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The Electron Transport System of the Anaerobic *Propionibacterium shermanii*

Cytochrome and Inhibitor Studies*

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Abstract. 1. Electron transport particles obtained from cell-free extracts of *Propionibacterium shermanii* by centrifugation at 105000×g for 3 hrs oxidized NADH, D,L-lactate, L-glycerol-3-phosphate and succinate with oxygen and, except for succinate, with fumarate, too.

2. Spectral investigation of the electron transport particles revealed the presence of cytochromes *b*, *d* and *o*, and traces of cytochrome a_1 and a *c*-type cytochrome. Cytochrome *b* was reduced by succinate to about 50%, and by NADH, lactate or glycerol-3-phosphate to 80–90%.

3. The inhibitory effects of amytal and rotenone on NADH oxidation, but not on the oxidation of the other substrates, indicated the presence of the NADH dehydrogenase complex, or "site I region", in the electron transport system of *P. shermanii*.

4. NQNO inhibited substrate oxidations by oxygen and fumarate, as well as equilibration of the flavoproteins of the substrate dehydrogenases by way of menaquinone. The inhibition occurred at low concentrations of the inhibitor, and reached 80–100%, depending on the substrate tested. The site of inhibition of the respiratory activity was located between menaquinone and cytochrome *b*. In addition, inhibition of flavoprotein equilibration suggested that NQNO acted upon the electron transfer directed from menaquinol towards the acceptor to be reduced, either cytochrome *b* or the flavoproteins, which would include fumarate reductase.

5. In NQNO-inhibited particles, cytochrome *b* was not oxidized by oxygen-free fumarate, but readily oxidized by oxygen. It was concluded from this and the above evidence that the branching-point of the electron transport chain towards fumarate reductase was located at the menaquinone in *P. shermanii*. It was further concluded that all cytochromes were situated in the oxygen-linked branch of the chain, which formed a dead end of the system under anaerobic conditions.

6. Antimycin A inhibited only oxygen-linked reactions of the particles to about 50% at high concentrations of the inhibitor. Inhibitors of terminal oxidases were inactive, except for carbon monoxide.

Key words: *Propionibacterium shermanii* — Anaerobic Electron Transport — Electron Transport Particles — Cytochromes — Menaquinone — Fumarate Reductase — Site of NQNO Inhibition.

Electron transport systems of anaerobic bacteria have found the investigators' interest mainly for two reasons: the search for primeval systems of oxidative phosphorylation, and the question, how much energy in terms of ATP may be derived from anaerobic electron transport by a fermenting organism. An example for the latter problem is presented by the genus *Propionibacterium*, since Bauchop and Elsden (1960) have concluded from molar growth yields that *Propionibacterium pentosaceum* has more ATP available for growth than can be accounted for from substrate-level phosphorylations only. The finding of cytochromes *a* and *b* by Chaix and Fromageot (1942) represents early evidence for an electron transport system in this organism; in the following it has repeatedly been confirmed in this and other species of the genus (Lara, 1959; Schwartz, 1970; Sone, 1972; DeVries *et al.*, 1972; Bonartzeva *et al.*, 1973). The isolation of menaquinones from *P. shermanii* (Schwartz, 1972, 1973a) adds another component to the electron transport system, which with regard to the number of constituents almost attains the composition of typical respiratory systems of aerobes. A small respiration can indeed be observed in *Propionibacterium* (Schwartz, 1970; DeVries *et al.*, 1972). This respiration, however, is too small to abolish the anaerobic properties of the genus, whereas it may protect against the inhibitory effects of oxygen under certain conditions (Schwartz, 1973b). As the electron transport system of *Propionibacterium* in this way seems

Unusual Abbreviations. ET = electron transport; G-3-P = glycerol-3-phosphate; NQNO = 2-n-nonyl-4-hydroxyquinoline-N-oxide; PMS = phenazine methosulfate; TMPD = tetramethyl-p-phenylene diamine.

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to be inadequate for a full aerobic function, the problem has arisen, how many of its constituents are necessary for the role ascribed to it in anaerobiosis, *i.e.* fumarate reduction. DeVries *et al.* (1972), Sone (1972), and Bonartzeva *et al.* (1973) suggested that cytochrome *b* is located on the direct pathway of fumarate reduction. In the present paper it will be demonstrated by the effects of inhibitors that all cytochromes are located in the aerobic branch of the electron transport system of *P. shermanii*.

Materials and Methods

Culture of Cells. The culture of stocks and inocula of *Propionibacterium shermanii PZ* 3 was performed as described by Schwartz (1973 b). For obtaining cell mass the strain was grown in a medium containing 30 g glucose (autoclaved separately), 5 g Difco yeast extract, 5 g Merck peptone from meat, 4 g K_2HPO_4, and 0.4 g KH_2PO_4 per liter, in 10 l bottles filled to the neck, for 3 days at 30° C. In some cases the culture was kept at a constant pH of 6.9 with sterile sodium carbonate in a pH-stat. The cells were centrifuged in a Sorvall RC2-B refrigerated centrifuge at 4° C and 17000 × g. Cells not used immediately were frozen in liquid nitrogen and stored at −25° C.

Preparation of ET Particles. Fresh or frozen cells were suspended in the same amount (w./v.) of 0.1 M potassium phosphate buffer pH 7.0 containing 0.3 M sucrose, hereafter called isolation medium. The suspension was twice passed through a French pressure cell (about 1200 kg/cm²). It was important to adjust the pH to 7.0 with 1 N KOH immediately after pressing. In some cases ET particles were prepared by sonication with a Branson Sonifier applied three times for 30 sec with intermittent cooling. Cell debris were sedimented in a Sorvall RC2-B centrifuge (SS-34 rotor) at 42000 × g for 30 min. The sediment was extracted with the isolation medium for a second time. The combined supernatants gave the crude extract. The particles were sedimented in a Beckmann ultracentrifuge at 105000 × g for 3 hrs, washed once and resuspended in the isolation medium. After this procedure the particles were completely oxidized and free from endogenous substrate. Protein was determined with the Biuret method using KCN (Kröger and Klingenberg, 1966).

