BBA 75636

THE CELL MEMBRANES OF A MARINE PSEUDOMONAD, PSEUDOMONAS BAL-31; PHYSICAL, CHEMICAL, AND BIOCHEMICAL PROPERTIES

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(Received February 8th, 1971)

SUMMARY

The marine pseudomonad, Pseudomonas BAL-31, is of special interest because it is the host cell of the lipid-containing bacteriophage PM2. The preparation of Pseudomonas BAL-31 membranes and their chemical composition is described in this paper. The buoyant density of the membranes is 1.30 in CsCl and 1.24 in sucrose. Electron micrographs of the intact membrane and the isolated cell membrane fraction are presented.

The membrane-bound ATPase has properties similar to mitochondrial F_1. It is Mg^{2+}-dependent and not activated by Na^+/K^+. On the other hand, very few inhibitors of mitochondrial F_1 inhibited this enzyme, *N,N'*-dicyclohexylcarbodiimide being a notable exception. Alkaline phosphatase activity was present at low levels in the membrane preparation but probably is not directly associated with the plasma membrane. Succinate dehydrogenase required no divalent cation cofactor and was inhibited by malonate. NADH oxidase activity was very low relative to ATPase and succinate dehydrogenase activities. The cytochromes of the electron transport system were characterized as o_{570}, a_{626}, b_{560}, and c_{554}.

INTRODUCTION

Pseudomonas BAL-31 is a marine pseudomonad isolated approximately one mile from the coast of Chile[1]. Although isolated within the littoral zone, this organism has a maximum growth rate at 25° and the ionic strength of the synthetic medium is 0.68, leaving no doubt as to its marine origin[1]. It is of particular interest as the host cell for a lipid-containing bacteriophage, PM2, also isolated from the same area[2].

As an introduction to an analysis of the cell-virus membrane system[3–8], certain properties of the membrane fraction from Pseudomonas BAL-31 will be described in this report. Of particular interest are the membrane-bound enzymes of oxidative

Abbreviations: BAL, 2,3-dimercaptopropan-1-ol; DCCD, *N,N'*-dicyclohexylcarbodiimide; NBT, 2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene)ditetrazolium chloride.

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phosphorylation because of their relatively high resistance to a number of typical inhibitors of the respiratory chain and the discrepancy between the rate of early steps in electron transport and the overall rate of NADH reduction.

MATERIALS AND METHODS

Growth of bacteria

Pseudomonas BAL-31 was grown at 25° in 2,3-dimercaptopropan-1-ol broth or BAL synthetic medium[1,2,8]. Thioglycolate broth contained AMS salts[8], 8 g/l of Bacto nutrient broth, and 1 g/l of sodium thioglycolate. Growth rates were measured by following $A_{610\ m\mu}$.

Preparation of membranes

The basic procedure for preparing membranes is described here; variations will be noted where appropriate. 200 ml of cells in logarithmic growth ($A_{610\ m\mu} = 0.25$) were collected by centrifugation (10400 × *g*, 10 min, 5°), washed once in NaCl–Tris–EDTA buffer (0.05 M Tris (pH 7.4, at 25°)–1 M NaCl–0.0025 M EDTA) and resuspended in 5 ml of this buffer. Lysozyme (EC 3.2.1.17) was added to a final concentration of 200–500 μg/ml and the cells were incubated for 30 min at 25°. As judged by phase-contrast microscopy, about 50 % of the cells rounded up, although nearly all of the cells lysed in a later step. 60 min of lysozyme treatment did not alter the percentage of spheroplasts or the specific activities of the enzymes in the isolated membranes.

The lysozyme-treated cells were pelleted (1240 × *g*, 20 min, 5°), resuspended in 5 ml NaCl–Tris buffer (0.05 M Tris (pH 7.4, at 25°)–1 M NaCl), and glucose was added to a final concentration of 2 % (w/v) to permit metabolic swelling[9]. This was done either by prewarming the cells to 37° for 2 min before bringing them into 2 % glucose for 10 min at 37°, or by incubating the cells in 2 % glucose (in NaCl–Tris buffer) for 20 min at 25° without prewarming. In both cases the cells were then chilled and lysed by diluting 1:1 with ice-cold Tris buffer (0.05 M, pH 7.4 at 25°).

The membranes, complexed with DNA, were centrifuged (9750 × *g*, 20 min, 5°), resuspended in 2 ml of 0.05 M Tris, 0.003 M $MgCl_2$ and 0.005 M $CaCl_2$, and treated with phenylmethyl sulfonylfluoride-reacted[3] deoxyribonuclease I (EC 3.1.4.5) at a final concentration of 10 μg/ml for 20 min at 5°. After low-speed centrifugation (1240 × *g*, 10 min, 5°) to remove unlysed cells, the membranes were washed extensively by centrifugation (Spinco Ti 50 rotor, 20000 rev./min, 10 min, 5°) and resuspended by Dounce homogenization in the appropriate buffer. Two washing procedures were used: (a) 3–5 washed with Tris buffer; (b) 1 wash with Tris buffer, 2 or 3 washes in Tris buffer with 0.01 M EDTA (Tris–EDTA), followed by three washes in Tris buffer with 0.0025 M $MgCl_2$ (Tris–Mg^{2+} buffer). The washed membranes were resuspended in either Tris or Tris–Mg^{2+} buffer.

Such membrane preparations are free of hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activities, two enzyme activities characteristically found in the cytoplasmic fraction[4].

CsCl and sucrose isopycnic density gradients of membranes

Cells were grown in synthetic medium containing 1 μC/ml [^{32}P]phosphoric acid and membranes from these cells were prepared and washed extensively with

Tris–EDTA. A small aliquot of the membrane fraction was diluted in 0.05 M Tris (pH 7.4), CsCl was added to obtain an average density of 1.3 g/cm^3, and the membranes were centrifuged at 50000 rev./min at 25° for 38 in the Spinco SW 65 rotor. Aliquots of each fraction were used for determining refractive index and radioactivity. The density of the fractions was calculated from the empirical relation between n_D^{25} and ρ^{25} as given by IFFT *et al.*[10]. Alternatively, labeled membranes in 0.1 ml buffer were layered onto preformed 30–60 % sucrose (in 0.05 M Tris, pH 7.4) gradients and spun at 36000 rev./min at 20° for 5.75 h in the Spinco SW 39 rotor. The membranes banded at the same density if the centrifugation was extended to 24 h. The refractive index and radioactivity was determined for each fraction and the density of the sucrose fractions at 20° was obtained from the International Critical Tables. A Bellingham and Stanley High Accuracy Abbe 60 refractometer, equipped with a constant temperature circulating water bath, was used for determination of n_D^{25} and n_D^{20}.

