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The Redox Reactions in Propionic Acid Fermentation

I. Occurrence and Nature of an Electron Transfer System in *Propionibacterium arabinosum**

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The redox enzymes in *Propionibacterium arabinosum* were examined. Succinate dehydrogenase was found mainly in the particulate fraction (membrane fragments), whereas NADH, α-glycerophosphate and D-lactate dehydrogenases [EC 1.6.99.3, 1.1.99.5 and 1.1.2.4] were distributed in both the particulate and soluble fractions. Comparison of the K_m values and pH optima indicated that both cellular fractions contained the same species of α-glycerophosphate and lactate dehydrogenases. The particulate fraction also contained a *b*-type cytochrome, flavoproteins, non-heme iron, and phospholipidis. These components, together with the membrane-bound dehydrogenases, constituted a particular type of electron transfer system which oxidized NADH, α-glycerophosphate and lactate actively. The oxygen uptake with these substrates was considerably inhibited by the addition of fumarate. The nature of the terminal oxidase functioning in the membrane fragments remained to be solved. Based on these observations, a scheme was presented for the electron-transfer system in the membrane fragments of *P. arabinosum*. This system is similar to the NADH-cytochrome *b* and succinate-cytochrome *b* segments of mammalian mitochondria. It is postulated that succinate dehydrogenases in this system acts as fumarate reductase and is responsible for the reduction step of propionic acid fermentation.

Bacteria belonging to the genus *Propionibacterium* grow anaerobically on such carbon sources as glycerol, lactate and glucose and produce propionate and acetate as the products (*2*). The mechanism of propionate formation by these organisms has been extensively studied by Wood and coworkers (*3–7*). They showed that the process is intrinsically cyclic and involves methylmalonyl-CoA transcarboxylase [EC 2.1.3.1] as a key enzyme (*3*, *4*). It was also shown that in the process oxaloacetate is reduced to succinate which is then converted to propionate (*7*), suggesting the importance of redox enzymes in propionic acid fermenta-

* Most part of this work was presented at the 42nd Meeting of the Japanese Biochemical Society (*1*). Abbreviations used are: α-GP, glycerol 1-phosphate; TMPD, N, N, N′, N′-tetramethyl-*p*-phenylenediamine; DCIP, 2,6-dichlorophenol indophenol; PCMB, *p*-chloromercuribenzoate; NOQNO, 2-*n*-nonylhydroxyquinolin N-oxide; DHAP, dihydroxyacetone phosphate; PMS, phenazine methosulfate.

tion. However, only fragmentary information is available at present concerning the redox systems in these bacteria. Apart from van Niel's earlier demonstration of oxidative activities in this group of organisms (*2*), Chaix and Fromageot (*8*) reported the occurrence of cytochrome pigments in *P. pentosacium*, and Delwiche and Carson (*9*) obtained evidence suggesting the operation of citric acid cycle in the same species. Partial purification and some properties of succinate dehydrogenase [EC 1.3.99.1] and D-lactate dehydrogenase [EC 1.1.2.4] from *P. pentosacium* were reported by Lara (*10*) and Molinari and Lara (*11*), respectively.

It is the purpose of this series of studies to elucidate the redox reactions involved in propionic acid fermentation in *Propionibacterium* and to compare the results with those obtained for various aerobes and anaerobes. This paper reports the occurrence and some properties of an electron-transfer enzyme system in the membrane fragments from *P. arabinosum* and presents evidence that this system is functioning in fumarate reduction.

EXPERIMENTAL PROCEDURE

Microorganism and Cultivation Condition—A strain of *Propionibacterium arabinosum* (IAM 1714) was grown at 30°C without shaking in deep cultures of the following composition (glycerol medium); glycerol, 8 g; yeast extract, 4 g; polypeptone, 2 g; K_2HPO_4, 5 g; $NaH_2PO_4 \cdot 2H_2O$, 1 g; $FeSO_4 \cdot 7H_2O$, 10 mg; $Co(CH_3CO_2)_2 \cdot 4H_2O$, 2 mg; thiamine-HCl, 1 mg; calcium pantothenate, 1 mg; biotin, 0.5 mg; tap water, 1 liter; pH 7.6. When indicated, glycerol was replaced by glucose (8 g) or sodium lactate (10 g) to obtain glucose-grown cells or lactate-grown cells, respectively. Inocula were obtained from 3 to 5 day-old cultures in the lactate medium. Usually 40 ml of inoculum was added to 5 liters of the culture medium. The cells were harvested at late log phase, *i.e.* about 40 hr in the glycerol medium, 30 hr in the glucose medium and 64 hr in the lactate medium after inoculation. The cells were washed once with 20 mM Tris-HCl buffer (pH 7.4) containing 0.9% NaCl and then with the buffer without NaCl. The cells could be kept frozen without causing any changes in the results.