Assays. NADH, D,L-lactate, L-glycerol-3-phosphate, and succinate oxidation were assayed by determining the initial rate of O_2 consumption with a Beckmann Clark electrode at 30° C as described by Estabrook (1967). The particles were diluted with isolation medium. Substrate concentrations were NADH 1 mM, D,L-lactate 7.7 mM, L-glycerol-3-phosphate 3.8 mM, succinate 7.7 mM. Succinate oxidase was assayed with and without 5 mM phenazine methosulfate. NADH oxidation by fumarate was assayed in a Hellma anaerobic cuvette with two bulbs for additions with an Eppendorf photometer at 366 nm and 30° C. NADH was 0.1 mM, fumarate 13 mM. Cuvette and contents were flushed with oxygen-free nitrogen (Stickstoff 4.5, Messer Griesheim Co., O_2 below 5 ppm) for 10 min. Oxidation of lactate and glycerol-3-phosphate by fumarate were estimated from the decrease at 260−290 nm in a Perkin-Elmer M 156 dual wavelength photometer in an open standard cuvette under anaerobic conditions at 25° C (Kröger *et al.*, 1971). The rate of fumarate decrease was corrected for the activity of fumarase, which was estimated in the absence of donor substrate. Substrate concentrations were D,L-lactate 7.7 mM, L-glycerol-3-phosphate 3.8 mM, fumarate 1.4 mM. An absorbance coefficient for fumarate of $\varepsilon_{mM} = 0.78$ at 260−290 nm was used (A. Kröger, personal communication). The cuvette containing buffer and non-foaming additions was partially deoxygenized by bubbling with oxygen-free nitrogen for 1 min. The residual oxygen, as well as the oxygen added with the particle suspension and the starting substrate, was consumed at the start of the reaction. This method is suitable for measuring the anaerobic activity of preparations containing sufficient oxidase activity, but not for inhibitor studies, obviously.

Spectrophotometry. Spectra at room and liquid nitrogen temperature were recorded with the split beam photometer of the Institut für Physikalische Biochemie, University of München. The carbon monoxide spectrum was recorded with an Aminco DW 2 photometer. Time-dependent reactions were observed with an Aminco Chance dual wavelength photometer, and a Perkin-Elmer M 156 dual wavelength photometer in the UV region. The spectral data on cytochromes were taken from the summary of Kamen and Horio (1970), unless special literature is cited. For quantitative determinations the following millimolar absorbance coefficients were used; $\varepsilon_{mM} = 11.0$ at 465−510 nm for flavoproteins (Chance and Williams, 1955), $\varepsilon_{mM} = 17.5$ at 560−575 nm for cytochrome *b* (in analogy to Deeb and Hager, 1964), and $\varepsilon_{mM} = 170$ at 417−429 nm in the CO spectrum for cytochrome *o* (Daniel, 1970). The steady state of menaquinones was observed at 248−253.6 nm and the menaquinone contents of the particles was determined as described by Kröger *et al.* (1971).

Inhibitors. Amytal, NQNO (2-n-nonyl-4-hydroxyquinoline-N-oxide), rotenone (all from Serva, Heidelberg), antimycin A (Nutritional Biochemical Co.), 1,10-phenanthroline (Merck) were dissolved in absolute ethanol. Dicoumarol (Sigma) was dissolved in 0.01 mM KOH and then adjusted to pH 8.0 with HCl. Unless indicated otherwise inhibitors were generally added to the reaction vessels after the uninhibited reaction had proceeded for 1 min. Control measurements with inhibitor-free ethanol indicated that the amounts of ethanol added were not inhibitory by themselves. Biochemicals were from Boehringer, Mannheim; PMS from Sigma; TMPD from Fluka, Switzerland.

Results

Activities of the Particles

The particles prepared by ultracentrifugation contain all enzymes necessary for the oxidation of the natural substrates NADH, lactate, glycerol-3-phosphate, and succinate by either fumarate or oxygen (Table 1). The addition of phenazine methosulfate (5 mM) increases the oxidation by oxygen of glycerol-3-phosphate twofold, of lactate threefold, and of succinate 70 fold. At this concentration PMS directly links the dehydrogenases to oxygen, as will be shown later by the complete absence of inhibition by NQNO under this condition. The elevated activities in the presence of PMS thus indicate that the rate-limiting steps of these oxidations

Table 1. Activities of electron transport particles prepared from *Propionibacterium shermanii* by sonication

Substrate tested	Substrate conc. mM	Fumarate reduced μmoles/min/mg protein	Oxygen consumed μatoms/min/mg protein	
			without phenazine methosulfate	with phenazine methosulfate 5 mM
NADH	0.3	0.56	1.34	—
D,L-Lactate	7.7	0.51	1.74	5.2
L-Glycerol-3-phosphate	3.8	0.32	0.24	0.46
Succinate	7.7	—	0.024	1.68
Ascorbate + TMPD	0.9 +0.03	0	0.018	—

are located in the electron transport chain and not in the dehydrogenases. The particularly high increase of succinate oxidation by PMS even suggests, that the rate-limiting steps may be different ones for the various substrates. The artificial hydrogen donor system ascorbate-TMPD is also oxidized at a very low rate.

The numerical values of the activities given in Table 1 are those of the purest particles obtained, which incidentally were prepared by sonication and came from a batch culture not kept at a constant pH of 6.9. Particles prepared with the French pressure cell displayed about one third to one half of these specific activities, and cells grown at a constant pH of 6.9 were more reliable in giving good preparations. The relations of the activities with various substrates were about the same in all sorts of preparations.

Cytochromes

The difference spectra of ET particles from *Propionibacterium shermanii* (dithionite-reduced minus oxidized) are dominated by the absorption maxima of *b*-type cytochromes, whereas other cytochromes are present only in smaller amounts (Fig. 1). The maximum of the *b*-type cytochromes at 560 nm (room temperature) is not homogeneous, as evident from its asymmetry, and shoulders at 558, and 569 nm. At the temperature of liquid nitrogen (−196°C = 77°K) the maximum is shifted to 555 nm, with the high shoulder appearing at 559 nm. This pattern indicates the presence of more than one *b*-type cytochrome. The other peaks belonging to cytochromes *b* are situated as follows: β peak 531 nm at room temperature, 527 nm at 77°K; Soret peak 429 nm at room temperature, 428 nm at 77°K.

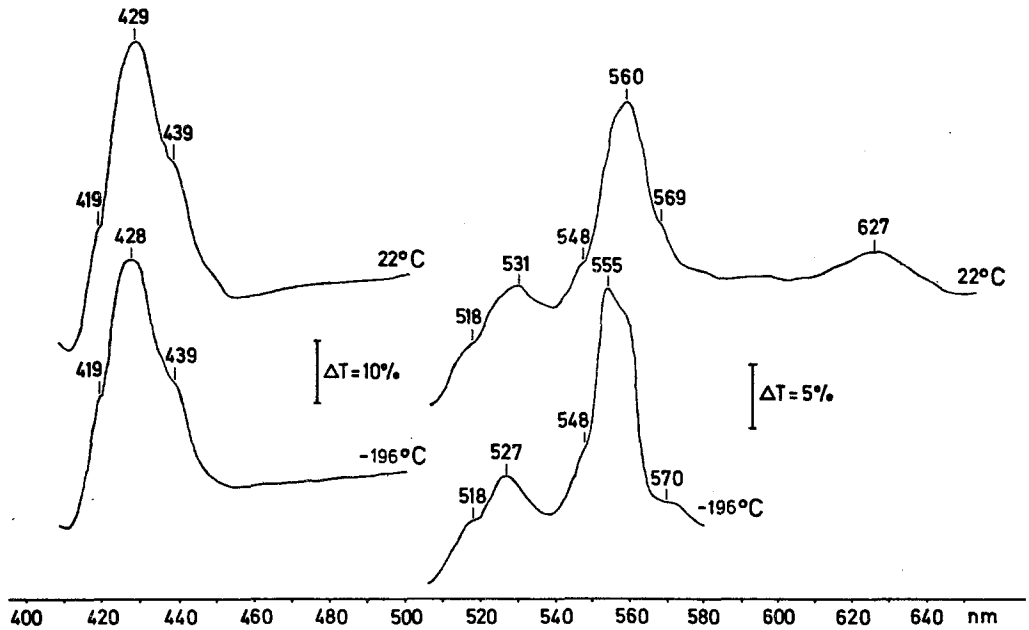

Fig. 1. Cytochrome spectra of electron transport particles from *Propionibacterium shermanii*. Particles reduced with dithionite in the sample cuvette, oxidized particles in the reference cuvette, protein 4 mg/ml; 22°C recorded at room temperature, optical path 0.5 cm; −196°C recorded at the temperature of liquid nitrogen, optical path 0.1 cm; split beam photometer of the Institut für Physikalische Biochemie, München. *T* transmission