Electron microscopy

Membranes washed in either Tris buffer or Tris–Mg^{2+} buffer were pelleted by centrifugation. The pellet was washed with 0.1 M sodium phosphate buffer (pH 7.0) containing 0.01 M $MgCl_2$ (phosphate–Mg^{2+} buffer) and then fixed for 30 min with 2.5 % neutralized glutaraldehyde in phosphate–Mg^{2+} buffer. After one wash phosphate–Mg^{2+} buffer, the pellet was post-fixed overnight with 1 % OsO_4 (see ref. 11) in phosphate–Mg^{2+} buffer, washed three times with Michaelis buffer without $CaCl_2$ or NaCl (see ref. 12) and stained fro 3 with 1 % uranyl acetate in Michaelis buffer. The pellet was dehydrated in ethanol and propylene oxide, cut into 1-mm^3 blocks, and mounted in Epon 812 (see ref. 13). Sections were stained with alcoholic uranyl acetate and lead citrate[14], and observed in a Philips EM 300 electron microscope operated at 80 kV.

Whole cells were prepared for electron microscopy by pelleting at 5000 × *g* for 10 min at 5°. The pellets were fixed for 2 min with 2.5 % glutaraldehyde containing 0.1 M phosphate buffer (pH 7.3) and 20 % sucrose. The pellets were then washed briefly with Michaelis buffer (with NaCl and $CaCl_2$) and post-fixed overnight with 1–2 ml 1 % OsO_4. Subsequently, the pellets were treated as described for the membrane pellets.

Enzyme assays

Our standard assays for ATPase (EC 3.6.1.3), succinate dehydrogenase (EC 1.3.99.1), 2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride reductase*, NADH oxidase (EC 1.6.99.3), and alkaline phosphatase (EC 3.1.3.1) have been described[4]. All assays were carried out at 25°. In some cases different buffers were used in the assays and these are reported in the table legends.

NADH oxidase: For the fluorescence assay of NADH, an interference filter at 465 mμ was used as a secondary filter instead of the less sensitive 496-mμ filter originally used[4]. Using the 465-mμ filter and the lowest light intensity, full scale reading (100) was equivalent to 2.0 nmoles NADH per ml. A Gilson oxygraph equipped with a Clark electrode was used to test the inhibitory effects of antimycin and *n*-heptylquinoline, since these compounds gave extremely high blanks in the fluorometer.

* This enzyme activity is not classified in "Enzyme Nomenclature", Elsevier Publishing Company, 1965.

ATPase: The conversion of ATP to ADP was followed by three procedures, including the standard procedure which measures the appearance of inorganic phosphate[4].

(a) [^{3}H]ATP was incubated at 25° in the presence of membranes for a given period of time. The final volume was 100 μl. The mixture was then chilled and 2 μl of 70% perchloric acid was added to yield a final concentration of 0.2 M perchloric acid. After about 10 min in perchloric acid, the sample was neutralized with KOH, centrifuged, and the supernatant collected. 5-μl aliquots were mixed with 5 μl of a mixture of carrier ATP, ADP, and AMP and a 5-μl aliquot of this mixture was chromatographed on thin-layer plates of DEAE-cellulose using 0.02 M HCl as solvent[15]. The spots were located by using a short-wave mineralight, scraped off the supporting plastic film into scintillation vials, and the radioactivity was measured by scintillation spectrometry.

(b) ADP was determined by a coupled enzyme reaction involving pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27)[16]. The initial reaction to form ADP was carried out by incubating the membranes at 25°. The reaction was stopped and the samples were neutralized as in (a). In this case, however, the total volume was 1 ml. A 200-μl aliquot was mixed in a cuvette to a final volume of 1 ml containing 50 μmoles potassium phosphate buffer (pH 7.0), 10 μmoles KCl, 5 μmoles $MgCl_2$, 1 μmole phospho(enol)pyruvate, and 0.15 μmole NADH. The $A_{340\,m\mu}$ was determined, then 5 μg of pyruvate kinase was added and the decrease in $A_{340\,m\mu}$ was followed until it stopped. The difference between the $A_{340\,m\mu}$ readings was a measure of the decrease in NADH, which was equivalent to the amount of ADP present. In addition, a calibration curve was constructed using known amounts of ADP ($\varepsilon_m = 15.4 \cdot 10^3$ at 259 mμ) and resulted in a conversion factor of 1 $\Delta A_{340\,m\mu}$ = 173 nmoles ADP per ml.

Chemical determinations

Membranes were prepared from 8 l of cells grown in synthetic medium to 0.2 $A_{610\,m\mu}$ and from 4 l of cells grown in broth to 0.4 $A_{610\,m\mu}$. The membranes were extensively washed in Tris–EDTA, resuspended in 0.05 M Tris (pH 7.4) and aliquots dried *in vacuo* over P_2O_5 for several days. Lipids were extracted frome the dried aliquots by the method of BLIGH AND DYER[17], as described by KATES[18], and dried under a stream of nitrogen and then *in vacuo*. Dry weight determinations were corrected for water uptake during manipulation of the samples and for the presence of buffer salts.

DNA was measured by the diphenylamine procedure[19], using calf-thymus DNA as a standard; RNA was measured by the orcinol reaction[20], using yeast RNA as a standard, and protein was determined by the procedure of LOWRY *et al.*[21], using bovine serum albumin as a standard.

Carbohydrate was determined by the phenol–sulfuric acid method[22], and hexosamine was measured by the Boas modified Elson–Morgan procedure[23–25]. Glucose and glucosamine served as standards in the respective assays. There were no significant differences in carbohydrate and hexosamine values determined on membranes hydrolyzed in 4 M HCl at 100° for 2, 4, or 6 h.