Preparation of Particulate and Soluble Fractions—The washed cells, suspended in 2–3 volumes of 20 mM Tris-HCl buffer (pH 7.4), were disrupted in a mill-type glass homogenizer (*12*), chilled with ice water, in the presence of a small amount of deoxyribonuclease [EC 3.1.4.5]. The homogenate was centrifuged at $10,000 \times g$ for 15 min and the supernatant fluid was recentrifuged at $104,000 \times g$ for 40 min. The supernatant thus obtained was used as the soluble fraction. The precipitate, once washed with the buffer, was suspended in 0.3 M sucrose containing 10 mM Tris-HCl buffer (pH 7.4) and 0.1 mM EDTA and used as the particulate fraction (membrane fragments).

Reagents—Deoxyribonuclease I, sodium DL-α-GP, NOQNO and NADH were obtained from Sigma Co., FAD and FMN from Tokyo Kasei Co., and antimycin A from Kyowa Hakko Co. Piericidin A and rotenone were kindly supplied by Dr S. Tamura of the University of Tokyo and Dr. A. Asano of Osaka University, respectively. Piericidin A, antimycin A, NOQNO and rotenone were used as methanolic solutions. Although effect of methanol (below 2%) on the enzyme reaction was slight, the same amount of methanol was added to the control to obtain inhibition percent.

Assay Methods—All enzyme reactions were carried out in a standard reaction medium (pH 7.4) of the following composition at 25°C unless otherwise described; Tris-HCl, 20 mM; potassium phosphate buffer, 10 mM; $MgSO_4$, 5 mM; EDTA, 0.1 mM. NADH dehydrogenase [EC 1.6.99.3], α-GP dehydrogenase [EC 1.1.99.5], D-lactate dehydrogenase and succinate dehydrogenase (in the presence of 0.17 mM PMS) activities were measured in a Hitachi EPS-3T recording spectrophotometer by following the reduction of DCIP by the corresponding substrate at 600 mμ. The absorbancy index used was 21 $mM^{-1} \cdot cm^{-1}$ (*13*). The final concentration of DCIP was 0.04–0.08 mM, which was saturating for the enzymes. NADH oxidation by fumarate was similarly followed at 340 mμ. Lactate oxidation and α-GP oxidation by fumarate were measured by determining pyruvate (*14*) and DHAP (*15*), respectively. Oxygen uptake was measured in a Clark-type oxygen electrode in a semi-closed cell (3 ml). Difference spectra were measured as described previously (*16*). Protein was determined by the Lowry method (*17*). Qualitative identification of methyl esters of organic acids was carried out at 80–165°C with a Hitachi model K53 gas chromatograph (hydrogen flame detector), fitted with a column (0.3×187 cm) of 15% polyethyleneglycol adipate on chromosorb W.

RESULTS

A Survey for Dehydrogenases—Active reduction of DCIP occurred when NADH, lactate, α-GP, and succinate plus PMS were added to the 10,000×*g* supernatant of cell homogenates of *P. arabinosum* grown in the glycerol, glucose, and lactate media (*18*). However, pyruvate, citrate, fumarate, formate, and malate failed to cause significant reduction of the dye. Table I shows the distribution of these dehydrogenase activities between the particulate and soluble fractions of the extracts. Regardless of the carbon sources used for growth, NADH, D-lactate, and α-GP dehydrogenase activities were detected both in the particulate and soluble fractions, whereas virtually all of the succinate dehydrogenase activity was recovered in the particulate fraction. Although not shown in Table I, NADH-dependent malate dehydrogenase [EC 1. 1. 1. 37] was mostly present in the soluble fraction. Table I also shows that more α-GP dehydrogenase was formed in the cells grown in the glycerol medium, and more D-lactate dehydrogenase in the cells grown in the lactate medium. There was no dependence of both NADH and succinate dehydrogenase activities on the carbon sources used for cultivation.