The broad maximum around 627 nm points to the presence of cytochrome *d* (previously a_2); the shoulders at 419 and 518 nm may belong to the same cytochrome (418 and 523 nm in *Pseudomonas aeruginosa*; Yamanaka and Okunuki, 1963). The shoulder observed at 439 nm indicates cytochrome a_1, the α peak of which should be seen at 585—600 nm (Kamen and Horio, 1970). In the numerous spectra recorded, small peaks occasionally appeared in this region of the wavelength scale (*e.g.* Fig. 2, I). Also, cytochrome a_1 was already observed by DeVries *et al.* (1972) in cell suspension of *P. shermanii* and other species of this genus. The shoulder at 548 nm may belong to a trace of a *c*-type cytochrome.

The 560 nm band is reduced to different degrees by various substrates of the particles. This suggested to investigate the cytochromes *b* by recording a series of difference spectra. As succinate gave the lowest percentage of reduction, the basic spectrum was obtained from particles made anaerobic with succinate *minus* oxidized particles (Fig. 2, I). The very slender peak shows a maximum at 562 nm. When the particles made anaerobic with succinate are placed in the reference cuvette, and particles rendered anaerobic with lactate (or NADH) in the sample cuvette, the maximum is shifted only slightly to the left (Fig. 2, II and III). Thus, better resolution of the peak at 560 nm is not obtained from these spectra. However, the existence of two cytochromes *b* reduced by different substrates cannot be excluded, and remains to be tested by other experiments. Fig. 2 also provides information on other cytochromes: Cytochrome *d* is completely reduced by succinate already. Upon reduction by lactate or NADH a broad shoulder becomes visible at 570 nm.

The presence of terminal oxidases was investigated by recording a difference spectrum of dithionite-reduced and CO-treated minus dithionite-reduced particles according to the technique introduced by Warburg. CO was applied by bubbling the cuvette until saturation, which took one minute. Three CO-binding cytochromes are present in *P. shermanii* (Fig. 3; the difference between the control spectrum of Fig. 3 and the spectrum of Fig. 1 is due to the different instruments used). The minima of the spectrum obtained in the presence of CO indicate the positions of maxima of cytochromes, which have been abolished by binding carbon monoxide. The CO-complexes form new maxima at different wavelengths. The minimum in the CO spectrum at 560 nm is due to cytochrome *o* (Castor and Chance, 1959). Comparing the depth of the minimum caused by CO with the height of the peak at 560 nm in the control spectrum, cytochrome *o* accounts for about 10% of the total absorption at this wavelength. Another minimum due to cytochrome *o* is observed at

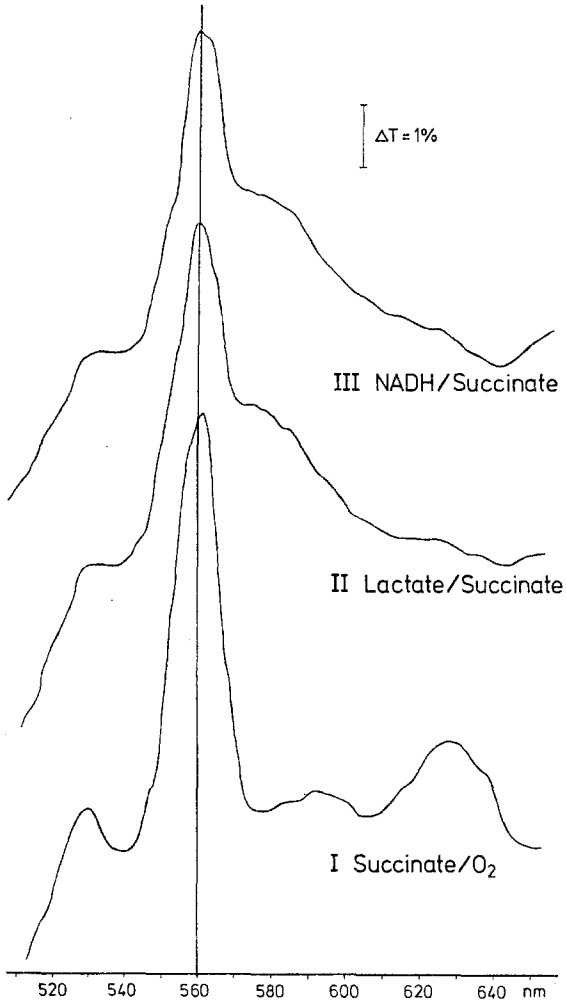

Fig. 2. Difference spectra of the region around 560 nm of electron transport particles from *Propionibacterium shermanii*. *I* Particles made anaerobic with succinate *minus* oxidized; *II* particles anaerobic with lactate *minus* anaerobic with succinate; *III* particles anaerobic with NADH *minus* anaerobic with succinate; protein 4.2 mg/ml, optical path 0.5 cm, room temperature. *T* transmission; photometer as in Fig. 1

429 nm. Peaks ascribed to the CO-compound of this cytochrome are visible at 417, 541 and 572 nm, in accordance with the observations in the literature (for a summary see Daniel, 1970). A spectrum of particles made anaerobic with ascorbate-TMPD minus oxidized particles revealed a broad peak around 570 nm (Fig. 4), which coincides with the shoulders at 569 and 570 nm in Figs. 1 and 2, and may be the α peak of cytochrome *o*. It probably still contains a small contribution from cytochrome *b* around 560 nm.