Total phosphate was measured on membranes and membrane lipid by the AMES method[26].

Materials

Enzymes: Pyruvate kinase (rabbit skeletal muscle, as an ammonium sulfate suspension) was obtained from Sigma Chemical Co., St. Louis, Mo. Lactate dehydrogenase (rabbit muscle, as a crystalline suspension in 2.2 M ammonium sulfate) was obtained from C. F. Boehringer, Mannheim, Germany. Lysozyme and electrophoretically purified deoxyribonuclease 1 were obtained from Worthington Biochemical Corp., Freehold, N. J..

Radioactive materials: [^{3}H]ATP, tetralithium salt, was obtained from Schwartz Bioresearch Inc., Orangeburg, N.Y. ^{32}P, as phosphoric acid, was obtained from New England Nuclear Corp., Boston, Mass..

Reagents: Rotenone was obtained from S. B. Penick and Co., New York, N.Y.. *N*-Heptyl-quinoline, antimycin A, ouabain, oligomycin, phospho(enol)-pyruvate, tricyclohexylamine salt, and phenylmethyl sulfonylfluoride were obtained from Sigma Chemical Co., St. Louis, Mo. BAL was obtained from British Drug Houses Ltd., Poole, Dorset, England. Sodium amytal was obtained from Eli Lilly and Co., Indianapolis, Ind. *N*,*N'*-dicyclohexyl-carbodiimide, and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate were obtained from Aldrich Chemical Co., Milwaukee, Wisc.. 2-Mercaptoethanol was obtained from Eastman Organic Chemicals, Rochester, N.Y.. NADH was obtained from C. F. Boehringer, Mannheim, Germany. *p*-Chloromercuribenzoic acid (sodium salt), 2,4-dinitrophenol and sodium deoxycholate, enzyme grade, were obtained from Mann Research Laboratories, New York, N.Y.. ADP was obtained from Miles Laboratory, Elkhart, Ind.. Sinnopal NP-12, a non-ionic detergent, was obtained from Melle-Bezons (Sinnovia), 95 Bezons, France.

RESULTS

Preparation of membranes

The effects on membrane preparations of metabolic swelling of the spheroplasts[9] and different procedures for washing the membranes is summarized in Table I. In Expt. 1, membranes were prepared from cells grown in synthetic medium to 1.10^8–$2 \cdot 10^8$/ml using metabolic lysis at 25°, while in Expt. 2, the cells were lysed by rapidly diluting the spheroplasts, which were in NaCl–Tris buffer at 0°, 1:1 in 0.05 M Tris buffer at 0°. In each experiment one aliquot of membranes was washed three times with Tris buffer and then once with Tris–Mg^{2+} buffer and the second aliquot was washed once with Tris buffer, twice with Tris–EDTA buffer, and once with Tris–Mg^{2+} buffer. The two different washing procedures yielded comparable specific activities for ATPase, succinate dehydrogenase, and NADH oxidase (Table I). Membranes from cells lysed after metabolic swelling, however, had significantly higher NADH oxidase and ATPase specific activities compared to untreated cells, and therefore this technique was used routinely.

Chemical composition

The chemical composition of membranes from cells grown in synthetic medium and broth is given in Table II. Only the carbohydrate content varied significantly in the two media. The lack of hexosamine indicates that the EDTA–lysozyme treatment removed most of the mucopeptide R-layer component of the cell wall, since this cell wall component usually contains the bulk of the cell membrane sugar amines[27].

In general, the composition is similar to that reported for another Gram-negative pseudomonad, *Pseudomonas aeruginosa*[28].

The phospholipids of membranes isolated from cells grown in synthetic medium were identified by previously published methods[5]. Only two major phospholipids were found: phosphatidylethanolamine (74 %) and phosphatidylglycerol (26 %).

Buoyant density

The buoyant density of the membranes differed considerably when measured in CsCl and sucrose (Fig. 1). In CsCl the density at 25° is 1.30, and in sucrose the density at 20° is 1.24. This difference is probably due to binding of Cs^+ to either the membranes or associated nucleic acid[29].

Morphology of membranes

The morphology of a membrane preparation washed with Tris–EDTA is shown in Fig. 2. Despite extensive washing there is some particulate matter still associated

TABLE I

EFFECT OF EDTA WASHING ON MEMBRANE-BOUND ENZYMES

In Expt. 1 membranes were isolated from spheroplasts incubated in 2% (w/v) glucose for 20 min at 25° before lysis at 0°. In Expt. 2 spheroplasts were lysed by dilution at 0° without prior incubation with glucose at 25°.

Expt. No.	*Conditions*	*ATPase**	*Succinate dehydrogenase**	*NADH oxidase**
1	Tris washed	107	12.9	1.26
	Tris–EDTA washed	151	12.0	1.08
2	Tris washed	34	11.2	0.81
	Tris–EDTA washed	33	11.2	0.87

* Enzyme specific activities are expressed in nmoles/min per mg membrane protein.

TABLE II

COMPOSITION OF PSEUDOMONAS BAL-31 MEMBRANES

MATERIALS AND METHODS gives references for the procedures. All values except for phosphorus are expressed as % dry wt. of cell membrane.

	Synthetic medium	*Broth*
Protein	49.3	51.9
DNA	0	2.9
RNA	9.6	12.9
Lipid	24.7	31.9
Carbohydrate	16.8	4.3
Hexosamine	0	0
Total %	100.4	103.9
Total phosphorus (μmoles/mg membrane)	0.22	0.49
Lipid phosphorus (μmoles/mg membrane)	0.20	0.41
Lipid phosphorus (μmoles/mg lipid)	0.86	1.28

with the membranes, although less than that seen associated with membranes washed only in Tris buffer.