Lactate and α-GP Dehydrogenases in Both Fractions—Lactate and α-GP dehydrogenases present in both the supernatant and particulate fractions did not seem to be NAD-dependent. NAD was not reduced by the enzyme preparations in the presence of lactate or α-GP even under anaerobic conditions, and it did not acceralate the reduction of DCIP by lactate or α-GP. As shown in Figs. 1 and 2, the same K_m value (0.12 mM for DL-lactate) and optimum pH (7.4) were obtained for lactate dehydrogenases in both fractions. Similarly as shown in Figs. 3 and 4, the same K_m value (0.14 mM for DL-α-GP) and optimum pH (7.4) were obtained for α-GP dehydrogenases in both fractions. It was thus likely that the same lactate and α-GP dehydrogenases were present both in the soluble and particulate fractions.

Absorption Spectra of Membrane Fragments—The dithionite minus oxidized difference

TABLE I. Distribution of dehydrogenases in *P. arabinosum* cells grown on various carbon sources. Each dehydrogenase activity was determined by following the reduction of DCIP by the corresponding substrate spectrophotometrically in 2.5 ml of the standard reaction medium. Final concentrations of substrates were as follows: NADH, *ca.* 0.2 mM; lactate and α-GP, *ca.* 2 mM; succinate and PMS, *ca.* 8 mM and 0.17 mM, respectively. Other conditions are described in "EXPERIMENTAL PROCEDURE."

Carbon source	Fraction	Protein recovered (mg)	Dehydrogenase activity (μmoles/min/mg protein)			
			NADH	Lactate	α-GP	Succinate
Glucose	P[1]	14.6	0.31	0.11	0.28	1.15
	S[2]	88.4	0.19	0.11	0.10	0.046
Glycerol	P[1]	33.3	0.36	0.15	1.0	1.11
	S[2]	203	0.16	0.11	0.14	0.050
Lactate	P[1]	195	0.22	0.33	0.22	1.13
	S[2]	1020	0.067	0.31	0.068	0.049

[1] The precipitated fraction of the cells after centrifuged at 104,000×*g* for 40 min. [2] The supernatant fraction of the cells after centrifuged at 104,000×*g* for 40 min.

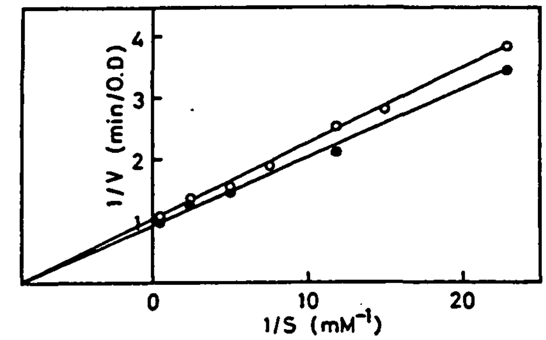

Fig. 1. Double reciplocal plots of lactate dehydrogenase activities of particulate fraction (●) and supernatant enzyme (○). The supernatant, prepared from lactate-grown cells as described in "EXPERIMENTAL PROCEDURE," was fractionated by polyethyleneglycol precipitation. The fraction between 4 and 12% (w/v) of polyethyleneglycol G-6,000 in the presence of 20 mM Tris-HCl buffer (pH 7.4) and 10 mM magnesium acetate was collected and used as the supernatant enzyme. The activity was measured as indicated in Table I.

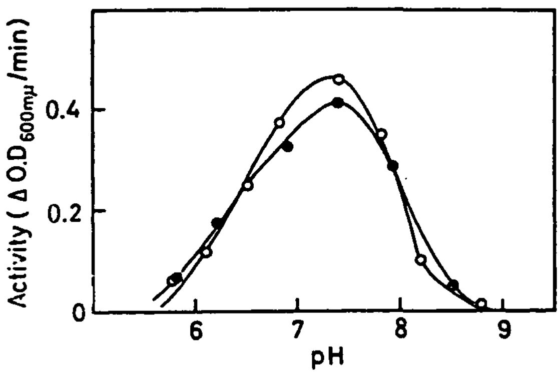

Fig. 2. Effect of pH on lactate dehydrogenase activities of particulate fraction (●) and supernatant enzyme (○). The enzyme samples and assay conditions were the same as described in Fig. 1. The reaction medium was adjusted to the indicated pH by adding a minimum amount of 1 N HCl or NaOH.

