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OXIDATIVE PHOSPHORYLATION IN *MICROCOCCUS DENITRIFICANS*

I. PREPARATION AND PROPERTIES OF PHOSPHORYLATING MEMBRANE FRAGMENTS

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SUMMARY

A procedure was described to prepare stable membrane fragments from aerobically grown cells of *Micrococcus denitrificans*. This preparation contained flavins, cytochromes *b*, *c*, *a* and *o*, and catalyzed the synthesis of ATP coupled to the oxidation of NADH and succinate. The P:O ratios were about 1.0 for NADH and 0.4 for succinate oxidation. The electron-transfer pathways responsible for these oxidations were similar to, though not identical with, those of mammalian mitochondria in their construction and sensitivity to inhibitors. Oxidative phosphorylation by the membrane fragments was uncoupled by the usual uncouplers and energy-transfer inhibitors, though 2,4-dinitrophenol was much less effective and higher concentrations of oligomycin and tributyltin chloride were required for complete inhibition as compared with the mitochondrial system. Oleate also caused uncoupling, which was relieved by serum albumin. Treatment with high concentrations of LiCl yielded an essentially uncoupled preparation, but this treatment as well as many other procedures failed to yield soluble coupling factors. Unlike the mitochondrial ATPase activity, ATP hydrolysis by the membrane fragments was inhibited to about 50 % by uncouplers and energy-transfer inhibitors. It seems that the bacterial preparation possessed two types of ATPase, one of which was sensitive to these reagents as well as to LiCl treatment and probably to high concentrations of ADP. The advantage of this preparation for the study of the mechanism of oxidative phosphorylation is discussed.

INTRODUCTION

Although oxidative phosphorylation has been studied mostly with mammalian mitochondria, important contributions have also come from studies on bacteria. For example, the requirement of soluble coupling factors for oxidative phosphorylation was first recognized in bacterial systems[1–4] and later has been extended to disrupted mitochondria[5,6]. Studies on high-energy intermediates of phosphorylation were also

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide trifluoromethoxyphenylhydrazone; NOQNO, 2-*n*-nonylhydroxyquinoline-*N*-oxide; PMS, phenazine methosulfate; TMPD, tetramethyl-*p*-phenylenediamine.

initiated on bacterial preparations[7,8]. However, the use of bacterial systems for the study of oxidative phosphorylation is disadvantageous in that our knowledge of the respiratory chain and the energy-transfer mechanism in these systems is still limited.

ASANO AND BRODIE[9–11] have elucidated the constitution of the respiratory chain and the approximate sites of phosphorylation in an obligate aerobe, *Mycobacterium phlei*, and revealed their close resemblance to those of mammalian mitochondria, though several important differences were noticed. To extend this line of approach to other types of bacteria, we have selected *Micrococcus denitrificans* as material in the present work. This organism grows not only aerobically in synthetic media but also anaerobically with nitrate as oxidant[12] and even autotrophically under appropriate conditions[13,14]. This organism therefore offers an opportunity to study variations in phosphorylation mechanism under widely different growth conditions. Another advantage is that the cytochrome system of nitrate-grown cells of this organism has been studied in detail[14–17]. Furthermore, VERNON AND WHITE[17] have reported the isolation of a phosphorylating preparation from this organism, though this system has not yet been characterized.

This paper describes a procedure for preparing stable phosphorylating membrane fragments from aerobically grown cells of *M. denitrificans* and reports some of their basic properties. The preparation exhibited P:O ratios higher than 1.0 with NADH and about 0.4 with succinate as substrates. The effects of electron-transfer inhibitors, uncouplers, and energy-transfer inhibitors on this system were similar to those observed with mitochondria. However, the ATPase activity of the membrane fragments was considerably different from that of mitochondria. This work has been preliminarily reported elsewhere[18,19].

MATERIALS AND METHODS

Preparation of phosphorylating membrane fragments

A strain of *M. denitrificans*, kindly supplied by Dr. J. LASCELLES of Oxford University, was grown aerobically for 16 h in a chemically defined medium described by CHANG AND MORRIS[12] with succinate as carbon source and NH_4Cl as nitrogen source. The cells were harvested at late log phase, washed with cold water, weighed and then washed with a medium ("sonication medium") containing 0.5 M sucrose, 20 mM $MgCl_2$ and 50 mM Tris–HCl buffer (pH 7.4). The washed cells were suspended in the sonication medium (10 g wet cells per 10 ml), and sonicated for 2 min in a Kubota 10-kcycles/sec sonic disintegrator with maximum output. The sonication vessel was cooled by circulating ice water. After removal of cell debris by centrifugation at $15000 \times g$ for 25 min, membrane fragments were sedimented by centrifuging in a Hitachi Model 40P ultracentrifuge at $105000 \times g$ for 90 min. The precipitate was suspended in either 0.5 M sucrose containing 10 mM $MgCl_2$ and 50 mM Tris–HCl buffer (pH 7.4), or 0.15 M KCl containing 10 mM $MgCl_2$ and 50 mM Tris–HCl buffer (pH 7.4), and the suspension was recentrifuged at $105000 \times g$ for 60 min. The precipitate was suspended in 0.5 M sucrose containing 10 mM $MgCl_2$ and 10 mM Tris–HCl buffer (pH 7.4) and used as membrane fragments, which could be stored for several weeks at $-15°$ without appreciable loss of the phosphorylative activity.

Chemicals and biochemicals

Crystalline yeast alcohol dehydrogenase (EC 1.1.1.1) was a gift from Dr. A.