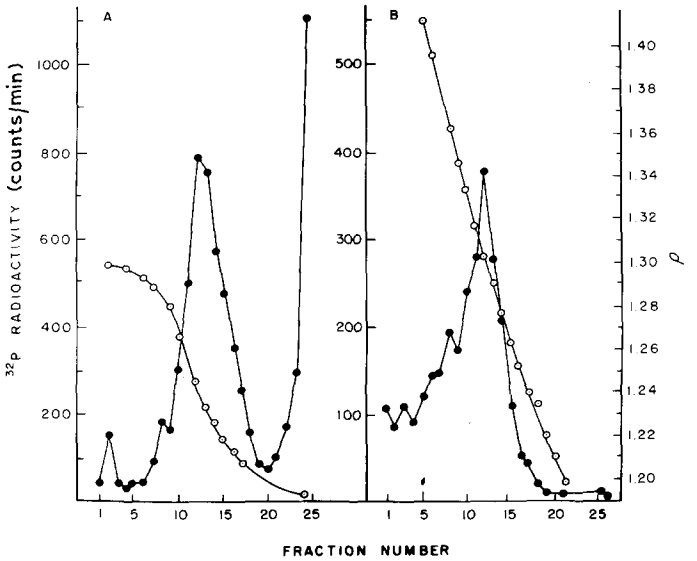

Fig. 1. Equilibrium distribution of [^{32}P]phosphoric acid labeled BAL-31 membranes in sucrose and cesium chloride. (A) 0.1 ml of labeled membranes were layered onto a 4.4-ml sucrose gradient (30%–60%, w/w, sucrose-Tris, pH 7.4) and centrifuged in a Spinco SW 39 rotor at 36000 rev./min at 20° for 24 h. (B) An aliquot of labeled membranes was centrifuged in cesium chloride with an approximate initial density of 1.3 g/cm^3 in a Spinco SW 65 rotor at 50000 rev./min at 25° for 38 h. ●—●, radioactivity; ○—○, density at 4°.

Pseudomonas BAL-31 is Gram-negative[1], and spheroplast formation using lysozyme–EDTA should not result in the complete removal of cell-wall material[30]. Therefore the physical and chemical properties of the membranes must be interpreted in light of the presence of cell wall components. To observe the effect of lysozyme–EDTA treatment on the Pseudomonas BAL-31 cell wall, cells grown in minimal medium were pelleted and resuspended in 1 M NaCl and 0.05 M Tris (pH 7.4). After the usual lysozyme–EDTA treatment, $MgCl_2$ was added to halt the enzyme action, and the cells were then pelleted and prepared for electron microscopy. Figs. 3a shows a typical untreated cell while Fig. 3b shows a cell treated with lysozyme. Although the mucopeptide layer is not resolved into a 20–30 Å electron dense component, as is often the case for other Gram-negative bacteria[31], it is clear that the space between the inner and outer membranes is less electron dense in the treated cell. We may therefore conclude that the lysozyme appears to be acting in the usual way, and the lipoprotein and lipopolysaccharide layers remain intact[31].

Membrane-bound enzymes

ATPase: The conditions for assaying ATPase were studied in a preliminary experiment. Using Mg^{2+} as a cofactor, and Sinnopal NP-12 to dissociate the membranes, the specific activity was 0.91 μmole P_i per 60 min per mg membrane protein. The concentration of ATP was 5 μmoles/ml. When 0.5 μmole/ml of ADP was added

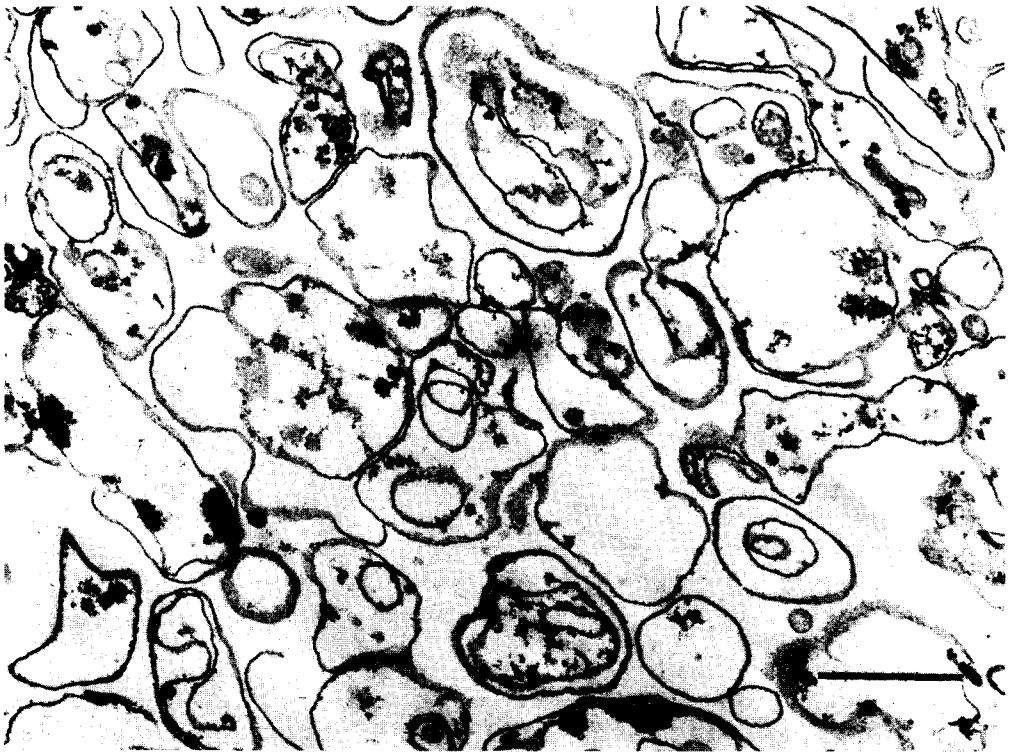

Fig. 2. Membranes from lysozyme-treated Pseudomonas BAL-31 cells were prepared and washed with Tris–Mg^{2+} buffer (*cf.* MATERIALS AND METHODS). Pellets of the washed membranes were prepared for electron microscopy and thin sections were stained with alcoholic uranyl acetate and lead citrate. The scale line represents 0.5 μ.

to the reaction, the specific activity dropped 40 % to 0.54. When a regenerating system consisting of phospho(enol)pyruvate and pyruvate kinase was added to the original assay mixture, the specific activity increased 50 % to 1.37. If NP-12 was removed from the reaction system containing the regenerating system, the specific activity was 0.77, a decrease of 44 % if based on 1.37. From these preliminary experiments, it appeared that ADP is a product of the reaction, and that solubilization of the enzyme enhanced its activity since the P_i assay is not affected by the detergent.

