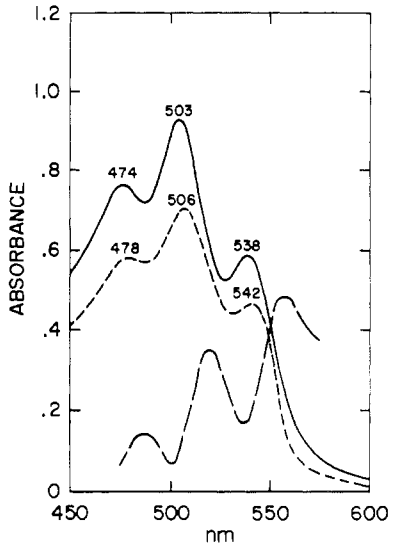


FIGURE 1: Influence of Triton X-100 on the absorption spectrum of bacterioruberin. A methanol solution of *H. cutirubrum* lipids was dispersed in buffer containing 3.4 M NaCl and basal salts (as described in the text): extract alone, ——; extract *plus* Triton X-100, - - - - - -; difference spectrum, – – –, shown on 5× expanded scale.

termined in a Petroff-Hauser chamber. At these cell densities the turbidity (Klett reading with filter 6225) was proportional to the number of cells.

Time-dependent turbidity changes were determined in a Klett photometer, using 250-ml flasks equipped with a side arm and containing 40 ml of cell suspension. The cells were kept aerobic by shaking at room temperature on a rotary shaker. All Klett readings over 150 were corrected for nonlinearity.

Absorption spectra were recorded with a Phoenix Model PMD-1010 spectrophotometer in the split beam mode. In the kinetic studies, the wavelength range of interest was scanned in less than 1 min, with the time constant set at 10 msec, to permit the resolution of changes within 5-min intervals. Cell suspensions were kept aerobic over long periods of incubation by circulating between a flow cuvet (for determining spectra) and an aeration chamber at flow rates of approximately 20 ml/min.

The determination of menadione reductase enzyme activity has been described before (Lanyi, 1969). Time-dependent release of various intracellular components on addition of detergent was followed by analyzing supernatant fractions obtained after centrifuging 2-ml cell suspension aliquots in a Misco microcentrifuge. Sedimentation of cells and debris was complete in 30 sec at maximum speed. DNA and RNA contents (including any soluble deoxyribose and ribose compounds) were determined according to Schneider (1957). Potassium was determined by atomic absorption (Lanyi and Silverman, 1972).

Buffers contained *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) at 0.05 M and pH 7.0. The term "basal salts" refers to salts other than NaCl, which were added to the growth medium, and consist of $MgCl_2 \cdot 6H_2O$, 20 g/l.; KCl, 2 g/l.; and $CaCl_2 \cdot 2H_2O$, 0.2 g/l.

Results

Lipids containing the major red pigment in *H. cutirubrum*, bacterioruberin (Kelly *et al.*, 1970; Gochnauer *et al.*, 1972), were extracted from cells with chloroform–methanol (3:1), the solvent was evaporated under nitrogen, and the residue was redissolved in chloroform. The absorption spectra of this preparation in various organic solvents were recorded. The typical three-peak appearance of carotenoid spectra (Rabinovich, 1951; Davies, 1965) was observed in all cases; the positions of the absorption maxima are shown in Table I. As discussed by Suzuki (1967), the absorption spectrum of such highly conjugated compounds is dependent on the polarizability of the solvent. In Table I solvents of low polarizability (such as hexane) are seen to give rise to blue shifts and those of high polarizability (such as carbon disulfide) cause red shifts. For bacterioruberin, the magnitude of such shifts amounts to more than 30 nm.

TABLE I: Absorption Maxima of *H. cutirubrum* Total Lipid Extract, Containing Bacterioruberin, in Various Solvents.[a]

Solvent	Wavelength (nm)		
Hexane	461	490	522
Ethanol	464	493	526
Chloroform	472	503	538
Benzene	475	505	541
Pyridine	478	510	546
Phenol	484	513	548
Carbon disulfide	494	524	562
Saline buffer (dispersed)	474	503	538
Freeze-thawed cells in saline buffer	474	503	539

[a] The extraction procedure for obtaining the lipid preparation is given in the text. The spectrum of cells was determined after freeze-thaw without further treatment.