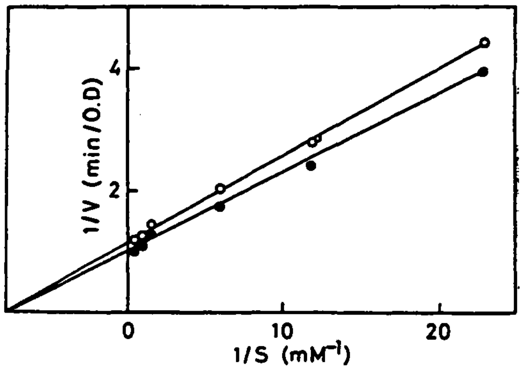

Fig. 3. Double reciprocal plots of α-GP dehydrogenase activities of particurate fraction (●) and supernatant enzyme (○). Assay system was the same as described in Fig. 1 except that α-GP was used instead of lactate.

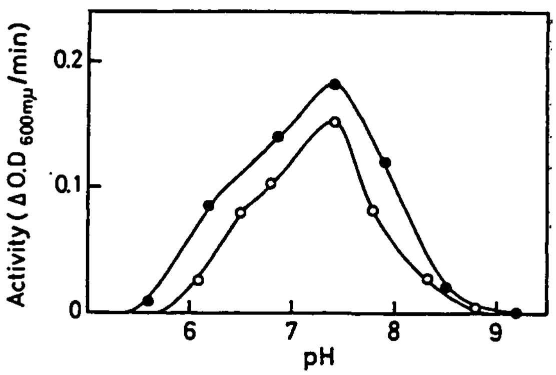

Fig. 4. Effects of pH on α-GP dehydrogenase activities of particulate fraction (●) and supernatant enzyme (○). Assay system was the same as described in Fig. 2 except that α-GP was used instead of lactate.

spectrum of the particulate fraction (membrane fragments) shown in Fig. 5 (curve A) indicates the presence of a cytochrome of the *b*-type and flavoprotein(s) in the membrane fragments of *P. arabinosum*. No other types of cytochrome could be detected in this spectrum. No spectral change was induced when CO was added to the dithionite-reduced sample. Both the *b*-type cytochrome and the flavoprotein were reduced upon addition of α-GP as shown in Fig. 5 (curve B). Similar degrees of reduction of the electron carriers were also observed upon addition of NADH, lactate and succinate.

Assuming that these pigments possessed the same molar extinction coefficients as the corresponding mitochondrial pigments, their concentrations in the membrane fragments were calculated from the difference spectrum. The results obtained are summarized in Table II, together with the iron and phospholipid contents which were colorimetrically measured.

Relatively high concentrations of the components were observed in the membrane fragments from *P. arabinosum* cells grown on different carbon sources. Most of the iron found in the membrane fragments was regarded as non-heme iron, in view of the relatively low cytochrome contents.

Fumarate as Terminal Electron Acceptor—As shown in Fig. 6 (curve A), the membrane fragments catalyzed the oxidation of NADH under aerobic condition and addition of fumarate accerelated the rate of NADH oxidation. When the membrane fragments were prepared unadequately or were aged, the rate of NADH oxidation by oxygen increased and its stimulation by fumarate was abolished. As shown in Fig. 6 (Curve B), NADH oxidation was fully dependent on fumarate under anaerobic conditions. As summarized in Table III, similar results were observed for lactate and α-GP oxidation. Pyruvate formation from lactate was fully dependent on fumarate under anaerobic conditions and partially dependent under aerobic conditions. DHAP formation from α-GP under anaerobic conditions was dependent on fumarate. Under aerobic conditions DHAP formation occurred in the absence of fumarate. In these experiments succinate formation from fumarate was qualitatively confirmed by gas chromatography after drying the reaction mixture and esterification with diazomethane in ether (*23*). The detection of a small amount of malate, besides succinate and fumarate, was probably due to contamination of the membrane fragments by fumarase [EC 4.2.1.2]. It is thus apparent that *P. arabinosum* possesses an electron transfer system which utilizes fumarate as a terminal oxidant.

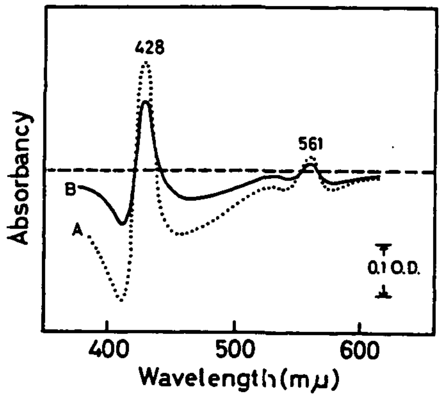

Fig. 5. Difference spectra of the membrane fragments of *P. arabinosum*. Both sample and reference cuvettes (10 mm light path) contained 5.8 mg protein/ml of the membrane fragments from glycerol-grown cells suspended in 50 mM Tris-HCl buffer (pH 7.4). ----, base line; ——, α-GP reduced minus oxidized; ······, dithionite reduced minus oxidized.