Based on these findings, our standard assay system consisted of an aliquot of membrane containing 50–500 μg protein, 2.5 μmoles ATP (Tris form), 2.5 μmoles $MgCl_2$, 0.25 μmole Tris buffer (pH 7.4 at 25°), 20 μl of 1 % (w/v) Sinnopal NP-12, 5.0 μmoles phospho(enol)pyruvate and 50 μg pyruvate kinase, in a final volume of 0.50 ml.

The kinetics of the reaction are illustrated in Fig. 2. The reaction is not linear with time; there is a slight but reproducible increase in rate after about 30 min. The effect of ADP and the absence of the phospho(enol)pyruvate/pyruvate kinase regenerating system are clearly shown (Fig. 4).

The cation requirement was investigated using the standard assay system with or without $MgCl_2$ (Table III). Although the enzyme requires divalent cations, Mg^{2+}

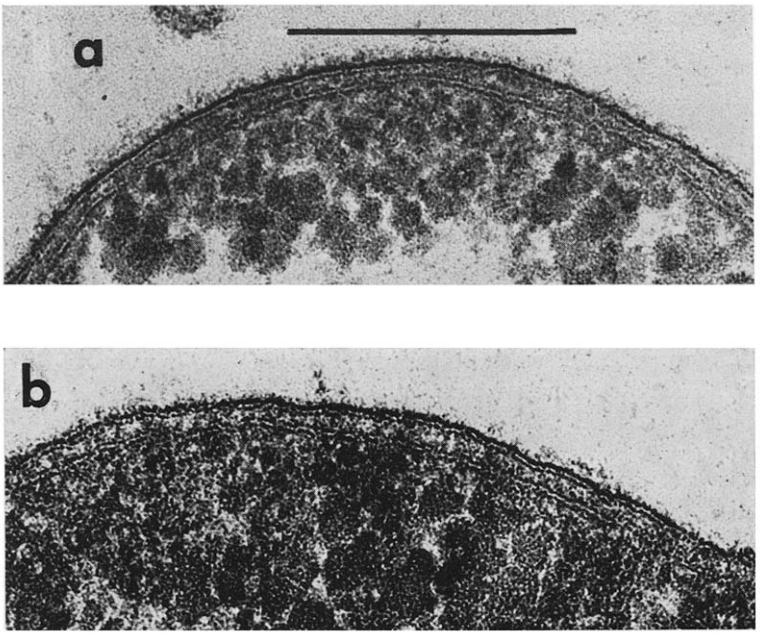

Fig. 3. Pseudomonas BAL-31 cells were pelleted and resuspended in 1 M NaCl, 0.0025 M EDTA, and 0.05 M Tris (pH 7.4). Half of the sample was treated with 400 μg/ml of lysozyme at 25° for 30 min. After treatment, both samples were pelleted, fixed, and prepared for electron microscopy. Thin sections were stained with alcoholic uranyl acetate and lead citrate. The scale line represents 1.0 μ. a, control cells; b, lysozyme-treated cells.

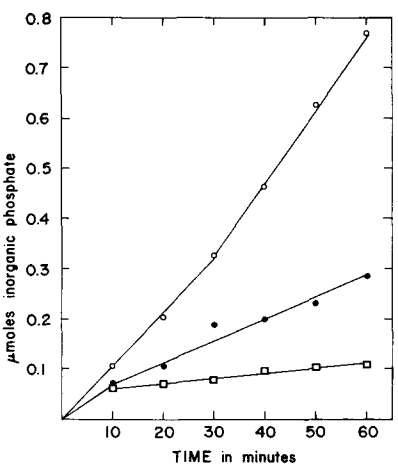

Fig. 4. Rate of formation of inorganic phosphate from ATP: ○—○, complete reaction mixture including the phospho(enol)pyruvate–pyruvate kinase regenerating system; ●—●, incubation mixture *minus* the regenerating system; □—□, incubation mixture *minus* the regenerating system and in the presence of 5 mM ADP.

is 11.5 times and Mn^{2+} is 3 times as effective as Ca^{2+}. There was no activity in the presence of Na^+/K^+ alone. In order to study the possibility of a Na^+–K^+-stimulated ATPase, Mg^{2+} must also be present and assays are usually carried out in the presence

of an inhibitor of Mg^{2+}-dependent ATPase[32]. Both DCCD and ouabain were used as inhibitors of the Mg^{2+}-dependent ATPase (see below and Table V) and no stimulation of ATPase activity was found when Na^+/K^+ was added to the inhibited system. Thus we are clearly dealing with a Mg^{2+}-dependent ATPase.

The stoichiometry of the reaction (Table IV) was followed in the absence of both regenerating system and detergent since the effect of detergents on the ADP assay was not studied. After 30 and 60 min, aliquots were collected and assayed for P_i and ADP as described in Table IV and in MATERIALS AND METHODS. The correlation between moles of P_i and ADP suggest that ATP is being hydrolyzed stoichiometrically to P_i and ADP.

The effect of inorganic phosphate on the reaction was studied by following the hydrolysis of [^{3}H]ATP to [^{3}H]ADP when potassium phosphate buffer was added to the usual Tris buffer. The reaction was carried out in the absence of detergent and

TABLE III

EFFECT OF MONOVALENT AND DIVALENT CATIONS ON ATPase ACTIVITY

Concentration of cations in μmoles/ml: Mg^{2+}, 10; Ca^{2+}, 10; Mn^+, 10; Na^+, 200; Li^+, 100; Na^+/K^+, 10/2. Concentration of inhibitors in μmoles/ml: DCCD, 0.4; ouabain, 10. Specific activity in μmoles P_i/60 min per mg protein.