Water-dispersed lipids were obtained by dissolving the lipid preparation in a few drops of methanol and adding 10 ml of buffer containing 3.4 M NaCl and basal salts. The absorption spectrum of the resulting red, slightly opalescent, solution was similar to the spectrum of cells, which were freeze-thawed to decrease turbidity (Table I). The spectrum of the dispersed lipid preparation is given in Figure 1. Adding Triton X-100 (final concentration, 1 mg/ml) caused the absorption peaks to shift by 3–4 nm toward higher wavelengths. This shift gave rise to a characteristic difference spectrum (Figure 1) with peaks at 480, 520, and 560 nm.

The appearance of a Triton difference spectrum, such as observed for chloroform-extracted bacterioruberin, was also tested in intact *H. cutirubrum* cells. For this purpose, cells were harvested by centrifugation at 12,000*g* for 10 min and were resuspended in 1:4 volume buffer containing 3.4 M NaCl and basal salts. After adding KCN (final concentration, 10 mM), the suspension was divided into two portions, Triton X-100 was added to one (final concentration, 1.5 mg/ml), and difference spectra were recorded at 5-min intervals. The results (Figure 2a) indicate that a difference spectrum develops within 30–40 min, which resembles that in Figure 1. In a similar experiment, when the cells were resuspended in buffer lacking added salt, they disintegrated to give a clear red solution devoid of turbidity (Kushner, 1964; Onishi and Kushner, 1966). Adding Triton to these lysates yielded the difference spectrum shown in Figure 2b. The peak height at 520 nm, drawn over a base line through the adjacent troughs, was

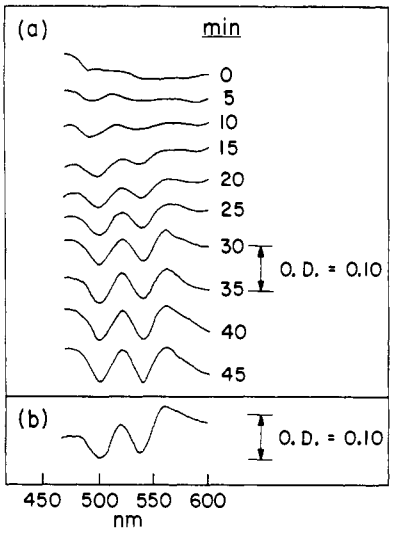


FIGURE 2: Triton X-100 difference spectra of nonrespiring *H. cutirubrum* cells. Logarithmic phase cells were suspended in 1/4 volume of (a) buffer containing 3.4 M NaCl and basal salts or (b) buffer without added salts. After adding KCN (10 mM), the cell suspension was divided into two portions and Triton X-100 (final concentration, 1.5 mg/ml) was added to one. Difference spectra were recorded at 5-min intervals for a; maximal difference spectrum was obtained immediately for b.

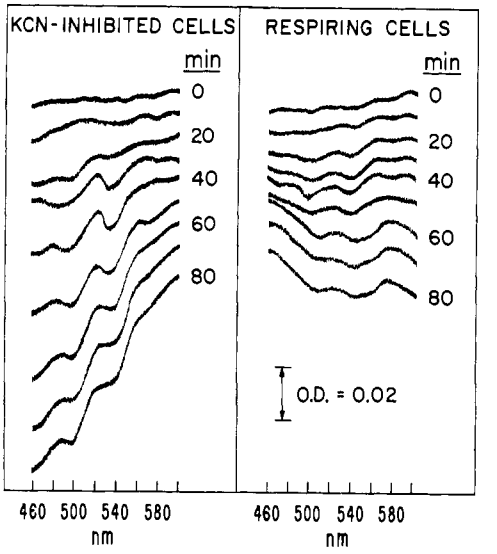


FIGURE 3: Triton X-100 difference spectra of KCN-inhibited and respiring *H. cutirubrum* cells. Cells were suspended in 1/2 volume of buffer containing 3.4 M NaCl and basal salts and Triton X-100 was added to half the suspension. Difference spectra were recorded as in Figure 2. To keep the cells aerobic, the cell suspension was circulated between a flow cuvet and an aeration chamber.

proportional to the amount of cells suspended. Since the cells are extensively dispersed in the absence of salt (Kushner, 1964), the peak heights obtained with the low-salt disrupted cells probably correspond to the spectral shift of the entire pigment content of the cells. The good agreement between this peak intensity, in the absence of salt, and the development of the peak in the presence of NaCl and basal salts (Figure 2) indicates that the absorbance change at 520 nm can be used as an estimate of the amount of bacterioruberin in the cells that is in association with the detergent.