YOSHIMOTO. Crystalline hexokinase (EC 2.7.1.1) was obtained from Sigma Chemical Co., or kindly supplied by Professor B. HAGIHARA. Adenine nucleotides, NAD^+, NADH and 2-*n*-nonylhydroxyquinoline-*N*-oxide (NOQNO) were obtained from Sigma Chemical Co. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and carbonyl cyanide trifluoromethoxyphenylhydrazone (FCCP) were kindly supplied by Dr. P. G. HEYTLER of DuPont de Nemours Co. Antimycin A, rotenone, tributyltin chloride and oligomycin were gifts from Dr. S. KINOSHITA, Dr. S. MATSUNAKA, Professor B. HAGIHARA and Dr. T. KAWASAKI, respectively. Octylguanidine and 2-ethylhexylguanidine were synthesized and recrystallized five times[20].

Measurement of oxidation and coupled phosphorylation

A conventional Warburg manometer was used. The main compartment of each vessel contained washed membrane fragments (1.5–3.0 mg protein), 10 μmoles of $MgCl_2$, 12 μmoles of P_i, 190 μmoles of sucrose, 20 μg of crystalline yeast hexokinase, 30 μmoles of glucose, 1 μmole of EDTA, 10 μmoles of Tris–HCl (pH 7.4), and water to a final volume of 1.0 ml. Crystalline yeast alcohol dehydrogenase (300 μg) and 10 μmoles of semicarbazide were added when the NADH-generating system was employed. The side arm of the vessel contained 2.5 μmoles of ATP and 1 μmole of NAD^+ *plus* 40 μmoles of ethanol or 25 μmoles of succinate. Tetramethyl-*p*-phenylenediamine (TMPD) and phenazine methosulfate (PMS) were added to the side arm when used. The reaction was started by tipping, and O_2 consumption was measured for 20–25 min at 30° and terminated by the addition of 1.0 ml of cold 10 % trichloroacetic acid. P_i in the deproteinized supernatant was determined by the method of FISKE AND SUBBAROW[21].

Assay of enzymatic activities

NADH oxidation was also measured spectrophotometrically. Experiments were carried out in cuvettes of 1 cm light path, and the measurements were made in a Cary Model 14 recording spectrophotometer at 20–25°. The assay system usually consisted of 100 μmoles of Tris–HCl (pH 7.4), 20 μmoles of $MgCl_2$, 500 μmoles of sucrose, washed membrane fragments (160–300 μg protein) and water to a final volume of 2.0 ml. Reaction was started by the addition of 0.3 μmole of NADH, and followed at 340 mμ. NADH oxidation by fumarate was measured in the system described above except that 1.25 μmoles of KCN and 1–3 mg protein of the membrane fragments were added to the system. Reaction was started by the addition of 10 μmoles of fumarate. ATPase (EC 3.6.1.3) activity was measured with a system consisting of 50 μmoles of Tris–HCl (pH 7.4 or 8.2), 10 μmoles of $MgCl_2$, 190 μmoles of sucrose, washed membrane fragments (0.5–1.5 mg protein) and water to a final volume of 1.0 ml. Reaction was started by the addition of 2.5 μmoles of ATP. After incubation for 20 min at 30°, it was terminated by the addition of 1.0 ml of 10 % trichloroacetic acid; the P_i liberated was determined in the deproteinized supernatant.

Other determinations

Protein was determined by the biuret method as modified for mitochondria[22]. Difference spectra were determined in a Cary Model 14 recording spectrophotometer.

360-mμ-light irradiation

Irradiation of the membrane fragments by ultraviolet light at 360 mμ was

accomplished with a combination of a Toshiba model SHLS-1002 mercury lamp and a Toshiba ultraviolet-D2 filter which had a transmission maximum at 360 mμ with 77 % efficiency and no transmission below 295 mμ and above 415 mμ. The preparation was irradiated for 30–60 min from a distance of 10 cm. The protein concentration employed for irradiation was 3–6 mg/ml.

Electron microscopy

Electron-microscopic observation of intact cells and isolated particles was carried out essentially as described by KELLENBERGER, RYTER AND SECHAND[23]. The materials fixed in a cold 1 % OsO_4–veronal–acetate buffer (pH 6.1) were dehydrated with ethanol of increasing concentration and embedded in methacrylate resin. After sectioning with a LKB microtome, the thin sections were observed with a Japan Electron Optics' electron microscope at 80 kV.

Determination of flavin contents

FAD and FMN (*plus* riboflavin) contents of isolated particles were determined fluorimetrically according to the method described by BURCH[24].

Determination of pyridine nucleotides

The amounts of bound pyridine nucleotides were determined fluorimetrically as described by PURVIS[25].

Treatments of membrane fragments with various reagents and enzymes

Sonication of membrane fragments in the presence of several reagents was performed at 10 kcycles/sec for 2 min. Enzymatic treatments were performed at 30°. After these treatments, particles were washed once with the sucrose–$MgCl_2$–Tris solution by centrifugation and resuspended with the same buffer solution for assays.

RESULTS

Preparation of phosphorylating membrane fragments

After a preliminary search, the procedure described in MATERIALS AND METHODS was adopted to prepare phosphorylating membrane fragments from aerobically grown cells of *M. denitrificans*. A change in pH of sonication medium (20 mM buffer) from 7.4 to 9.5 had little effect on the quality of the resultant preparation, though the use of acid pH's (5.0–6.5) resulted in lower P:O ratios. The cell concentration (wet cells:medium, 0.5–2, w/v) and presence of Mg^{2+} (0–20 mM) during sonication showed no appreciable effects. However, the concentration of sucrose in the medium as well as sonication time were important. The phosphorylating efficiency of the preparation was maximal when sonicated for not more than 2 min in a medium containing 0.5 M sucrose. Replacement of sucrose by KCl resulted in a preparation showing lower P:O ratios. The growth phase of the cells affected the results only slightly, though the cells from late stationary phase yielded poorer preparations.