Cation	*Inhibitor*	*Specific activity*	%
Mg^{2+}	—	3.43	100
Ca^{2+}	—	0.30	8.7
Mn^{2+}	—	1.17	34.1
Mg^{2+} + Ca^{2+}	—	2.28	66.5
Mg^{2+} + Na^+	—	1.17	34.1
Mg^{2+} + Li^+	—	4.03	118
Mg^{2+}	—	2.36	100
Mg^{2+} + Na^+/K^+	—	1.94	82.2
Mg^{2+}	DCCD	0.788	33.4
Mg^{2+} + Na^+/K^+	DCCD	0.586	24.8
Mg^{2+}	Ouabain	2.12	89.8
Mg^{2+} + Na^+/K^+	Ouabain	1.88	79.6

TABLE IV

STOICHIOMETRY OF THE ATPase REACTION

Membrane aliquots were incubated under standard conditions except that the regenerating system and detergent were omitted. The volume of the incubation mixture was 0.5 ml. After the given time of incubation, the aliquot was chilled and adjusted to 0.2 M perchloric acid. After 10 min at 0°, the pH was adjusted to 7 with KOH and then the protein and potassium perchlorate were removed by centrifugation. Inorganic phosphate was measured on one aliquot and ADP was measured on another aliquot using the pyruvate kinase–lactate dehydrogenase coupled system.

Time (min)	*Membrane protein (μg/0.5 ml)*	P_i *(nmoles/0.1 ml)*	*ADP (nmoles/0.1 ml)*	$[P_i]/[ADP]$
30	180	27.0	20.1	1.34
60	180	42.0	47.0	0.90
30	360	55.0	51.8	1.07
60	360	69.6	60.0	1.16

TABLE V

EFFECT OF VARIOUS INHIBITORS AND ACTIVATORS ON ATPase ACTIVITY

The assays were carried out under standard assay conditions with Sinnopal NP-12 and regenerating system.

Conditions	*Volume methanol (µl)*	*Concentration of inhibitor (µmoles/ml)*	*µg inhibitor per mg membrane protein*	*µM P_i/60 min per mg protein*	*% of control*
Control	0	—	—	2.93	—
Methanol	1	—	—	3.09	—
Methanol	2	—	—	3.38	—
Methanol	10	—	—	3.36	—
Methanol	20	—	—	3.36	—
Ouabain	—	0.1	—	3.10	106.0
	—	1.0	—	2.37	80.8
Oligomycin	10	10	36.2	3.04	90.4
	20	20	72.4	2.21	65.8
Water-soluble carbodiimide*	—	2	—	2.36	80.7
	—	20	—	1.49	50.8
DCCD	1	0.2	—	0.90	29.1
	2	0.4	—	0.48	14.2
Protein inhibitor	—	36	180	2.85	97.2
	—	72	360	2.82	96.5
	—	114**	720	2.72	93.0
2,4-Dinitrophenol	1	0.2	—	3.47	112.5
	2	4	—	3.62	107.0

* 1-Cyclohexyl-3(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate.
** µg/ml.

regenerating system. In the control reaction, 15.0 % and 23.2 % ADP was formed in 30 and 60 min respectively. When 0.1 M potassium phosphate buffer (pH 6.6) was included, 19.0 % and 32.2 % ADP formed in 30 and 60 min respectively. Thus P_i does not inhibit the hydrolysis of ATP by this Mg^{2+}-dependent ATPase.

A number of inhibitors or activators of mitochondrial Mg^{2+}-dependent ATPase, *i.e.* coupling factor (F_1), were tested for their ability to inhibit our Pseudomonas ATPase (Table V). There was slight inhibition with ouabain at very high concentrations. Oligomycin also inhibited at very high concentrations or very high ratios of inhibitor to membrane protein. The water-soluble carbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate, was moderately effective as an inhibitor, while DCCD was far more inhibitory. The naturally occurring protein inhibitor of mitochondrial ATPase[33] was only slightly inhibitory, even at high ratios of protein inhibitor to membrane protein. 5 µg of this particular preparation of inhibitor was sufficient to inhibit 20 µg of yeast F_1 by greater than 90 % (A. TZAGOLOFF, personal communication). Finally, there was only a slight activation in the presence of 2,4-dinitrophenol.

Alkaline phosphatase: Alkaline phosphatase activity occurred in our membrane preparations. Although the initial rates of 21–31 nmoles/min per mg protein were similar to those sometimes found with ATPase, the reaction continued for only 8–10 min. No Mg^{2+} or other added cations were required. Furthermore, 0.1 or 0.2 µmole DCCD per ml caused the alkaline phosphatase activity to increase to 1.8

and 1.5 times that of the control, respectively. Therefore the alkaline phosphatase activity appears to be distinct from that of the Mg^{2+}-dependent ATPase.

Succinate dehydrogenase: This enzyme did not require Mg^{2+} for optimal activity and since the activity was actually enhanced in the presence of EDTA, no divalent cation appeared to be required (Table VI). KCN, which is a commonly used inactivator of cytochrome oxidase, slightly enhanced the activity of succinate dehydrogenase. Malonate, a competitive inhibitor of succinate dehydrogenase, effectively inhibited the Pseudomonas membrane-associated enzyme.

TABLE VI

PROPERTIES OF SUCCINIC DEHYDROGENASE

The assays were carried out under standard conditions (see MATERIALS AND METHODS).

Conditions	*nmoles/min per mg protein*	*% of control*
Control	11.68	—
+ 15 μmoles $MgSO_4$	11.36	97.3
Less KCN	9.76	83.6
+ 0.25 μmole EDTA	16.20	138.7
+ 0.50 μmole EDTA	16.36	140.0
+ 0.10 μmole sodium malonate	3.50	30.0
+ 1.00 μmole sodium malonate	2.12	18.2

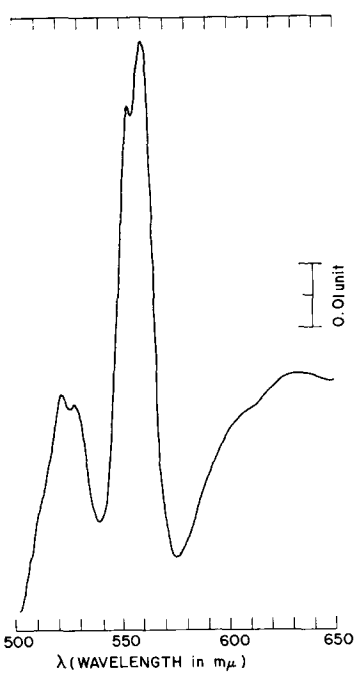

Fig. 5. Difference spectrum of Pseudomonas BAL-31 membranes, sodium hydrosulfite reduced *minus* potassium ferricyanide oxidized. The membranes were in 0.05 M Tris buffer (pH 7.4, 25°) with 0.5% sodium deoxycholate, and were scanned on the Cary 14 spectrophotometer.