The kinetics of the development of Triton difference spectra were determined also under respiring conditions. Respiring cells, in contrast to KCN-inhibited cells, exhibit very little increase in peak intensity at 520 nm after adding Triton X-100 (Figure 3). These results are presented more quantitatively, as fractions of absorbance change at 520 nm, relative to the total expected (Figure 4). In this graph the relative change in A_{520} is plotted as a function of time after adding the detergent, in the presence and absence of KCN. Also plotted is the turbidity decrease and menadione reductase enzyme activity, the latter parameter indicating the accessibility of substrates to the interior surface of the cell membrane where this enzyme is located (Lanyi, 1972b). It is apparent in Figure 4a that the spectral shift, which indicates that the detergent is in the vicinity of the pigment molecules, begins to develop soon after Triton X-100 is added to KCN-inhibited cells and the intracellular enzyme becomes rapidly accessible. As turbidity begins to decrease the entire pigment content of the cells becomes exposed to the detergent. During and after these events, turbidity decreases to about 5% of the initial value. In respiring cells (Figure 4b), the increase in menadione reductase activity is slower, but after an hour of incubation more than three-fourths of this enzyme becomes accessible to externally added substrates. However, under these conditions both the absorption change at 520 nm and the turbidity decrease are greatly inhibited. Examination of the cell suspensions (Figures 4a and b) in the light microscope, after 2-hr incubation with Triton X-100, showed that the KCN-inhibited cells disintegrated completely, while the respiring cells appeared relatively undamaged.

The data in Figure 4a indicate that, at an early time after the addition of Triton, the cell membrane loses integrity. To explore this point further, the release of various cell constituents was studied. Figure 5 shows the amounts of DNA, RNA, and intracellular K^+ (Lanyi and Silverman, 1972) released during incubation with Triton X-100, relative to the total cell contents. In the presence of KCN (Figure 5a), the

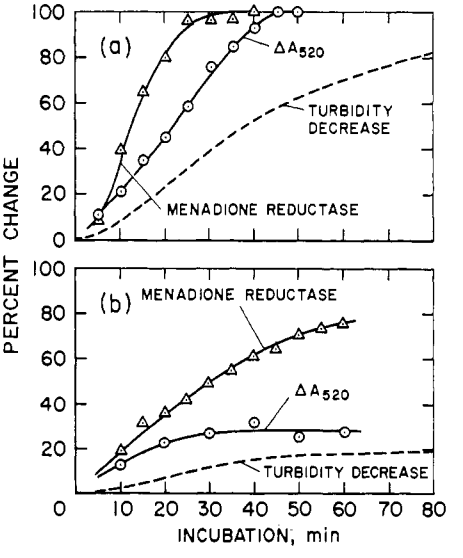


FIGURE 4: Changes during incubation of KCN-inhibited (a) and respiring (b) *H. cutirubrum* cells with Triton X-100. Conditions as in Figure 3. Turbidity decrease, - - -; absorption change at 520 nm, determined from difference spectra (such as in Figures 2 and 3) by measuring peak heights above a base line drawn between two adjacent troughs, ⊙; menadione reductase enzyme activity, assayed in cell suspension aliquots, △.

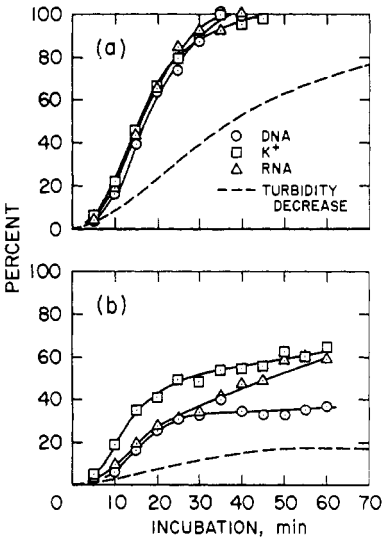


FIGURE 5: Release of intracellular components from KCN-inhibited (a) and respiring (b) *H. cutirubrum* cells during incubation with Triton X-100. Conditions as in Figure 3. At the indicated times after the detergent was added, aliquots of cell suspension were centrifuged for 30 sec in a Misco microcentrifuge. The supernatants were assayed for DNA (⊙), RNA (△), and K^+ (⊡), as described under Methods.