The other important CO-binding cytochrome is cytochrome *d*, which has been identified in Fig. 1 already. Its maximum is shifted to 638 nm by CO

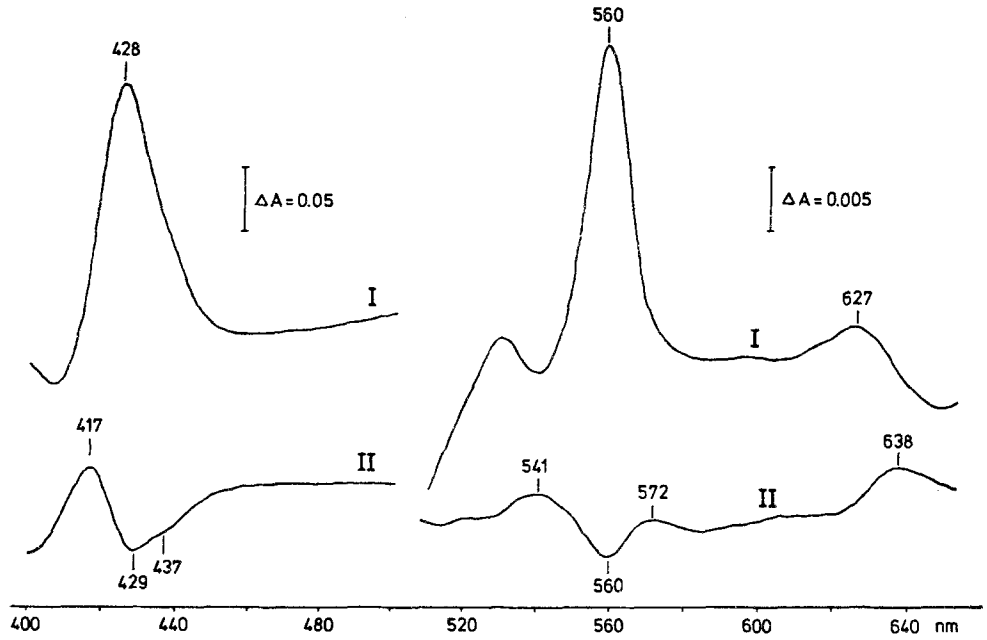

Fig. 3. Carbon monoxide spectrum of electron transport particles from *Propionibacterium shermanii*. *I* Control spectrum, dithionite-reduced *minus* oxidized particles; *II* sample and reference reduced with dithionite, sample saturated with CO; protein 4 mg/ml, optical path 0.5 cm, room temperature. *A* absorbance; photometer DW-2, American Instrument Co

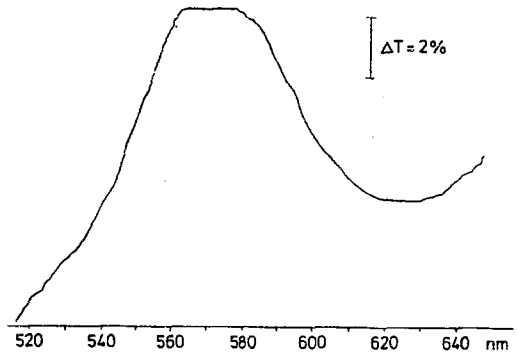

Fig. 4. Difference spectrum of electron transport particles from *Propionibacterium shermanii* reduced with TMPD-ascorbate. Reference oxidized particles, protein 8.4 mg/ml, optical path 0.5 cm, room temperature. *T* transmission; photometer as in Fig. 1

Table 2. Concentrations of electron transport components in electron transport particles prepared from *Propionibacterium shermanii* by sonication

Electron transport component and reductant	Concentration nanomoles/mg protein
Cytochromes $b+o$ (dithionite)	1.8
Cytochrome b (succinate)	0.80
Cytochrome b (lactate, NADH, G-3-P)	0.63 [a]
Cytochrome o	0.19
Cytochrome d	$2.6 \cdot 10^{-3}$ [b]
Cytochrome a_1	trace
C-type cytochrome	trace
Flavoproteins	2.4
Menaquinones	18.3

[a] Corrected as indicated in the text; value in addition to the amount reduced by succinate.
[b] Absorbance at 627−605 nm per mg protein.

(Fig. 3, II), in agreement with observations made in CO action spectra by Castor and Chance (1959). The trace of cytochrome a_1 is confirmed by the distinct shoulder at 437 nm in the CO spectrum, which corresponds to the shoulder at 439 nm in Fig. 1.

The estimation of the various cytochromes in ET particles confirms the overwhelming amount of *b*-type cytochromes (Table 2). It should also be noticed that the determination of cytochrome *b* reduced by lactate, NADH, and G-3-P at the wavelength pair 560−575 nm may be subject to an error due to the shoulder at 570 nm (Fig. 2). For example, if the wavelength pair 560−537 nm were chosen, a ratio of 1:0.79 of cytochrome *b* reduced by succinate to cytochrome *b* reduced

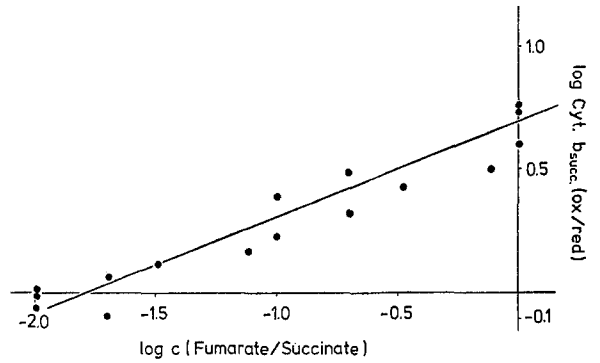

Fig. 5. Redox titration of cytochrome *b* reduced by succinate in electron transport particles of *Propionibacterium shermanii*. Protein 1.8 mg/ml, cytochrome *b* (succ.) 0.5 nanomoles/mg protein, values from two experiments

by lactate, NADH and G-3-P would be obtained, instead of 1:0.46 obtained with the reference wavelength of 575 nm. This procedure is feasible, because interfering amounts of cytochrome *c* are absent. From the estimations in Table 2, a value of 12.8 is calculated for the ratio of menaquinone: cytochrome *b*, excluding cytochrome *o*.

The amount of cytochrome *b* reduced by succinate was shown to be in equilibrium with the fumarate-succinate system according to the Nernst equation (Fig.5). A slope of 0.38 was obtained instead of the theoretical value of 0.5. The titration was performed under nitrogen at 562—575 nm and at various ratios of fumarate (between 1 and 10 mM) to succinate (between 10 and 100 mM). A redox potential of −30 mV for the cytochrome *b* reduced by succinate was calculated from this titration.

Inhibitors of the NADH Dehydrogenase Complex

Amytal and rotenone inhibited NADH oxidation both by fumarate and oxygen, but did not inhibit lactate or succinate oxidation by oxygen (Table 3). Complete inhibition was not obtained with either inhibitor. In cases when maximal inhibition was particularly low, sonication of the particles for 1 min with the inhibitor present resulted in better inhibition. Apparently, the site of inhibition is not freely accessible for the inhibitor.

NADH oxidation was the activity most sensitive towards o-phenanthroline, an inhibitor of non-heme iron centers (Kurup and Brodie, 1967). The inhibition of the other activities was rather weak.