Respiratory components

The difference spectra shown in Fig. 1 indicate the presence in the membrane fragments of cytochromes of the *b*, *c*, *a* and *o* types. The presence of an *a*-type cyto-

chrome was evident from a small band at 605 mμ and a shoulder at 445 mμ in the reduced *minus* oxidized difference spectrum as well as from a trough at 443 mμ in the CO difference spectrum. The peak at 421 mμ in the CO difference spectrum further showed the presence of a pigment probably of the *o* type[26].

Assuming that these pigments possessed the same molar extinction coefficients as mitochondrial cytochromes, their concentration in the membrane fragments was

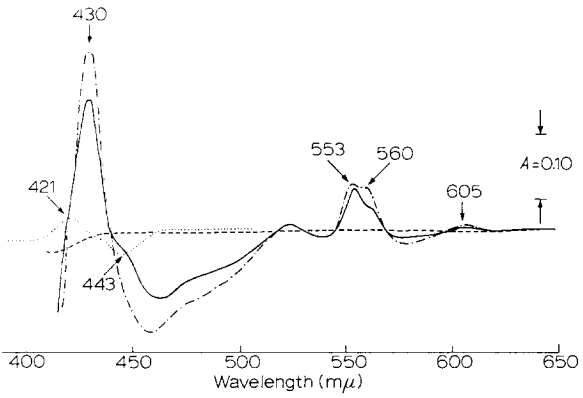

Fig. 1. Difference spectra of the membrane fragments of *M. denitrificans*. 3.0 ml each of the mixture consisting of 200 μmoles of Tris–HCl buffer (pH 7.4), 40 μmoles of $MgCl_2$, 50 μmoles of sucrose and 9.4 mg protein of the membrane fragments were placed in both the sample and reference cuvettes (optical path, 1.0 cm). Succinate (25 μmoles) was added to the sample cuvette, and the reduction of cytochromes was followed at 430 mμ until the O_2 in the medium was exhausted. The spectral difference between the two cuvettes was then recorded (succinate-reduced *minus* oxidized). Dithionite was then added to the sample cuvette and the difference spectrum was again recorded (dithionite-reduced *minus* oxidized). For the measurement of CO difference spectra, both the sample and reference cuvettes were treated with dithionite and a stream of O_2-free CO was bubbled into the sample cuvette for about 2 min. –––––, base line; ———, succinate-reduced *minus* oxidized; –·—·–, dithionite-reduced *minus* oxidized; ·····, reduced *plus* CO *minus* reduced.

TABLE I

CONTENTS OF RESPIRATORY AND OTHER COMPONENTS IN MEMBRANE FRAGMENTS OF *M. denitrificans*

Cytochrome contents were calculated from experiments similar to that shown in Fig. 1, and molecular extinction coefficients employed were the same as those applied for the other bacterial system[9]. Phospholipids and RNA contents were determined as described previously[52]. DNA content was determined by DISHE's method[53]. The other determinations were performed as described in MATERIALS AND METHODS.

Component	*Content (mμmoles/mg protein)*
NAD^+	0.015
$NADP^+$	0.055
FAD (acid-extractable)	0.50
FMN	0.50
Cytochrome *c*	0.53
Cytochrome *b*	0.47
Cytochrome *a*	0.035
Phospholipid	85 μg/mg protein
DNA	7 μg/mg protein
RNA	150 μg/mg protein

calculated from Fig. 1. The results obtained are shown in Table I, together with the contents of other components. While the concentrations of FAD, FMN, and cytochromes *b* and *c* were comparable, that of the *a*-type pigment was exceedingly low. This might be related to the presence of another terminal oxidase, cytochrome *o*. The content of pyridine nucleotides was very low, as in the case of submitochondrial particles. Correspondingly, most of the pyridine nucleotide-linked dehydrogenases were detected only in the soluble fraction.

Electron-microscopic observation

Fig. 2 shows an electron micrograph of thin-sectioned membrane fragments. It can be seen that the preparation consisted mostly of profiles of membraneous nature. Contamination by cell-wall-like materials and ribosomes was also evident; this may be responsible for the high RNA content of the preparation (Table I).

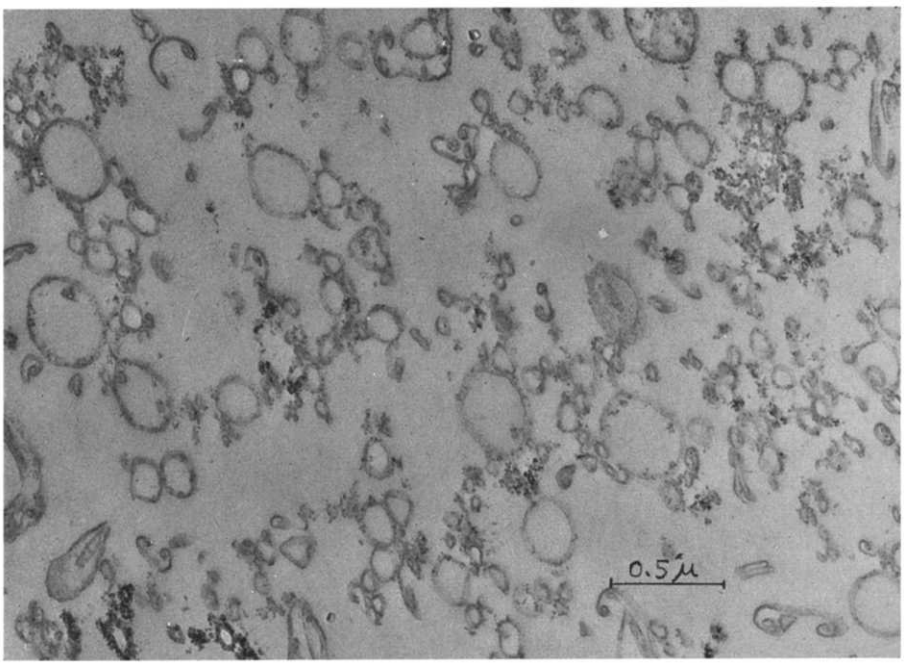

Fig. 2. Electron micrograph of a thin section of membrane fragments of *M. denitrificans*.