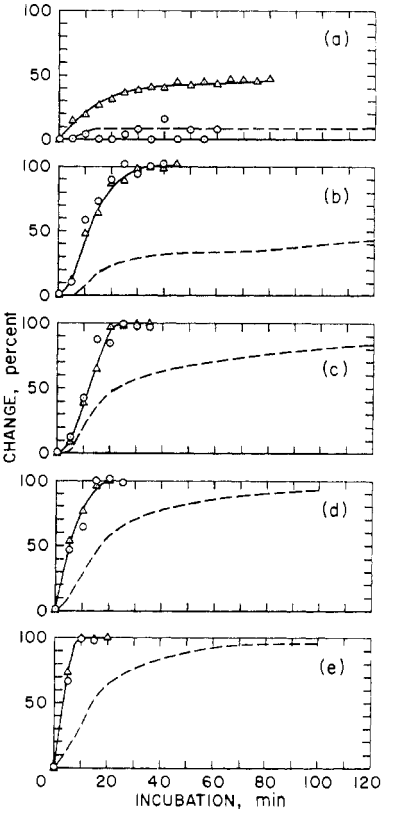


FIGURE 6: Changes during incubation of KCN-inhibited *H. cutirubrum* cells with Triton X-100. The cells were suspended in 1/2 volume of buffer containing 3.4 M NaCl, but no basal salts. Turbidity (- - -), absorbance change at 520 nm (⊙), and menadione reductase enzyme activity (△) were determined after adding Triton X-100 in the following amounts: (a) 10, (b) 20, (c) 30, (d) 40, and (e) 50 μg/ml.

time course of the leakage of all three cytoplasmic constituents studied corresponds well with the appearance of menadione reductase activity (Figure 4a). In all experiments where the release of DNA was determined, the results were qualitatively confirmed by an increase in viscosity when DNA release was found. In respiring cells (Figures 4b and 5b), the loss of cell envelope integrity coincides with the release of intracellular contents, although DNA appears to be retained more than RNA.

The effect of detergents on the cell envelope is greatly influenced by divalent cations (Lanyi, 1972a). Thus, omitting the basal salts from the buffer in which the cells were suspended made it possible to use very low concentrations of Triton, below 50 μg/ml instead of over 1 mg/ml, and obtain kinetics similar to those in Figures 4 and 5. Under these conditions, the amount of detergent present limits the extent of the detergent action as well as the rate of dissolution of the cells. Figure 6 shows turbidity changes, increase in A_{520}, and menadione reductase activity at Triton X-100 concentrations of 10–50 μg/ml in the presence of KCN. At 10 μg/ml of Triton X-100 (Figure 6a), very little turbidity change occurs and the increase in absorbance at 520 nm is lower than the detection limit. The cells are seen to become leaky, however, and nearly half the menadione reductase activity can be assayed. This concentration of detergent is below the threshold described previously (Lanyi, 1972a) for the amount of cells present. At 20 μg/ml of Triton X-100 (Figure 6b), both menadione reductase activity and the absorbance change at 520 nm are complete by 30 min, but the light scattering decrease is only 30% at this time and does not reach 50% even after 2 hr. At higher concentrations of Triton (Figures 6c–e) all three processes reach completion, at rates dependent on the concentration of the detergent. Unlike in the experiments where basal salts are added (Figure 4a), however, the pigment spectral shift under these conditions proceeds concurrently with the increase in the amount of accessible menadione reductase.