Inhibitors of the Cytochrome Region

Preliminary experiments showed that the aerobic and anaerobic branches of the ET system reacted in a different way towards the inhibitors antimycin A and NQNO. Therefore the effects of these inhibitors on several activities was tested over a wide concentration range. Antimycin A only partially inhibited the aerobic oxidation of NADH, lactate, and succinate at very high concentrations of the inhibitor (Fig. 6). The site of inhibition is situated on the oxygen side of cytochrome *b*, as the reduction of this compound is increased upon the addition of antimycin A (figure not shown). The site is identical to that of mitochondria (Chance, 1952, 1958).

NQNO inhibited the anaerobic oxidation of NADH and lactate by fumarate as well as all aerobic activities (Fig. 7). The oxidation of succinate (without PMS present) is much more sensitive towards NQNO than

Table 3. Effect of inhibitors of the NADH dehydrogenase complex on the electron transport system of *Propionibacterium shermanii*

Inhibitors	Oxidation of NADH by fumarate		Oxidation by oxygen of					
			NADH		D,L-lactate		Succinate	
	Maximal inhibition %	Conc. of inhibitor mM	Maximal inhibition %	Conc. of inhibitor mM	Maximal inhibition %	Conc. of inhibitor mM	Maximal inhibition %	Conc. of inhibitor mM
Amytal	25	1.0	50	1.0	0	1.0	0	1.0
—, sonicated with the particles	—	—	78	1.0	—		—	
Rotenone	55	0.17	37	0.17	0	0.17	0	0.17
—, sonicated with the particles	—	—	55	0.17	—		—	
o-Phenanthroline	60	2.0	65	1.0	22	2.0	16	2.0

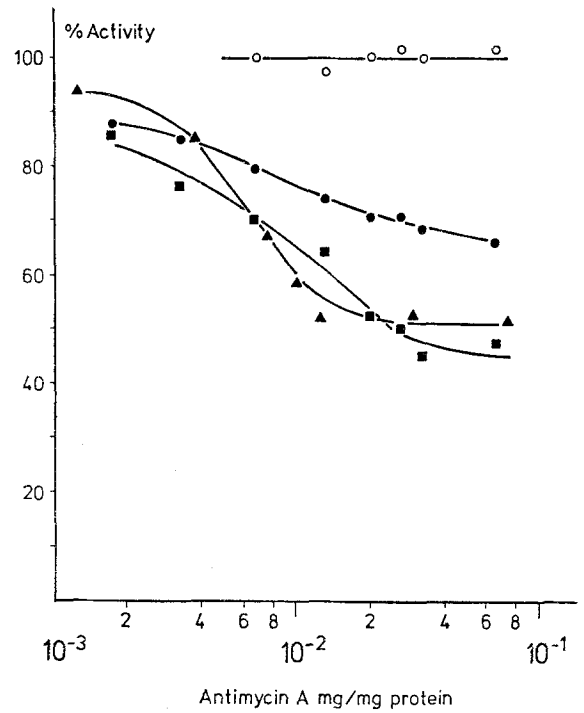

Fig. 6. Inhibition by antimycin A of the electron transport particles from *Propionibacterium shermanii*. Oxidation of NADH by fumarate (O——O) and by oxygen (●——●), of D,L-lactate by oxygen (■——■), and of succinate by oxygen (▲——▲)

the other reactions; it is the sole activity completely inhibited by this agent. The NADH-dependent reactions and lactate oxidation by O_2 are only inhibited up to 80%. The inhibition curves of these two substrates are located at slightly different positions between themselves, but the inhibition curve of succinate oxidation is clearly located quite out of this range.

The oxidation of succinate in the presence of PMS is not affected by NQNO. This proves that the electrons can bypass the normal transport chain in the presence of 5 mM PMS.

Dicoumarol inhibited the anaerobic and aerobic reactions of *P. shermanii* particles in a similar manner as NQNO, around concentrations of 10^{-7} moles/mg protein (figure not shown). Succinate respiration again was the activity most sensitive to the inhibitor, but it differed from the other substrates not more than those between themselves. Maximal inhibition obtained was 70% or less.

Of the inhibitors of cytochrome oxidase, azide was ineffective with all substrates, including ascorbate-TMPD. Cyanide only inhibited at concentrations of 10^{-2} M, which is not typical for terminal oxidases. The inhibition of oxygen consumption by carbon monoxide was tested in a preliminary manner at half saturation in the buffer each of CO and O_2. The respiration of all

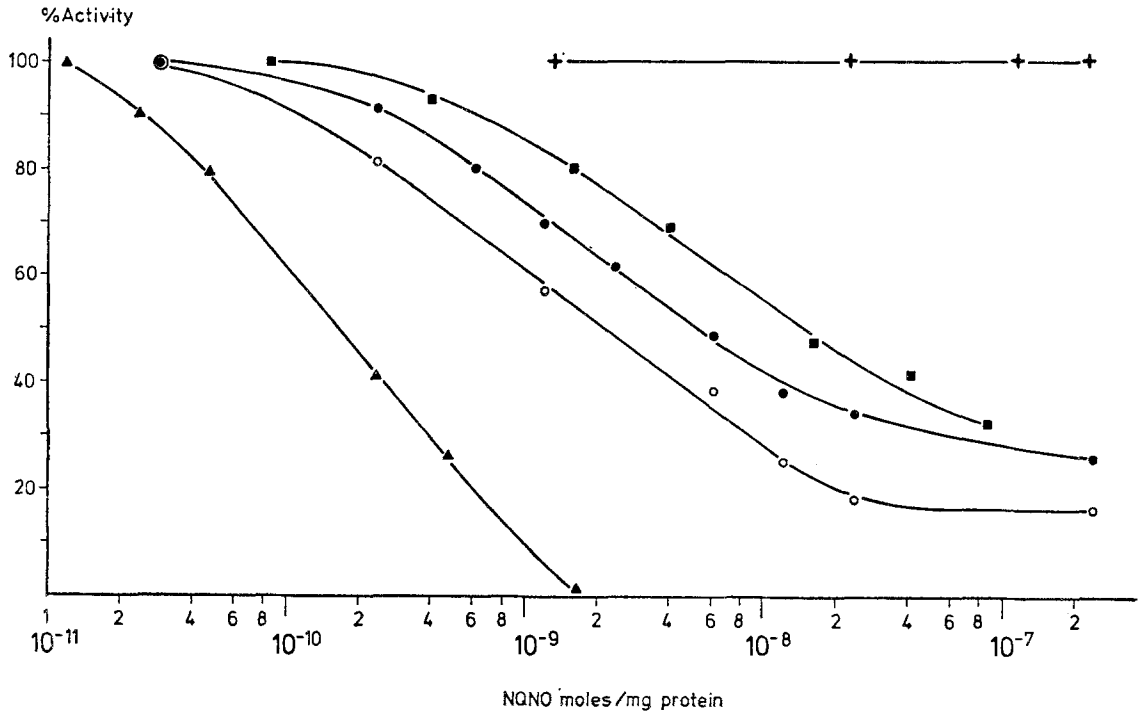

Fig. 7. Inhibition by NQNO of the electron transport particles from *Propionibacterium shermanii*. Oxidation of NADH by fumarate (O——O) and by oxygen (●——●), of D,L-lactate by oxygen (■——■), of succinate by oxygen without PMS present (▲——▲) and in the presence of 5 mM PMS (+——+)

substrates was inhibited from 40 to 70% on this condition.