Oxidase Activity of Membrane Fragments—The oxygen uptake by the membrane fragments was measured in the presence of various substrates. As shown in Table IV, NADH and α-GP were oxidized rapidly in accordance with the results shown in Fig. 6 and Table III. NADH oxidation was inhibited by cyanide but not by azide. Oxidation of α-GP was inhibited by neither cyanide nor azide. Lactate was slowly oxidized and the activity was again

TABLE II. Contents of redox components and phospholipid in the membrane fragments. Flavin and cytochrome *b* contents were calculated from experiments similar to that shown in Fig. 5 according to the method of Chance (*19*). Total iron was determined colorimetrically according to the method of Doeg and Ziegler (*20*), with a slight modification. Phospholipid was extracted by the method of Folch (*21*) and determined according to the method of Bartlett (*22*).

Carbon source	Redox components (mμmoles/mg protein)			Phospholipid (mg/mg protein)
	Flavin	Cyt. *b*	Total Fe	
Glucose	1.33	1.31	96	0.22
Glycerol	1.30	0.75	93	0.22
Lactate	1.42	1.32	56	0.21

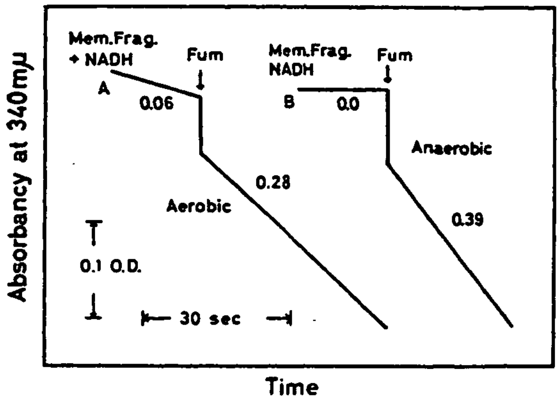

Fig. 6. NADH oxidation by oxygen and fumarate as terminal electron acceptors. NADH oxidation of the membrane fragments (0.5 mg protein in curve A and 0.91 mg protein in curve B) from glycerol-grown cells was spectrophotometrically measured in the standard reaction medium (2.5 ml). Final concentrations of NADH and fumarate was about 0.2 and 3 mM, respectively.

TABLE III. Dependency of lactate oxidation and α-GP oxidation on fumarate under anaerobic condition. In exp. 1 lactate (10 μmoles) was added to 2.5 ml of the standard reaction medium containing the membrane fragments (2.25 mg protein) from lactate-grown cells. After 20 min incubation, 0.5 ml aliquot was pipetted out and assayed for pyruvate according to the method of Shimizu (*14*), scaled down to 4 ml. In exp. 2, α-GP (1.6 μmoles) was added to 1.0 ml of the reaction medium containing the membrane fragments (0.19 mg protein) from lactate-grown cells. After 10 min incubation, 0.2 ml aliquot was pipetted out and assayed for DHAP according to the method of Lowry *et al.* (*15*), scaled up to 7 ml. The concentration of fumarate was 4 mM, when added.

Air	Fumarate	Exp. 1 Pyruvate formed (μmoles)	Exp. 2 DHAP formed (μmoles)
+	−	0.20	0.130
+	+	0.41	0.146
−	−	0.018	0.025
−	+	0.27	0.111

TABLE IV. Respiratory rate of the membrane fragments and effects of azide and cyanide on it. Oxygen uptake of the membrane fragments (1.25 mg protein) from lactate-grown cells was polarographically measured in the standard reaction mixture (3 ml). Final concentration of each substrate was about 3 mM except NADH (0.4 mM) and TMPD (0.1 mM). Final concentrations of NaN_3 and KCN were 4 mM and 1 mM, respectively.