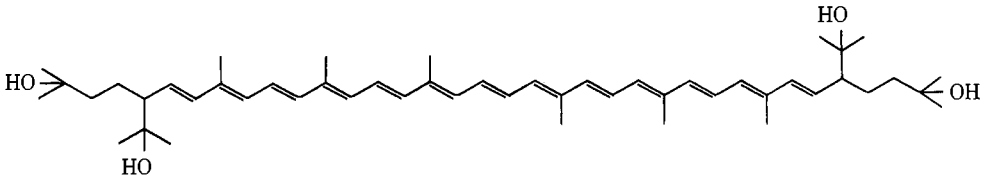
Discussion

It has been recognized that the interaction of detergents with artificial lipid bilayers and biological membranes includes, as a critical step, the insertion of the hydrophobic portions of the detergents into the hydrocarbon-like interior of the lipid phase (Pethica and Schulman, 1953; Pethica, 1958; Kondo and Tomizawa, 1966). However, the observation that the dissolution of some membranes by detergents seems to depend on the functional state of membrane proteins (Bolle and Kellenberger, 1958; Lanyi, 1972a) necessitated another examination of the mechanism of the detergent action. Thus, we studied various experimentally accessible parameters related to the structural integrity of *H. cutirubrum* cell envelopes and their time-dependent changes after adding the nonionic detergent, Triton X-100.

The assay of menadione reductase provided the means to detect the loss of integrity of the cell envelopes. The release of intracellular constituents of various molecular weights (including K^+, RNA, and DNA) was seen to take place concurrently with the increase of detectable menadione reductase activity (Figures 4a and 5a). The decrease in turbidity, measured after Triton was added to the cells, on the other hand, was not correlated with the appearance of menadione reductase activity or cytoplasmic leakage. Thus, in aerobic cells only about 10% turbidity decrease was observed while lysis and leakage were extensive (Figures 4b and 5b). In the absence of basal salts, at very low concentration of detergent, a considerable amount of menadione reductase could be

assayed without much change in turbidity (Figure 6a). It appears, therefore, that the turbidity decrease observed when the cells are dispersed with Trition is not primarily due to the loss of cytoplasmic material, but may be caused by the disintegration of the cell envelopes and associated structures.

The red pigment of *H. cutirubrum* has been identified as bacterioruberin (Kelly *et al.*, 1970) of the following structure



Such highly conjugated molecules exhibit solvent dependence in their absorption spectra due to an induced dipole moment in adjacent solvent molecules which reduces the energy of the excited state (Suzuki, 1967). On the basis of the shifts of the absorption maxima of a bacterioruberin extract in various solvents, particularly in phenol (Table I), we expected that the phenoxy group in Triton X-100 (octylphenoxypolyethoxyethanol) would cause a small red shift when associated with the pigment molecule. Such a red shift was indeed observed (Figure 1); thus, bacterioruberin could be used as a reporter molecule to indicate the presence of the detergent in its immediate neighborhood. Bacterioruberin is a highly apolar molecule of 36 Å length, a size appropriate for spanning the width of a lipid bilayer, and one might expect that it is located in the lipid phase of the membranes. This assumption is supported by the observations that the pigment is easily extracted with chloroform–methanol, and that the lipid extract, which contains the pigment, exhibits the same absorption spectrum in aqueous suspension as do cells. Thus, the red shift observed in the absorption peaks when Triton X-100 is added to cells should reflect the penetration of the detergent into the lipid regions of the membrane or the subsequent extraction of lipids from the membrane into detergent micelles.

The kinetics of changes in turbidity, menadione reductase activity, release of cytoplasmic constituents, and increase in the absorption of bacterioruberin at 520 nm after adding Triton X-100 to *KCN-inhibited* cells suggest the following sequence for the action of the detergent: (1) the loss of integrity of the cell envelope, resulting in release of cell contents, is an early event in the disintegration of the cells; (2) the association of the major portion of membrane lipids with Triton is coincident with lysis in the absence of divalent cations (Figure 6) and follows lysis in the presence of basal salts (Figure 4a); and (3) decrease in turbidity, apparently reflecting the dispersion of cell envelope components, is a late process, occurring much slower than steps 1 and 2 (Figures 4a and 6). When Triton is added to *respiring* cells, the cell envelope becomes leaky (Figures 4b and 5b), but the association of the detergent with the membrane lipids (step 2) and the subsequent disintegration of the cell envelope (step 3) are greatly impeded. Thus, the source of the greater resistance of the cells to Triton under respiring conditions seems to be at or near the level of lipid–detergent interaction. It is premature to speculate on the kind of protein–lipid interaction that would confer resistance to the lipid phase to perturbation by nonionic detergents. The effect does not seem to be related to respiration-dependent transport of ions or other substances since respiring cells are maintained in the presence of Triton even when the intracellular enzyme assayed is freely available to the external medium (Figure 4b). It may be relevant to point out, therefore, that proteins and polypeptides have been shown to affect the physical state of the lipid bilayer, resulting in some loss of motional freedom, as determined by proton magnetic resonance (pmr) (Chapman and Dodd, 1971) and with spin labels (Tourtellotte *et al.*, 1970; Seelig and Hasselbach, 1971). In *H. cutirubrum* membranes, where spin-labeled fatty acid probes indicate dramatic restriction of motion in the lipid phase as compared to vesicles prepared from the lipids alone (Esser and Lanyi, 1973), protein–lipid interactions may be of overriding importance.