Sites of Inhibition by NQNO

The degree of reduction of cytochrome *b* during the aerobic state is not increased, and in the case of NADH slightly decreased, in particles preincubated with high amounts of NQNO (Fig. 8). This excludes that NQNO interacts on the oxygen side of cytochrome *b*, and puts the inhibitor site to the substrate side of this component of the chain. The decrease of reduced cytochrome *b* in the anaerobic state of inhibited particles underlines this conclusion, as it represents cytochrome *b* no more accessible to reducing equivalents due to the inhibitor. Further proof is supplied by the observation of menaquinone: With increasing amounts of NQNO applied, the amount of reduced menaquinone in the aerobic state rises from about one third (control) to two thirds at a NQNO concentration of 130 nanomoles/mg protein (Fig. 9). Thus, NQNO interacts between menaquinone and cytochrome *b* in the aerobic branch of the chain.

The inhibition by NQNO provides the means to investigate the participation of cytochromes in fumarate reduction. By adding a saturating amount of oxygen-free NQNO during the anaerobic state the reduced cytochrome *b* is blocked from the electron-supplying substrate. When oxygen is then stirred into the cuvette, oxidation of cytochrome *b* down to the level of the aerobic state is observed for inhibited as well as for control particles (Fig. 10). This is followed by slow re-reduction according to the residual activity of the inhibited particles, whereas the control quickly returns to the anaerobic state. The same results are obtained for the substrates NADH and succinate (figure not shown). In a similar experiment oxygen-free fumarate instead of oxygen is added to the cuvette with a gastight syringe: In this case cytochrome *b* is only slightly reduced in the NQNO-treated particles, but to an appreciable degree in the control (Fig. 10). Evidently, after interrupting the chain with NQNO the reduced cytochrome *b* is no longer freely accessible for fumarate, whereas it is completely oxidized by oxygen. Again, the same results are obtained for NADH and succinate. Cytochrome *d*, which has been reported to be oxidized by fumarate (DeVries *et al.*, 1972) is not oxidized by oxygen-free fumarate in our experiments (wavelengths 627—605 nm).

The degree of reduction of flavoproteins was estimated at the wavelengths 465—510 (Chance and Williams, 1955). In the anaerobic states the sum of the degrees of reduction obtained with various substrates exceeded the dithionite value (Table 4). This observation suggests that the flavoproteins of the substrate dehydrogenases equilibrate across the menaquinone pool, irrespective of the substrate added. Upon preincubation with NQNO the degree of reduction of both aerobic and anaerobic states is decreased in most cases: The equilibration of the flavoproteins is inhibited by NQNO. In the presence of the inhibitor only that flavoprotein, which belongs to the dehydrogenase of the

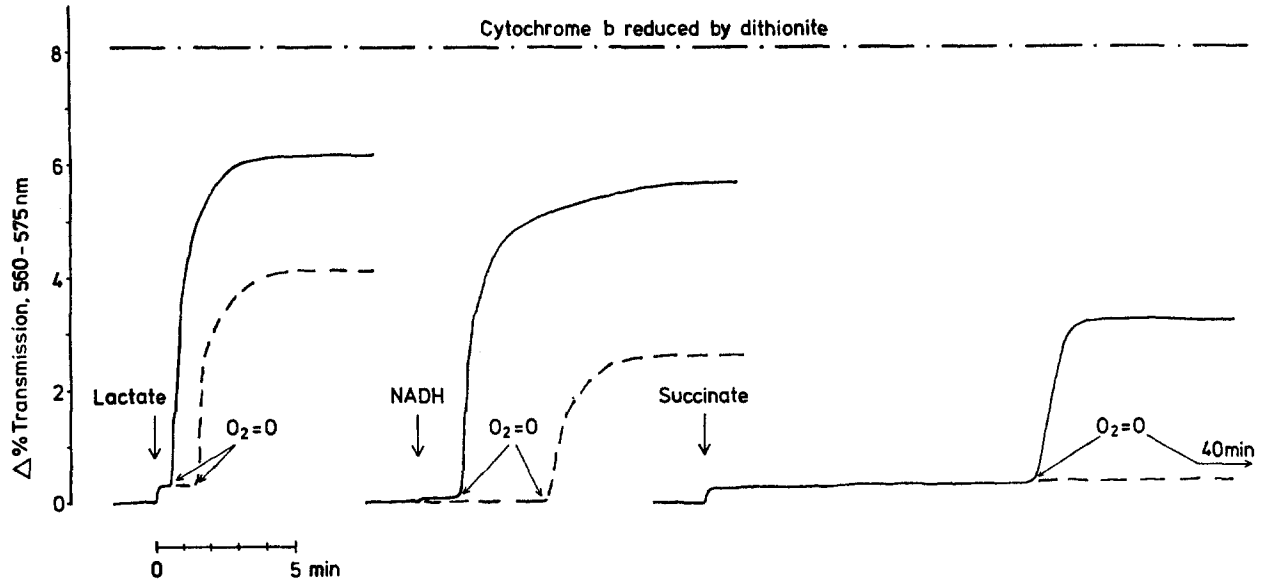

Fig. 8. Effect of NQNO on the aerobic and anaerobic states of cytochrome *b* in electron transport particles from *Propionibacterium shermanii*. Protein 1.4 mg/ml, optical path 1 cm; —— untreated particles, --- particles preincubated with NQNO $2.5 \cdot 10^{-7}$ moles/mg protein

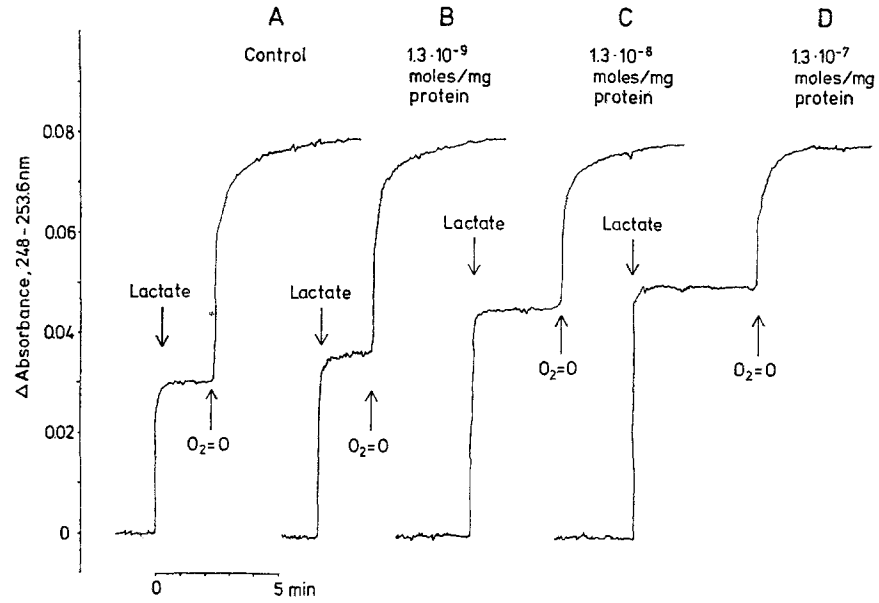

Fig. 9. Effect of NQNO on the aerobic state of menaquinone in electron transport particles from *Propionibacterium shermanii*. Particles preincubated with various concentrations of NQNO, protein 0.6 mg/ml, optical path 1 cm