Electron-transfer pathway

Fig. 3 shows that the NADH oxidase activity of the membrane fragments was strongly inhibited by such inhibitors of the mitochondrial respiratory chain as cyanide, sulfide, rotenone and NOQNO; only partial inhibition was attained by antimycin A even at relatively high concentrations. Ultraviolet irradiation of the preparation (0.7 mg/ml) at 360 mμ for 30 and 60 min caused 75 and 95 % inhibition, respectively, of the NADH oxidase activity; vitamin K_1 dispersed with Asolectin[27] could not restore the inactivated activity. The irradiation, however, did not inhibit the NADH–

coenzyme Q_0 reductase* activity appreciably. The quinone present in the membrane fragments was recently identified as coenzyme Q_{10}. The addition of NADH or succinate caused its reduction as in the mitochondrial system[28]. Direct estimation of coenzyme Q_{10} by the method of PUMPHREY AND REDFEARN[29] showed that ultraviolet irradiation at 360 mμ resulted in a destruction of the quinone. These observations will be described in detail in a later publication. Dicumarol (5 mM) inhibited the NADH oxidation by only 26 %. The succinate oxidase activity was similarly inhibited by cyanide, NOQNO and antimycin A, but was insensitive to rotenone. This activity was also inhibited by thenoyltrifluoroacetone (41 % inhibition at 1.7 mM).

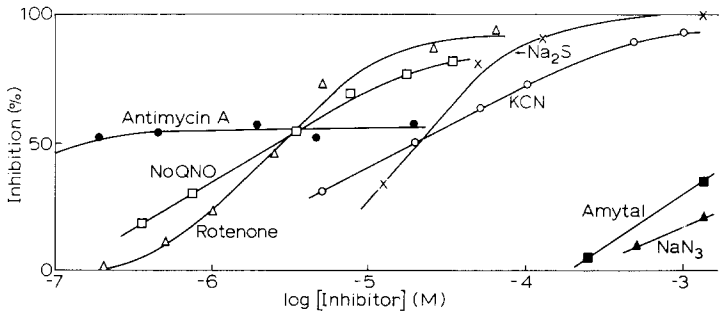

Fig. 3. Effects of inhibitors on NADH oxidation. The reaction system employed was the same as described in MATERIALS AND METHODS, except that 0.73 mg protein of the membrane fragments was used and indicated concentrations of various inhibitors were added.

The addition of antimycin A to an aerobic suspension of the membrane fragments in the presence of NADH or succinate caused rapid reduction of cytochrome *b*, keeping the other cytochromes in the oxidized state. It was therefore concluded that this antibiotic inhibits the respiratory chain between cytochrome *b* and the next pigment (probably cytochrome *c*) of the chain. No cytochromes were reduced in the presence of rotenone and the addition of succinate to this system caused reduction of all cytochromes in the cyanide-treated preparation. Thus it was clear that the site of action of rotenone is located between NADH and cytochrome *b*.

From these findings it appears that the electron-transfer pathway as well as the sites of inhibitor actions in the membrane fragments of *M. denitrificans* may be similar to those in mammalian mitochondria[30].

Oxidation and phosphorylation with various substrates

Of various substrates tested only succinate, NADH and, though slowly, L-lactate were oxidized by the membrane fragments; no O_2 uptake was observed with D-lactate, β-hydroxybutyrate, formate, glutamate, pyruvate and NADPH as substrates. As shown in Table II, phosphorylation was coupled to oxidation of NADH and succinate, but not to that of L-lactate. P:O ratios obtained with NADH as substrate were 0.8–1.4; higher values than 1 were obtained with preparations from small-scale cultures. With succinate as substrate, P:O ratios were always lower and ranged from 0.3 to 0.5. Although ascorbate in the presence of TMPD or PMS could also support a considerable uptake of O_2, no phosphorylation was coupled to this

* NADH–coenzyme Q_0 reductase activity was measured as described for NADH oxidation except that 2.5 μmoles of KCN and 0.5 μmole of coenzyme Q_0 (in 0.02 ml methanol) were added. Details of the properties of the reductase will be published elsewhere.

oxidation. Difference spectrophotometry indicated that ascorbate–TMPD could reduce cytochrome *c* in the preparation. It may therefore be tentatively concluded that phosphorylation sites are located at the NADH–coenzyme Q and coenzyme Q–cytochrome *c* regions, but the cytochrome *c*–O_2 region is not coupled to ATP formation. However, the possibility could not be excluded that the electrons from ascorbate–TMPD enter the chain after the phosphorylation site at the terminal region.

Requirements for oxidative phosphorylation

As shown in Table III, phosphorylation coupled to NADH oxidation by the membrane fragments was greatly decreased, without appreciably affecting the oxidation rate, when either one component of the phosphate acceptor system (ATP, hexokinase, and glucose) was omitted. Some residual phosphorylation in the absence

TABLE II

EFFICIENCY OF OXIDATIVE PHOSPHORYLATION WITH VARIOUS SUBSTRATES

The conditions employed were as described in MATERIALS AND METHODS except that 20 μmoles of L-lactate or 12.5 μmoles of ascorbate (pH 7.4) *plus* 0.2 μmole of TMPD were used as substrate when indicated.