Substrate	Oxygen uptake (μatoms/min/mg protein)	% Inhibition by	
		NaN_3	KCN
α-GP	0.145	9	10
NADH	0.135	28	97
Lactate	0.009	0	3
Succinate	0.0	—	—
Ascorbate plus TMPD	0.091	34	0

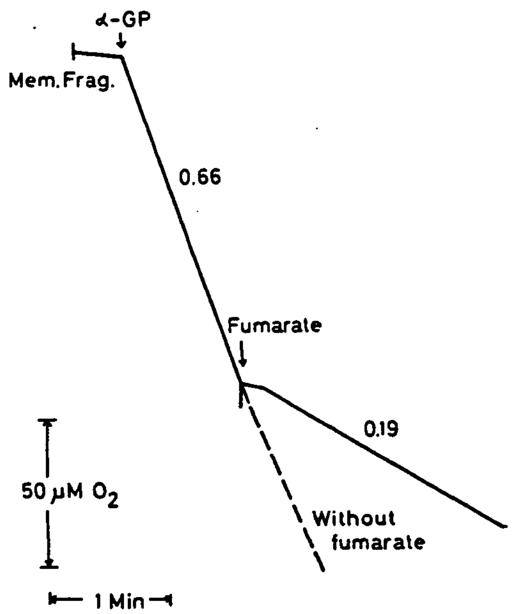

Fig. 7. Effect of fumarate on oxygen uptake by the membrane fragments. Oxygen uptake of the membrane fragments (1.0 mg protein) from glycerol-grown cells was polarographically measured in the standard reaction medium (3 ml). Final concentrations of α-GP and fumarate were about 3 and 5 mM, respectively.

inhibited by neither cyanide nor azide. Oxidation of ascorbate plus TMPD, though stimulatory effect of TMPD on ascorbate oxidation was slight in this case, was insensitive to cyanide and azide. Succinate, pyruvate, malate, formate and citrate were not oxidized significantly. As shown in Fig. 7, oxygen uptake with α-GP as substrate was largely blocked upon addition of fumarate. Since formation of DHAP was not blocked by fumarate as already shown in Table III, it was likely that the addition of fumarate caused a change in terminal electron acceptor from oxygen to fumarate. Oxygen uptake with NADH or lactate as substrate was similarly blocked by addition of fumarate. Thus the electron transfer system of *P. arabinosum* seems to use fumarate in preference to oxygen as terminal electron acceptor.

TABLE V. Effects of inhibitors on NADH-fumarate reductase and NADH oxidase activities. Assay conditions were the same as in Fig. 6 except that the membrane fragments (0.22 mg protein) from glycerol-grown cells were pre-incubated with the inhibitor for 5 min at 25°C and the reaction was started by adding NADH.

Inhibitor	(mM)	Inhibition (%) Electron acceptor Fumarate	O_2
Amytal	(1.0)	33	35
Amytal	(4.0)	70	60
Rotenone	(0.06)	35	33
Rotenone	(0.13)	42	44
Dicumarol	(0.16)	67	61
Dicumarol	(0.4)	90	89
Piericidin A	(0.0021)	74	73
Piericidin A	(0.011)	98	98
o-Phenanthroline	(0.8)	52	50
o-Phenanthroline	(2.0)	74	73
PCMB	(0.01)	74	65
PCMB	(0.02)	98	98
$CdCl_2$	(0.3)	95	94
KCN	(1.0)	13	74
KCN	(2.0)	43	89
NaN_3	(2.0)	5	16
NaN_3	(4.0)	18	20
Antimycin A	(0.03)	3	3
NOQNO	(0.07)	14	—

Effects of Inhibitors—As shown in Table V, amytal, rotenone, dicumarol, piericidin A, *o*-phenanthroline, PCMB and $CdCl_2$ were found to block both NADH-fumarate reductase and NADH oxidase activities of the membrane fragments. On the contrary, cyanide blocked only NADH oxidase activity but did not inhibit NADH-fumarate reductase activity appreciably. Azide caused only slight inhibition on both activities. Neither antimycin A nor NOQNO was found to be inhibitory. As shown in Table VI, neither amytal, dicumarol nor piericidin A was found to block the two primary dehydrogenases; neither DCIP reduction by NADH nor that by succinate plus PMS was inhibited. An appreciable inhibition of DCIP reduction by NADH occurred, when PCMB was added at a final concentration of 20 μM which caused complete inhibition in NADH oxidation and NADH-fumarate reduction. DCIP reduction by succinate *via* PMS was appreciably (36%) inhibited by the addition of 2 mM *o*-phenanthroline which caused 74% inhibition on NADH-fumarate reduction.