NADH oxidase: The oxidation of NADH using molecular oxygen as electron donor occurred at a lower rate than either succinate oxidation or oxidation of NADH in an early step in electron transport (measured as NBT reductase). In one experiment, the specific activities of these three enzymes were: NADH oxidase, 3.3 nmoles/min per mg protein; NBT reductase, 680 nmoles/min per mg protein; and succinate dehydrogenase, 150 nmoles/min per mg protein. Thus the NBT reductase reaction was 206 times faster and the succinic dehydrogenase reaction was 45 times faster than NADH oxidase. In the experiments of Table I, the rate of the succinate dehydrogenase reaction was 10–14 times the rate of the NADH oxidase reaction.

So far we have no explanation for this discrepancy in reaction rates. In searching for some explanation we have investigated the cytochrome system associated with the membranes and the effect of a number of possible inhibitors of NADH oxidase activity.

(a) *Cytochromes*: Cytochromes were measured by difference spectroscopy. Fig. 5 shows the spectra of oxidized *versus* reduced membranes. In this case potassium ferricyanide was used to obtain complete oxidation and sodium hydrosulfite was used for reduction. A cytochrome *c* with α and β bands at 554 mμ and 522 mμ respectively, and a cytochrome *b* with α and β bands at 560 mμ and 527 mμ respectively, were the prominent features of this spectrum. Troughs were present at 577 mμ and 539 mμ. The broad band with a peak at about 626 mμ and a rather flat region between 620 mμ and 630 mμ may be due to a cytochrome a_2 type terminal oxidase[34]. Using oxygen rather than ferricyanide to oxidize the cytochromes, and sodium hydrosulfite to reduce, it was possible to demonstrate a sharp Soret band at 429 mμ. Using a slow scan, the peak appears skewed in the direction of longer wavelengths, but it was not

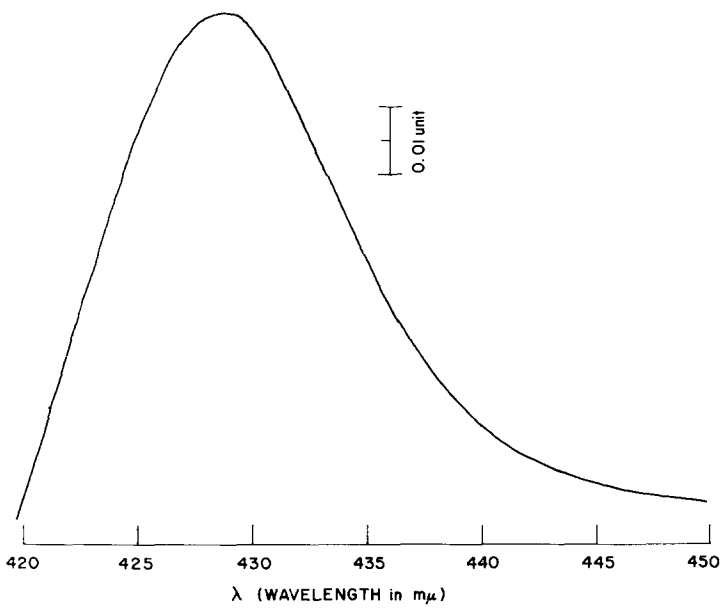

Fig. 6. Difference spectrum of Pseudomonas BAL-31 membranes, sodium hydrosulfite reduced *minus* oxygen oxidized run at a slow scanning speed (0.5 Å/sec) in the region of the Soret band; the buffer is described in the legend to Fig. 5.

possible to demonstrate individual Soret bands for cytochromes *b* and *c* (Fig. 6).

The CO-complex spectrum (Fig. 7) is characterized by two peaks at 570 mμ and 550 mμ with a trough at 431–432 mμ, typical of cytochrome *o* (see refs. 34, 35). The spectra for whole cells were similar to those for membranes.

(*b*) *NADH oxidase inhibition*: Overall oxidation was measured by the decrease in the fluorescence of NADH as it was converted to NAD. The rate of decrease in fluorescence remained linear only for 1 or 2 min and then started to decrease. This decrease was not due to depletion of the substrate, for when fresh NADH was added every 2 min to a deoxycholate extract of membrane, the following sequence of initial rates of oxidation (in nmoles/min per mg protein) were recorded: 0.825, 0.594, 0.561 and 0.417.

A number of compounds were tested for their effect on the initial (1–2 min) reaction rates of NADH oxidase, either as the membrane-bound complex or as the solubilized system. The oxidase proved almost completely resistant to sulfhydryl groups (BAL, 2-mercaptoethanol), a number of powerful inhibitors of electron transport in mitochondria (amytal, rotenone, Na_2S, KCN, antimycin), and the bacterial inhibitor *n*-heptylquinoline.

Oxidative capacity of whole cells

Because of the low level of NADH oxidase activity, the presumed requirement for aerobic growth conditions was reexamined. In standard broth with shaking, the

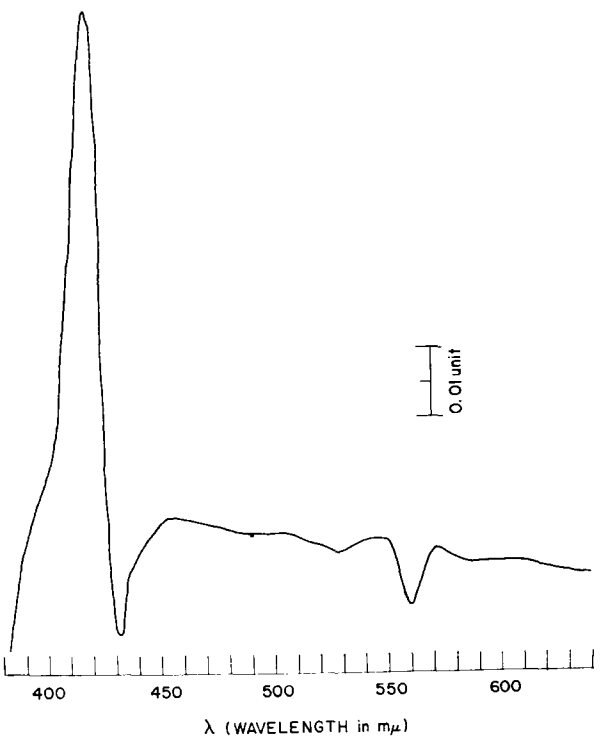

Fig. 7. Difference spectrum of Pseudomonas BAL-31 membranes, CO complex–sodium hydrosulfite reduced *minus* sodium hydrosulfite reduced. The buffer is described in the legend to Fig. 5.