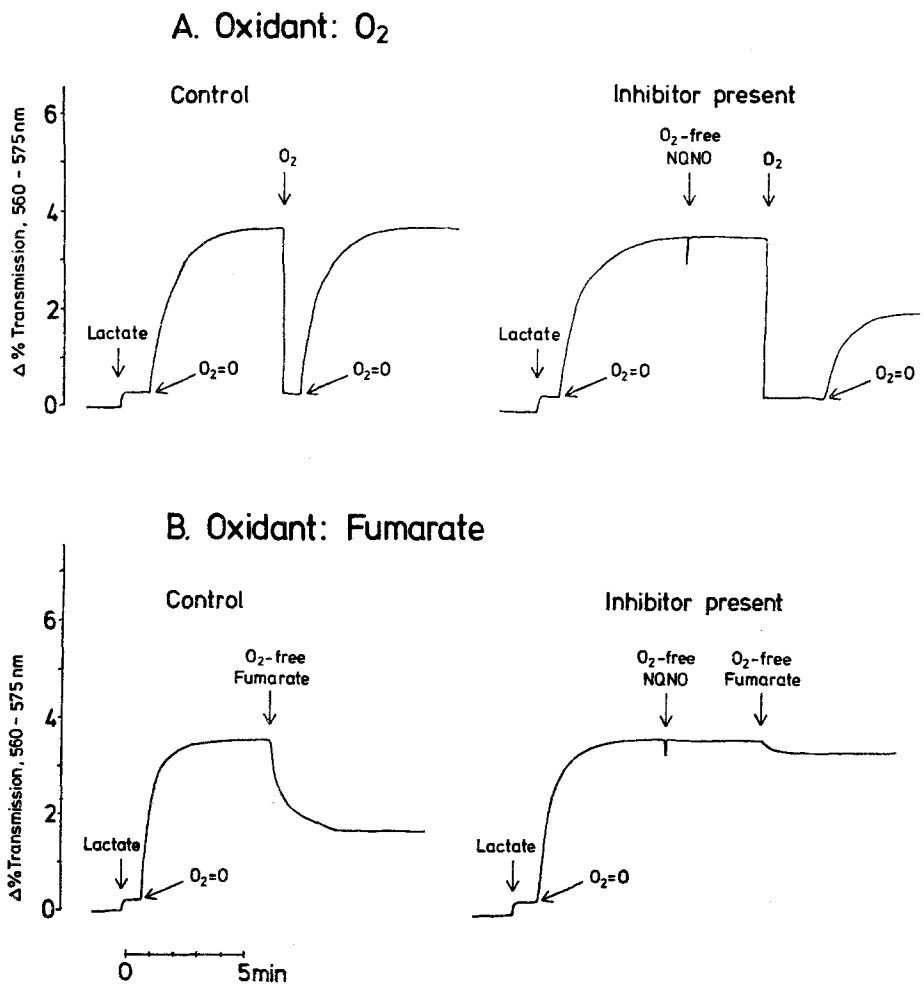

Fig. 10 A and B. Effect of NQNO on the reoxidation of reduced cytochrome *b* by oxygen and fumarate during the anaerobic state in electron transport particles from *Propionibacterium shermanii*. Protein 0.9 mg/ml, NQNO $8.7 \cdot 10^{-8}$ moles/mg protein

Table 4. Effect of NQNO on the reduction of flavoproteins with various substrates in electron transport particles from *Propionibacterium shermanii*

	Aerobic state % reduction	Anaerobic state % reduction	Anaerobic state in NQNO-inhibited particles [b] % reduction
Dithionite	100 [a]	100	100
NADH	9	58	15
D,L-Lactate	29	63	75
L-Glycerol-3-phosphate	14	75	40
Succinate	12	25	12

[a] Flavoproteins 2.2 nanomoles/mg protein (dithionite value).
[b] NQNO 0.1 μmole/mg protein.

substrate added, should preferably be reduced. The percentages of reduction obtained for NADH, G-3-P, and succinate in NQNO-inhibited particles concur with this conception, whereas the value obtained for lactate is higher in the inhibited particles. This may be due to the incomplete inhibition, which has already been shown in previous sections to be most evident for lactate. With succinate, the anaerobic state of inhibited particles is identical to the aerobic state of control particles. This would indicate that succinate dehydrogenase is already completely reduced during the aerobic state. The low value of 15% for NADH dehydrogenase coincides with the small amount of this enzyme (about 20%) residing on the particles. About 80% of the total NADH dehydrogenase activity are found in the supernatant of the ultracentrifugation (Schwartz and Krause, in preparation).

Discussion

In addition to the cytochromes a_1, *b*, and *d* already recognized in *Propionibacterium shermanii* (DeVries *et al.*, 1972; Bonartzeva *et al.*, 1973), cytochrome *o* as the third terminal oxidase is identified in carbon monoxide spectra in the present paper. Selective reduction by ascorbate-TMPD suggests that peaks or shoulders around 570 nm can be ascribed to cytochrome *o*. Cytochrome *b* is reduced only to about 50% by succinate, but to about 90% by lactate, NADH, or glycerol-3-phosphate. The question, whether more than one cytochrome *b* proper is present in *P. shermanii* cannot be decided from the asymmetry of the 560 nm band, and must be left to titration experiments covering the whole amount of cytochrome *b* in the particles. The high excess of menaquinone over cytochrome *b* (ratio 12.8) lies in the same order of magnitude as in *Bacillus megaterium* (6.2; Kröger and Dadak, 1969). In preliminary experiments with ET particles from *P. shermanii* oxygen- and fumarate-linked reactions were suspended upon extraction of the menaquinone, and partially reactivated upon its reincorporation (Schwartz, 1972). It has been shown in the present paper that menaquinone participates in the redox reactions of the ET chain. Therefore the same function can be assumed for menaquinone in *P. shermanii* that has been demonstrated for ubiquinone in mitochondria (Ernster *et al.*, 1969; Kröger and Klingenberg, 1973a, b), menaquinone in *Bacillus megaterium* (Kröger and Dadak, 1969), and both quinones in *Proteus rettgeri* (Kröger *et al.*, 1971): the collection of reducing equivalents from the substrate dehydrogenases and their distribution to the cytochrome system.