TABLE VI. Effects of inhibitors on NADH dehydrogenase and succinate dehydrogenase activities. Assay conditions were the same as in Table I except that the membrane fragments (0.22 mg protein in NADH dehydrogenation and 0.11 mg protein in succinate dehydrogenation) from glycerol-grown cells were preincubated with the inhibitor for 5 min at 25°C and the reaction was started by adding NADH or PMS.

Inhibitor	(mM)	Inhibition (%) NADH	Succinate
Amytal	(4.0)	17	—
Dicumarol	(0.24)	—	0
Dicumarol	(0.4)	20	—
Piericidin A	(0.0008)	10	0
o-Phenanthroline	(2.0)	0	36
o-Phenanthroline	(4.0)	—	55
PCMB	(0.02)	37	0

TABLE VII. Effects of inhibitors on lactate oxidation and α-GP oxidation by fumarate. Assay conditions were similar to those described in Table III. The membrane fragments from lactate-grown cells used were 0.41 mg protein in lactate oxidation and 0.10 mg protein in α-GP oxidation. The condition of preincubation was the same as in Table V.

Inhibitor	(mM)	Inhibition (%)	
		Lactate	α-GP
Amytal	(5.0)	75	17
Rotenone	(0.16)	0	18
Piericidin A	(0.0025)	2	17
o-Phenanthroline	(2.0)	59	24
PCMB	(0.08)	70	24

As shown in Table VII, rotenone and piericidin A did not appreciably inhibit both the oxidations of lactate and α-GP by fumarate. Amytal and PCMB inhibited lactate oxidation by fumarate appreciably and did not inhibit α-GP oxidation by fumarate. Since lactate dehydrogenase (DCIP assay) of *P. pentosacium* was inhibited by amytal and PCMB (*11*), the present inhibition of lactate oxidation may be due to a blocking of the lactate dehydrogenase segment of the electron transfer system. On the other hand, weak inhibition by o-phenanthroline may be due to an inhibitory effect of this chelator on succinate dehydrogenase, which has been shown in Table VI.

It is thus likely that the site(s) which is very susceptible to various inhibitors such as amytal, rotenone, piericidin A, PCMB, dicumarol and o-phenanthroline is localized in the NADH-cytochrome *b* segment of the electron transfer system of *P. arabinosum*. Similar susceptibility of the NADH-cytochrome *b* region (site 1) to the inhibitors was already reported in mammalian mitochondria (*24–26*) and in such bacterial membrane systems as that of *Micrococcus denitrificans* (*27*).

DISCUSSION

The present study has shown that the membrane fragments obtained from *P. arabinosum* contain NADH dehydrogenase, α-GP dehydrogenase, lactate dehydrogenase, succinate dehydrogenase (fumarate reductase), a *b*-type cytochrome, a flavin, and non-heme iron. Work now in progress in this laboratory also suggest the presence of a light-sensitive menaquinone-like factor. These components constitute a particular form of electron transfer system where succinate dehydrogenase probably acts as fumarate reductase* and NADH, lactate and α-GP oxidations are coupled with fumarate reduction. These facts, together with the effects of various inhibitors, suggest that an electron transfer system shown in Fig. 8 is functional in *P. arabinosum*. This scheme resembles those reported for mammalian mitochondria (see *28*, for review) and bacterial systems (*27*, *29*), although the *Propionibacterium* system seems to lack the chain of multiple cytochromes linking the system to oxygen. Although the spectrophotometric data presented do not indicate the occurrence of cytochrome *a* or *o* in the membrane fragments, it is not possible to exclude the possibility of the existence of these cytochrome in minute amounts. Active oxygen uptake can, however, be observed with both the membrane fragments

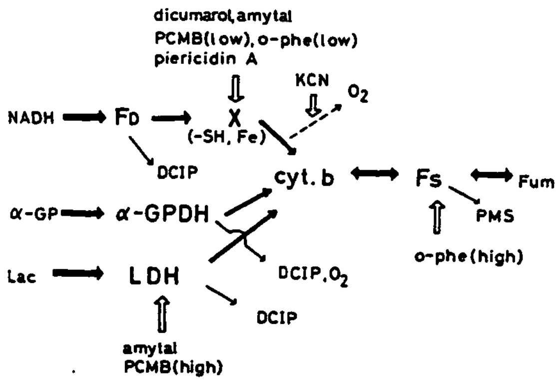

Fig. 8. A postulated scheme of the electron transfer pathway in *P. arabinosum*. ➡ indicates the flow of electron. ⇨ shows the site of inhibition. Abbreviations: F_D, NADH dehydrogenase; Fs, succinate dehydrogenase; LDH, lactate dehydrogenase; α-GPDH, α-GP dehydrogenase.