Substrate	*O_2 uptake (μatom O/min per mg protein)*	*Phosphate uptake (μmole P_i/min per mg protein)*	*P:O*
NADH	0.098 ± 0.017*	0.100 ± 0.019*	1.02 ± 0.205*
Succinate	0.167 ± 0.036*	0.068 ± 0.014*	0.40 ± 0.054*
L-Lactate	0.018	0.00	0.00
Ascorbate–TMPD	0.095	0.00	0.00

* Mean values obtained in 11 experiments ± S.D. of the mean.

TABLE III

REQUIREMENTS FOR OXIDATIVE PHOSPHORYLATION BY THE MEMBRANE FRAGMENTS

The conditions employed were as described in MATERIALS AND METHODS except that 2.70 mg protein of the membrane fragments were used in Expt. 1, and 3.60 mg protein of the membrane fragments and 3.45 mg protein of dialyzed supernatant fraction were used in Expt. 2. The duration of the reaction was 20 min in both experiments. NADH (generated by ethanol and alcohol dehydrogenase) was used as substrate.

Expt. No.	*System*	*O_2 uptake (μatoms O/20 min)*	*Phosphate uptake (μmoles P_i/20 min)*	*P:O*
1	Complete	6.22	4.49	0.72
	minus ATP, *plus* AMP	7.61	5.80	0.76
	minus ATP, *plus* ADP	6.89	5.12	0.75
	minus ATP	6.64	1.47	0.22
	minus hexokinase	6.90	0.10	0.01
	minus glucose	6.51	0.87	0.13
	minus ATP, hexokinase, glucose	5.97	0.41	0.07
	minus EDTA	6.82	3.82	0.56
	plus KF, 20 mM	5.66	3.09	0.55
	plus serum albumin, 1%	6.36	3.83	0.60
2	Complete	5.49	6.32	1.15
	plus dialyzed supernatant	6.36	7.48	1.17

of added adenine nucleotides could probably be due to endogenous nucleotides. In fact, the level of acid-soluble nucleotides, as measured from the absorbance at 260 mμ was more than six times the total amount of pyridine nucleotides and flavins present in the membrane fragments. AMP was as effective as ADP as phosphate acceptor, probably because of the presence of adenylate kinase in the system. Omission of EDTA from the complete system caused slight decrease in phosphorylation efficiency. The addition of serum albumin had no stimulatory effect. Sucrose in the complete system could be replaced by mannitol or KCl without impairing the phosphorylation efficiency, and mannose was as effective as glucose as phosphate acceptor. Table III also includes data showing that the soluble fraction of the cells caused no appreciable effects on both the oxidative and phosphorylative activities of the membrane fragments. It was thus clear that the membrane fragments were a self-contained unit for these activities.

As in the mitochondrial system[31–33], the time courses of NADH oxidation and coupled phosphorylation show that P:O ratios apparently higher than normal values were obtained at the initial stage of reaction. However, normal values were established after 15 min of incubation. The P:O ratios reported in this paper were all determined after 20–30 min of reaction.

Effects of uncouplers and energy-transfer inhibitors

Uncouplers and energy-transfer inhibitors of mitochondrial phosphorylation such as pentachlorophenol, CCCP, and FCCP were found to inhibit phosphorylation coupled to NADH oxidation by the membrane fragments almost as effectively as with the mitochondrial system (Fig. 4). The sensitivity of the bacterial system to 2,4-dinitrophenol, arsenate, tributyltin chloride, and oligomycin was considerably lower than that of mitochondria (Fig. 4 and Table IV). Inhibition by oligomycin was dependent on pH; 50 μg of the antibiotic per tube caused 40 % inhibition at pH 7.4, whereas 72 % inhibition could be attained at pH 8.6. All the reagents tested did not inhibit the NADH oxidase activity at concentrations sufficient to inhibit phosphorylation. The membrane fragments could, therefore, be regarded as a loosely coupled system. Neither 0.1 mM octylguanidine, 0.1 mM 2-ethylhexylguanidine nor 5 mM Ca^{2+} affected oxidation or phosphorylation.

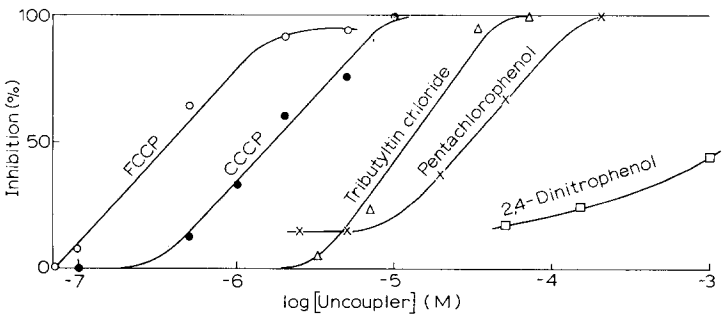

Fig. 4. Effects of uncouplers on the oxidative phosphorylation. The conditions employed were as described in MATERIALS AND METHODS except that uncouplers were added as indicated.

Attempts to remove coupling factors from membrane fragments

In attempts to resolve the system into a soluble coupling factor or factors and an insoluble fraction deficient in the factor(s), the membrane fragments were treated

under various conditions, some of which have been successfully applied to mitochondria. However, none of the treatments tested, except for the two methods which will be described below, caused any appreciable dissociation of oxidation from phosphorylation; the two activities were affected to similar extents by such treatments as digestion with phospholipase C, steapsin (see ref. 34 in which trypsin was used), and Nagarse, and sonication in the presence of Asolectin[35], EDTA (ref. 36), urea *plus* EDTA (ref. 34), ammonia[35], and acetic acid.

TABLE IV

EFFECTS OF ARSENATE AND OLIGOMYCIN ON OXIDATIVE PHOSPHORYLATION COUPLED WITH NADH OXIDATION

The conditions employed were as described in MATERIALS AND METHODS except that 10 μmoles of Tris–HCl buffer (pH 8.6 instead of pH 7.4) were used in case of oligomycin inhibition. Uncoupling by arsenate was measured by a radioactive phosphate assay essentially as described by NIELSEN AND LEHNINGER[54]. The amount of membrane fragments used was 0.52 mg protein in Expt. 1 and 2.68 mg protein in Expt. 2, respectively.