doubling time was 48 min; in thioglycolate broth, it was 68 min; and in thioglycolate broth in a nitrogen atmosphere there was no growth whatsoever. Thus we confirmed the original report of ESPEJO AND CANELO that this organism is a strict aerobe[1]. In BAL synthetic medium, the division time was 2.3 h. In the presence of 0.001 M sodium azide the division time increased to 6.6 h, and in 0.01 M sodium azide or 0.001 M KCN the cells did not divide. Thus, although the NADH oxidase of the membrane-bound electron transport system appeared to be insensitive to azide or cyanide, the cells themselves were very sensitive.

DISCUSSION

Until recently[36,37], purification of membranes from Gram-negative bacteria has rarely involved complete removal of cell-wall material. Lysozyme produces spheroplasts by digesting the inner mucopeptide R-layer of the cell envelope, but the lipoprotein and lipopolysaccharide layers are not affected[27,30]. Both cell-wall material and particulate matter trapped within some of the membrane-micelles are undoubtedly responsible for the high density values obtained for our membrane preparations (*cf.* Fig. 2). Although lysozyme is clearly acting on the cell envelope (*cf.* Fig. 3), only about 50 % of the cells round up during treatment. This may be due to the presence of 1 M NaCl in the medium, which might prevent swelling of the cells and permit maintenance of cell shape despite the weakened cell wall.

Our characterization of some of the membrane-bound enzymes of Pseudomonas BAL-31 has concentrated on electron transport and Mg^{2+}-dependent ATPase. The ATPase has many of the characteristics of mitochondrial F_1, which couples oxidation and phosphorylation[38,39]. In common with F_1, Mg^{2+} is the most effective divalent cation for our ATPase, while Li^+ and Na^+ had no effect on the maximal rates of ATP hydrolysis in the presence of Mg^{2+}. As with F_1, ADP was inhibitory, but P_i was not[38]. The classical uncoupler, 2,4-dinitrophenol, stimulated ATPase activity slightly. DCCD and the water-soluble carbodiimide were the best inhibitors of our ATPase, while ouabain and oligomycin[40] were largely ineffective. DCCD was effective whether or not the membranes were disrupted by the detergent Sinnopal NP-12. Since the membrane-bound ATPase of *Streptococcus faecalis* is no longer inhibited by DCCD when it is released from the membrane[41], the non-ionic detergent NP-12 may not convert the ATPase into a soluble form. The naturally occurring inhibitor of mitochondrial ATPase[33] was only slightly inhibitory at high concentrations. Although we have not completely characterized this activity, it appears similar to the membrane-bound Mg^{2+}-dependent or Ca^{2+}-dependent ATPase found in a number of Gram-positive bacteria[42–45].

Alkaline phosphatase activity associated with the membranes was shown to be distinct from ATPase activity. As in *Escherichia coli*[46,47], this enzyme probably occurs between the cell envelope and the plasma membrane and therefore should not be considered a component of the plasma membrane.

The enzymes of the electron-transport system include the cytochromes and NADH oxidase. The cytochromes were characterized only by difference spectra and we cannot offer a complete classification of the electron transport proteins. The Pseudomonas BAL-31 cytochrome *c* has, in the reduced form, typical α and β bands at 554 mμ and 527 mμ, respectively. The Soret band at 429 mμ is characteristic of

cytochrome *b* (see ref. 48). The broad peak at 626 mμ is tentatively assigned to cytochrome a_2 (see refs. 34, 48). A slight peak in the CO difference spectrum at 455 mμ might represent the Soret band of a_2. The CO complex of cytochrome *o* (see ref. 49) has α, β and γ absorption peaks at 565–570, 535, and 416–418 mμ, respectively. We find a peak at 570 mμ and a Soret band at 416 mμ, but the β peak appears to be at 550 mμ rather than at 335 mμ. Nevertheless, it would appear that the CO spectrum is most representative of an *o*-type terminal oxidase[35]. In summary, we tentatively describe the electron transport system in Pseudomonas BAL-31 as o_{570}, a_{626}, b_{560}, and c_{554}.

NADH oxidase was characterized by a relatively low specific activity compared to succinate dehydrogenase and NBT reductase. Comparison with NBT reductase is particularly noteworthy since this enzyme activity utilizes NADH as hydrogen donor in an early stage of electron transport[50]. Although all of the components of the electron-transport system appear to be present in the membrane fraction, NADH oxidase activity was so low that it could only be followed by a sensitive fluorometric assay, or by using mg amounts of membrane protein in the oxygen electrode system. Furthermore, a number of compounds which inhibit the mitochondrial respiratory chain[40] were without effect on our oxidase activity. Since Pseudomonas BAL-31 is an aerobe, and NADH oxidase is an essential function, there may be a preferential loss or inactivation of this enzyme during membrane preparation, which requires a large number of washings and centrifugation steps. It is also possible that the NADH oxidase is uncoupled from the earlier steps in oxidation because of physical alterations in the membranes during preparation.

ACKNOWLEDGEMENTS

The authors are particularly grateful for the advice and discussions of Drs. M. Pullman, H. Penefsky, and A. Tzagoloff. Dr. Tzagoloff kindly provided us with a sample of the protein inhibitor of mitochondrial F_1. This work was supported in part by U. S. Public Health Service Grant AI-07645 from the National Institute of Allergy and Infectious Diseases. J. E. D. was the recipient of National Cancer Institute Postdoctoral Fellowship No. 5 FO2-CA-31, 368-02. S. N. B. is a New York University School of Medicine M.D.–Ph.D. Fellow, supported by U. S. Public Health Service Medical Scientist Training Grant 5 TO5 GMO 1668 06.

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