* Fumarate reductase activity of the partially purified succinate dehydrogenase from *P. pentosacium* has been reported by Lara (*10*).

TABLE VIII. Respiratory rate of whole cells of *P. arabinosum* with various substrates. *P. arabinosum* cells grown on glycerol medium were kept frozen after harvested and washed as described in "EXPERIMENTAL PROCEDURE." Before use, the cells were washed with 20 mM Tris-HCl buffer (pH 7.4) to eliminate the endogenous respiration. Oxygen uptake of the cells (1.8 mg) was measured in the standard reaction medium (3 ml). Final concentration of each substrate was 5–10 mM except glycerol (0.8 mM).

Substrate	Q_{O_2} (μatoms/min/mg dry wt.)
None	2
Glycerol	63
Glucose	22
Lactate	18
Propionate	17
Citrate	2
Malate	2
Succinate	2
Fumarate	3
α-Oxoglutarate	4
Glutamate	5
Pyruvate	4

(Table IV) and whole cells. As summarized in Table VIII, rather restricted organic compounds such as glucose, glycerol and lactate can serve as substrates for oxygen uptake. Carboxylic acids involved in Krebs cycle do not significantly support oxygen uptake. Such behavior is in contrast with that of *P. pentosacium* which has been reported to oxidize various carboxylic acids involved in Krebs cycle (*9*). It seems likely that *P. arabinosum* is less adapted to aerobic life than *P. pentosacium*.

Oxygen uptake by the membrane fragments is not sensitive to cyanide and azide, except that NADH oxidation is blocked by cyanide. Furthermore, although the dehydrogenations of NADH, α-GP, lactate and succinate can proceed at appropriate rates (Table I), oxygen uptakes with NADH and α-GP are much faster than those with lactate and succinate as substrates (Table IV). These observations suggest that oxygen uptake in this case is catalyzed not by a usual terminal cytochrome oxidase but by a flavoprotein oxidase as in the cases of NADH (*30*) and α-GP oxidations (*31*) in lactic acid bacteria. However, K_m of this "oxidase" of *P. arabinosum* for oxygen is far lower than those for usual flavoprotein oxidases, and production of H_2O_2 is not detectable. More work is obviously needed to elucidate the nature of the "oxidase" in *P. arabinosum*.

Fumarate seems to serve predominantly as a terminal electron acceptor; its addition results in repression of oxygen uptake by α-GP and other substrates (Fig. 7). The electron transfer system involved in fumarate reduction is localized in the particulate fraction and probably serves as a redox system operating in propionic acid fermentation. Thus this fermentation takes place by a cooperation of the soluble fermentation enzymes studied by Wood *et al.* (*3–7*) and the membrane-bound redox system reported in the present paper. It is of interest that the fermentation process intrinsically involves a membrane-bound electron transfer system. We may call the membrane-catalyzed process "fumarate respiration" following the terminology of nitrate respiration (*32*).

In contrast to the *P. arabinosum*, cytochrome-independent electron transfer systems of *Streptococcus faecalis* (*33, 34*) and of *Micrococcus lactilyticus* (*35*) are known to use fumarate as terminal electron acceptor. However, those systems are localized in the soluble fraction of the cells and seem to be mediated by FAD (*33, 35*). The present fumarate-reducing system of *Propionibacterium* seems to be distinct from those of *S. faecalis* and *M. lactilyticus*. A fumarate respiration system similar to that in *Propionibacterium* may also be operative in succinate-producing strict anaerobes such as *Cytophaga succinicance* (*36*) and *Bacteroides ruminicola* (*37*). In *B. ruminicola* the occurrence of *b*-type cytochrome has actually been reported (*37*).

Large amounts of lactate dehydrogenase and α-GP dehydrogenase are found in the soluble as well as particulate fractions of *P. arabinosum* (Table I). Since K_m values for α-GP and lactate and pH-activity curves of the dehydrogenase activities in the soluble fraction are the same with those in the membrane fraction, the same enzyme are probably present in both fractions, rather than isozymes. Studies on the soluble enzymes are in progress in our laboratory.

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