Expt. No.	*Arsenate concentration (mM)*	*Oxidation (% of control)*	*P:O (% of control)*
1	3	100	92
	10	106	75
	30	112	53
	Oligomycin concentration (μg)		
2	10	99	86
	20	98	63
	50	80	28

One of the two procedures causing clearly selective inactivation of phosphorylation was treatment with high concentrations of LiCl. As shown in Table V, 2.5 M LiCl inactivated the phosphorylation activity and CCCP-sensitive ATPase activity almost completely and at the same time caused slight stimulation of the NADH and succinate oxidase activities. However, phosphorylation by the treated preparation could not be restored by soluble fractions obtained from the membrane fragments by several different procedures, even though energy-linked functions of the treated preparation were still operative[37]. Unlike the lithium salt, other neutral salts such as KSCN, KI and RbCl inhibited both oxidation and phosphorylation and KCl had no effect on either process.

Treatment of the membrane fragments with snake venom containing phospholipase A also uncoupled oxidative phosphorylation. In this respect, it was of interest that oleate, a product of phospholipase action, acted as an effective uncoupler and this uncoupling could be reversed by serum albumin. Lysolecithin also produced a similar uncoupling effect.

ATPase of membrane fragments

The membrane fragments showed an ATPase activity at pH 7.4 and in the presence of 10 mM Mg^{2+}, conditions employed for the assay of oxidative phosphorylation. This activity was much lower than that of usual mitochondrial preparations. When Mg^{2+} was omitted, the ATPase activity was increased by about 60 %. It was found, contrary to the case of mitochondrial ATPase, that ATP hydrolysis by the membrane fragments was inhibited, rather than stimulated, by uncouplers such as pentachlorophenol and CCCP (Table VI). It was also inhibited by energy-transfer

TABLE V

EFFECTS OF HIGH CONCENTRATIONS OF LiCl ON OXIDATIVE PHOSPHORYLATION AND ATPase ACTIVITY

Treatment with LiCl of the membrane fragments was performed as follows: the membrane fragments suspended in the usual Tris–Mg^{2+}–sucrose medium were mixed with 5 M LiCl (pH 7.4) at 0° to give final concentrations as indicated. The LiCl-treated membrane fragments were then sedimented by centrifugation at 105000 × *g* for 90 min, resuspended in the Tris–Mg^{2+}–sucrose medium, and centrifuged again. The washed precipitate was resuspended in the same medium and used for determinations of oxidative phosphorylation and ATPase activity.

Substrate	*Activity*	*LiCl concentration (M)*			
		0.0	*1.5*	*2.0*	*2.5*
NADH	Oxidation (μatoms/20 min per mg protein)	2.71	3.92	4.07	3.80
NADH	Phosphorylation (μmole/20 min per mg protein)	1.74	0.91	0.76	0.07
NADH	P:O ratio	0.64	0.23	0.19	0.02
Succinate	Oxidation (μatoms/20 min per mg protein)	1.23	1.78	1.97	1.68
Succinate	Phosphorylation (μmole/20 min per mg protein)	0.48	0.13	0.00	0.07
Succinate	P:O ratio	0.34	0.07	0.00	0.04
ATPase (mμmoles/min per mg protein)		9.1	4.3	4.4	—

TABLE VI

EFFECTS OF INHIBITORS ON ATPase ACTIVITY

The conditions employed were as described in MATERIALS AND METHODS except that inhibitors were added as indicated.

Addition	*Concentration (M)*	*Relative activity*
None	—	100
CCCP	$1 \cdot 10^{-5}$	47
FCCP	$2 \cdot 10^{-5}$	44
Tributyltin chloride	$7 \cdot 10^{-5}$	43
Pentachlorophenol	$5 \cdot 10^{-4}$	41
2,4-Dinitrophenol	$1 \cdot 10^{-3}$	61
Chlorpromazine	$1 \cdot 10^{-3}$	41
Oligomycin	25 μg/ml (pH 8.2)	34
NOQNO	$7 \cdot 10^{-5}$	32
NOQNO	$3.5 \cdot 10^{-5}$	63
Antimycin A	$1.8 \cdot 10^{-5}$	92
Rotenone	$1.3 \cdot 10^{-4}$	107

inhibitors such as tributyltin chloride and oligomycin. These inhibitions were, however, only partial; 50–60 % inhibition was attained by all the reagents tested, except for 2,4-dinitrophenol, at concentrations causing maximal uncoupling of oxidative phosphorylation. No further inhibitions were observed even when the inhibitor concentrations were increased. It seemed that the preparation contained two types of ATPase, one sensitive and the other resistant to uncouplers. Using CCCP as inhibitor, it was found that the inhibition of 'sensitive' ATPase paralleled the uncoupling of oxidative phosphorylation (Fig. 5). This parallelism suggested a close interrelationship between the 'sensitive' ATPase and phosphorylation mechanism.

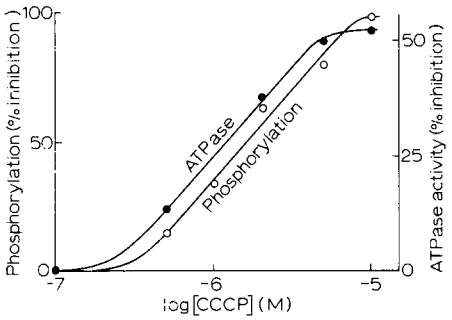

Fig. 5. Effects of CCCP on phosphorylation coupled with NADH oxidation and ATPase activity of the membrane fragments. Conditions employed were as described in MATERIALS AND METHODS except that CCCP was added as indicated and the amount of the membrane fragments used was 2.7 mg protein for both phosphorylation and ATPase assays.