The effects of various inhibitors on several activities of the electron transport system of *P. arabinosum* have been reported by Sone (1972). In accordance with his observations, and those of Bonartzeva *et al.* (1973), NADH oxidation by both fumarate and oxygen in *P. shermanii* is inhibited by rotenone and amytal to a high percentage. These findings, especially the sensitivity towards rotenone (Ohnishi, 1973), suggest that the first step of NADH oxidation is mediated by a NADH dehydrogenase complex similar to that of aerobic bacteria and mitochondria. The inhibition of several activities of the particles by o-phenanthroline observed by Sone (1972) and in our own experiments indicates the involvement of non-heme iron to a similar degree as in aerobes.

NQNO and antimycin A did not inhibit the electron transport system of *P. arabinosum* (Sone, 1972). Contrary to this, DeVries *et al.* (1972, 1973) reported that NQNO inhibited the oxidation of lactate, as well as glycerol-3-phosphate by both fumarate, and oxygen in *P. freudenreichii*. The authors also stated that NQNO interfered on the substrate side of cytochrome *b*. These findings are confirmed and extended in the present paper. All fumarate- and oxygen-dependent oxidations are inhibited by NQNO to a high percentage at low concentrations of the inhibitor. Succinate oxidation by oxygen can even be completely inhibited by NQNO. The observation of the aerobic steady states of mena-

quinone and cytochrome *b* reduction in the absence and presence of the inhibitor permitted to locate one site of interaction between menaquinone and cytochrome *b*. *Propionibacterium* obviously shares this position of the inhibitory site of NQNO with *Proteus vulgaris*, some strains of *Escherichia coli* (Lightbown and Jackson, 1956), *Bacillus megaterium* (Kröger and Dadak, 1969), and *Proteus rettgeri* (Kröger *et al.*, 1971), whereas a location on the oxygen side of cytochrome *b* has been postulated for *Bacillus subtilis*, *Staphylococcus aureus*, pig heart mitochondria (Lightbown and Jackson, 1956), *Mycobacterium phlei* (Brodie, 1965), and another strain of *Escherichia coli* (Cox *et al.*, 1970). The sites of inhibition of NQNO and antimycin A can be expected to be different in *P. shermanii*, as antimycin A, in contrast to NQNO, inhibits only oxygen-linked reactions.

Experiments with NQNO also provide information on the branching point of the electron transport chain towards fumarate reductase. The problem is whether the common inhibition of aerobic and anaerobic reactions by NQNO puts the branching point to cytochrome *b*, or whether NQNO inhibits at more than one site. In *P. shermanii*, NQNO inhibits between menaquinone and cytochrome *b*, and also between menaquinone and the flavoproteins, as shown by the NQNO-dependent inhibition of flavoprotein redox equilibration by way of the menaquinone. Thus, NQNO exerts its inhibitory effect always in those cases, when the electrons are passed from the reduced menaquinone in the lipid phase to a protein-bound carrier, either cytochrome *b* or flavoprotein. It was further shown in a set of crucial experiments that, in the presence of NQNO, reduced cytochrome *b* is not oxidized by oxygen-free fumarate, but completely oxidized by oxygen. As cytochrome *b* is not auto-oxidizable, this is a proof that cytochrome *b* is situated in the oxygen-linked branch of the chain. The branching-point must therefore be located at the level of the menaquinone. Under anaerobic conditions the whole cytochrome chain represents a dead end of the system. Cytochrome *b*, of course, has a redox potential sufficiently negative to fluctuate in its side position according to the state of the adjacent member of the chain, menaquinone, during fumarate reduction. Therefore, oxidation of cytochrome *b* by fumarate in uninhibited particles alone cannot clarify beyond doubt, whether cytochrome *b* is located in the direct sequence of reactions leading to fumarate reduction, or in a blind alley. This dead end position of cytochrome *b* can only be demonstrated by blocking between cytochrome *b* and menaquinone with a specific inhibitor, NQNO: After the inhibitor has become effective, cytochrome *b* is no longer oxidized by oxygen-free fumarate. As a consequence of these findings, the views of Sone (1972), DeVries *et al.* (1972, 1973), and Bonartzeva *et al.* (1973), which include cytochrome *b* into the direct sequence of reactions leading to fumarate reduction, can no longer be maintained on the basis of their experiments.

Our opinion seems difficult to reconcile with the results on *Bacillus megaterium* (Kröger and Dadak, 1969), which put the branching-point to cytochrome b_1. Some estimations on this organism, however, would support a branching-point at the menaquinone as well. In the case of the cytochrome b_1 kinetics, the argument has been based on the decrease of reduction of this compound in the anaerobic state of NQNO-inhibited membranes, when oxygen was applied together with fumarate. In the present paper it has been demonstrated that this decrease of reduction of cytochrome *b* in the anaerobic state is a general feature of the inhibition by NQNO, which has so far not been recognized. It was observed by us with the substrates NADH and lactate after NQNO inhibition, when no fumarate was present. These findings suggest further detailed studies on the inhibitory action of NQNO. Fumarate reductase is directly linked to menaquinone in *Proteus rettgeri* (Kröger *et al.*, 1971).

The position of cytochrome b_{560}, besides menaquinone-6, in the direct pathway of fumarate reduction by molecular hydrogen has been postulated for *Desulfovibrio gigas* (Hatchikian and LeGall, 1972; Hatchikian, 1974) on the basis of the inhibition by antimycin A and NQNO, but remains to be ascertained by detailed kinetic experiments.

Our present view of the electron transport system of *P. shermanii* is summarized in Fig. 11. In order to include flavoprotein equilibration, the scheme is drawn in one of several possible versions, with NADH for substrate. The relation between fumarate reductase and succinate dehydrogenase is an open question for *P. shermanii*. NQNO, of course, may act upon an unknown component such as non-heme iron, situated

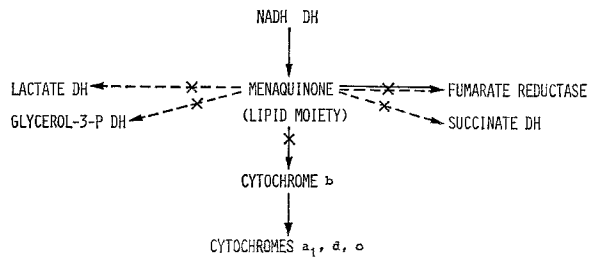

Fig. 11. Schematic presentation of the electron transport system of *Propionibacterium shermanii*, assuming, for example, NADH as the hydrogen donor. *DH* dehydrogenase; × sites of inhibition by NQNO; → electron flow; --→ flavoprotein equilibration

between menaquinone and cytochrome *b*, or the flavoproteins. The idea of an interaction of NQNO between the lipid-soluble quinone and protein-bound components of the chain is also favoured by its lipid solubility and molecular similarity to quinones as well as its iron-complexing properties (Cornforth and James, 1956).

In general, the electron transport system of *P. shermanii* is indeed rather similar in composition and organization to the respiratory chains of aerobic bacteria. Therefore it would be difficult to regard this genus as a primary anaerobe. It may rather be a descendant of aerobes, which has become an anaerobe from secondary causes.

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