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Respiration of Rat Lung Mitochondria and the Influence of Ca^{2+} on Substrate Utilization†

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ABSTRACT: Mitochondria were prepared from homogenates of rat lungs by modification of standard techniques. The mitochondrial preparation was characterized by its content of cytochromes, adenine and pyridine nucleotides, coenzyme A, and divalent cations. Rates of substrate oxidation were measured polarographically. Succinate was oxidized at a rate of 53 ± 9 nmol of O_2/min per mg of protein while other substrates were oxidized less rapidly. The Ca^{2+} content of the mitochondrial preparation was of major importance in determining rates of substrate oxidation. Addition of 100–200 μM Ca^{2+} during oxidation of NAD-linked substrates markedly decreased respiration rate; this effect was due to loss of free pyridine nucleotides from the mitochondria in the presence of Ca^{2+}. On the other hand, very low levels of Ca^{2+} (<1 μM) stimulated oxidation of α-glycerophosphate. This effect was due to increased glycerophosphate dehydrogenase activity in the presence of Ca^{2+}. Lung mitochondria were able to passively bind large amounts of Ca^{2+} and also actively accumulated Ca^{2+} from the suspending medium in the presence of exogenous ATP and during substrate oxidation in the presence of Mg^{2+} or rotenone. Although the effects on oxidation of NAD substrates may not be physiologically significant, cytosolic [Ca^{2+}] in the intact lung cell may be important as a determinant of mitochondrial glycerophosphate dehydrogenase activity. This activation may affect the rate of α-glycerophosphate incorporation into alveolar phospholipids or may regulate the activity of the α-glycerophosphate cycle for intramitochondrial transport of reducing equivalents.

Recent studies have shown the lung to be an actively metabolic organ with an oxygen consumption per unit weight almost as great as liver (Heinemann and Fishman, 1969; Weber and Visscher, 1969). Reiss and others have isolated mitochondria from lung homogenates (Reiss, 1966; Kyle and Riesen, 1970; Sayeed and Baue, 1971) and have suggested that these organelles are qualitatively similar to mitochondria derived from other tissues. However, basic information about the lung mitochondrial preparation such as content of cytochromes and nucleotides, factors affecting rates of substrate oxidation, and mechanisms for transport of reducing equivalents across the mitochondrial membrane have not been determined. Because this type of data obtained with mitochondria from other organs has provided important information with respect to control of tissue metabolism, we began similar studies with a lung mitochondria preparation. Although it is known that the lung is heterogeneous from a morphologic standpoint (Bertalanffy, 1964), preparation of mitochondria from the whole organ provided a reasonable starting point. Our initial results showed that respiration of lung mitochondria is very dependent on calcium ion content of the preparation and we have explored this area of lung mitochondrial metabolism in detail.

Materials and Methods

Preparation of Mitochondria. Eight to ten 180–220-g Sprague-Dawley male rats were killed by decapitation. The lungs were quickly removed, dissected free of large airways and blood vessels, and minced with scissors in ice-cold isolation medium containing 225 mM mannitol, 75 mM sucrose, and 2 mM EDTA. The minced tissue was transferred to isolation medium (approximately 1:10 tissue to medium ratio) containing in addition 5 mM Mops[1] (pH 7.3) and 1–2% (w/v) lipid-free bovine serum albumin (Chen, 1967). In some experiments, 20 mM EGTA was substituted for EDTA in the homogenization medium without demonstrable effect on characteristics of the mitochondrial preparations. The lung mince was

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[1] Abbreviations used are: Mops, morpholinopropane sulfate; EGTA, ethylene glycol bis(amino ethyl ether *N,N'*-tetraacetic acid); RCR, respiratory control ratio; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; α-GP, α-glycerophosphate; TMPD, *N,N,N,N*-tetramethyl-*p*-phenylenediamine.