Inhibitors of the electron-transfer pathway such as antimycin A and rotenone had no effect on ATP hydrolysis, whereas NOQNO inhibited both ATPase and electron transfer (Table VI). Treatment of the preparation with LiCl (Table V) also inhibited the 'sensitive' ATPase activity as well as oxidative phosphorylation. On the contrary, oleate stimulated ATP hydrolysis at concentrations which were inhibitory for phosphorylation. The presence of ADP during the reaction inhibited ATP hydrolysis as reported for mitochondria[38]; about 50 % inhibition was observed by 1–3 mM ADP.

DISCUSSION

The membrane fragments prepared from aerobically grown cells of *M. denitrificans* possess a number of features which are suitable for the study of the mechanism of oxidative phosphorylation. First, the phosphorylation efficiency of this preparation is higher than that of most other bacterial systems so far studied, though the P:O ratios observed are slightly lower than those of *M. phlei* preparations[9]. Second, its phosphorylation capacity is very stable. It can be stored at −15° for at least 3 months without appreciable loss of activity. Third, the system is self-contained; in other words, it does not require the addition of soluble factors for oxidative phosphorylation. Such an autonomy provides a good opportunity for the study of the mechanism of oxidative phosphorylation without serious disturbance by enzymes in soluble fraction, such as electron-transfer bypass enzymes, ATPase, *etc.* Finally, the electron-transport and energy-transfer systems of the preparation are characterized to such an extent that several specific inhibitors can be used as tools for further studies.

From the results reported in this paper it seems that the constitution and sensitivity to inhibitors of the electron-transport system of the bacterial preparation are rather similar to those reported for mammalian mitochondria, except for the O_2 side of cytochrome *c*. That the similarity is more extensive than expected from the present data will be described in a later publication. The electron-transport system of *M. denitrificans* preparation is, however, somewhat dissimilar to those of *M. phlei*[9] and *M. lysodeikticus*[39,40]. While the terminal region of the respiratory chain of *M. denitrificans* contains cytochrome *o* as well as a pigment of the *a* type, those of mammalian mitochondria and *M. phlei* contain only cytochrome $a + a_3$. This difference may be related to the lack of phosphorylation at this site in *M. denitrificans*. In contrast to the cases of most of the other bacterial systems[1–4,39,41–43], the entire energy-transfer system of *M. denitrificans* is firmly attached to the membrane fragments. In this respect, oxidative phosphorylation of the *M. denitrificans* system is similar to photophosphorylation[44] and oxidative phosphorylation[45,46] of chromatophores of a photosynthetic bacterium, *Rhodospirillum rubrum*. The energy-transfer system of the *M. denitrificans* preparation further resembles the mitochondrial system in the sensitivity to uncouplers and energy-transfer inhibitors, though the bacterial system requires higher concentrations of some of these reagents. The *M. denitrificans* membrane fragments, however, appear to contain at least two types of ATPase, one sensitive to energy-transfer inhibitors and the other insensitive. Uncouplers of the mitochondrial energy conservation also seem to be inhibitory to the former ATPase, and a parallelism can be observed between the uncoupling of oxidative phosphorylation and inhibition of the ATPase by these reagents. A similar parallelism also exists between the LiCl-sensitive ATPase activity and the LiCl-sensitive energy-transfer reactions. These facts, though indirect, strongly suggest the participation of a factor with an ATPase activity in the energy-transfer mechanism of the bacterial system. The ATPase activity of the membrane fragments is, however, much lower than that of disrupted preparation of mammalian mitochondria. Another difference is that the bacterial ATPase activity is inhibited, rather than stimulated, by the uncouplers of mitochondrial oxidative phosphorylation. Therefore, most of these uncouplers should be classified as energy-transfer inhibitors for the bacterial system, rather than as true uncouplers. The lack of respiratory control is a feature common to various bacterial preparations. But the procedures employed for preparation of those bacterial systems are quite similar to those for submitochondrial particles which are also devoid of respiratory control. Accordingly, comparison of the control mechanism of the energy-transfer system should be done using intact bacterial cells and carefully isolated mitochondria. As commented on by SENEZ[47], the occurrence of respiratory control in growing bacteria and resting organism has not yet been demonstrated. However, stimulation of electron transport in the intact cells by the addition of 2,4-dinitrophenol[48], dibromophenol[49] or a non-metabolizable thiomethylgalactoside[50] suggested the presence of some sort of regulatory mechanism in energy-generating metabolism. Further studies on the mechanism of energy transfer will provide information regarding the control of the energy conservation in bacteria.

The change in mechanism of oxidative phosphorylation of bacteria grown under different conditions is interesting, and we intend to start a study along this line in the near future. We have already prepared successfully similar membrane fragments from *M. denitrificans* cells grown anaerobically in the presence of nitrate, and demon-

strated that phosphorylation is coupled to the oxidation of NADH and succinate with O_2 by this preparation. Recent reports by NAIK AND NICHOLAS[51] have also demonstrated the phosphorylation coupled to nitrate reduction with similar preparation.

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REFERENCES

1 G. B. PINCHOT, *J. Biol. Chem.*, 205 (1953) 65.
2 A. F. BRODIE AND C. T. GREY, *Biochim. Biophys. Acta*, 19 (1956) 384.
3 A. TISSIÈRES AND E. C. SLATER, *Nature*, 176 (1955) 736.
4 H. G. HOVENKAMP, *Nature*, 184 (1959) 471.
5 M. E. PULLMAN, H. PENEFSKY AND E. RACKER, *Arch. Biochem. Biophys.*, 76 (1958) 227.
6 A. W. LINNANE, *Biochim. Biophys. Acta*, 30 (1958) 221.
7 P. J. RUSSELL, JR. AND A. F. BRODIE, in G. E. W. WOLSTENHOLME AND C. M. O'CONNOR, *Quinones in Electron Transport*, J. and A. Churchill, London, 1960, p. 205.
8 G. B. PINCHOT, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 929.
9 A. ASANO AND A. F. BRODIE, *J. Biol. Chem.*, 239 (1964) 4280.
10 A. ASANO AND A. F. BRODIE, *Biochem. Biophys. Res. Commun.*, 19 (1965) 121.
11 A. ASANO AND A. F. BRODIE, *J. Biol. Chem.*, 240 (1965) 4002.
12 J. P. CHANG AND J. G. MORRIS, *J. Gen. Microbiol.*, 29 (1962) 301.
13 W. VERHOEVEN, A. L. KOSTER AND M. C. A. VAN NIEVELT, *Antonie van Leeuwenhoek, J. Microbiol. Serol.*, 20 (1954) 273.
14 C. A. FEWSON AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 48 (1961) 208.
15 R. SATO, in W. D. MCELROY AND B. GLASS, *Inorganic Nitrogen Metabolism*, Johns Hopkins, Baltimore, 1956, p. 163.
16 L. P. VERNON, *J. Biol. Chem.*, 222 (1956) 1035.
17 L. P. VERNON AND F. G. WHITE, *Biochim. Biophys. Acta*, 25 (1957) 321.
18 K. IMAI, A. ASANO AND R. SATO, *Seikagaku*, 37 (1965) 646 (in Japanese).
19 A. ASANO, K. IMAI AND R. SATO, *Seikagaku*, 37 (1965) 646 (in Japanese).
20 R. PHILIPS AND H. J. CLARKE, *J. Am. Chem. Soc.*, 45 (1923) 1756.
21 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
22 E. E. JACOBS, M. JACOB, D. R. SANADI AND L. B. BRADLER, *J. Biol. Chem.*, 223 (1956) 147.
23 E. KELLENBERGER, A. RYTER AND J. SECHAND, *J. Biophys. Biochem. Cytol.*, 4 (1958) 671.
24 H. B. BURCH, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 960.
25 J. PURVIS, *Biochim. Biophys. Acta*, 38 (1960) 120.
26 B. CHANCE, *J. Biol. Chem.*, 202 (1953) 383.
27 A. ASANO, T. KANESHIRO AND A. F. BRODIE, *J. Biol. Chem.*, 240 (1965) 895.
28 E. R. REDFEARN AND A. M. PUMPHREY, *Biochem. J.*, 76 (1960) 64.
29 A. M. PUMPHREY AND E. R. REDFEARN, *Biochem. J.*, 76 (1960) 61.
30 A. L. LEHNINGER, *The Mitochondrion*, W. A. Benjamin, New York, 1964, p. 60.
31 G. LENAZ AND R. E. BEYER, *J. Biol. Chem.*, 240 (1965) 3653.
32 J. M. HASLAM, *Biochim. Biophys. Acta*, 105 (1965) 184.
33 G. P. BRIERLEY, *Biochem. Biophys. Res. Commun.*, 19 (1965) 500.
34 E. RACKER, *Proc. Natl. Acad. Sci. U.S.*, 48 (1962) 1659.
35 T. E. CONOVER, R. L. PRAIRIE AND E. RACKER, *J. Biol. Chem.*, 238 (1963) 2831.
36 C. P. LEE, G. F. AZZONE AND L. ERNSTER, *Nature*, 201 (1964) 152.
37 A. ASANO, K. IMAI AND R. SATO, *Biochim. Biophys. Acta*, 143 (1967) 477.
38 M. E. PULLMAN, H. S. PENEFSKY, A. DATTA AND E. RACKER, *J. Biol. Chem.*, 235 (1960) 3322.
39 S. ISHIKAWA AND A. L. LEHNINGER, *J. Biol. Chem.*, 237 (1962) 2401.
40 M. FUJITA, S. ISHIKAWA AND N. SHIMAZONO, *J. Biochem. Tokyo*, 59 (1966) 104.
41 A. F. BRODIE, *J. Biol. Chem.*, 234 (1959) 398.

42 E. R. KASHKET AND A. F. BRODIE, *Biochim. Biophys. Acta*, 78 (1963) 52.
43 T. YAMANAKA, A. OTA AND K. OKUNUKI, *J. Biochem. Tokyo*, 51 (1962) 253.
44 A. W. FRENKEL, *J. Biol. Chem.*, 222 (1956) 823.
45 D. M. GELLER, *J. Biol. Chem.*, 237 (1962) 2947.
46 J. YAMASHITA, S. YOSHIMURA AND T. HORIO, *Koso Kagaku Shimpoziumu*, 17 (1965) 178 (in Japanese).
47 J. C. SENEZ, *Bacteriol. Rev.*, 26 (1962) 95.
48 E. R. KASHKET AND A. F. BRODIE, *J. Bacteriol.*, 83 (1962) 1094.
49 W. P. HEMPFLING, S. STEINBERG AND R. W. ESTABROOK, *Abstr. 6th Intern. Congr. Biochem., New York, 1964*, Vol. 32, p. 779.
50 A. KEPES, *Compt. Rend.*, 244 (1957) 1550.
51 M. S. NAIK AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 113 (1966) 490.
52 K. IMAI, T. OMURA AND R. SATO, *J. Biochem. Tokyo*, 60 (1966) 274.
53 Z. DISHE AND K. SCHWARZ, *Mikrochim. Acta*, 2 (1937) 13.
54 S. D. NIELSEN AND A. L. LEHNINGER, *J. Biol. Chem.*, 215 (1955) 555.