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ELECTRON-TRANSPORT SYSTEM OF *VIBRIO SUCCINOGENES*

I. ENZYMES AND CYTOCHROMES OF THE ELECTRON-TRANSPORT SYSTEM

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SUMMARY

Enzyme systems responsible for the oxidation of formate and H_2 by fumarate and O_2 have been demonstrated and partially characterized in cell-free extracts of the cytochrome containing anaerobic vibrio, *Vibrio succinogenes*. The formic dehydrogenase and hydrogenase are similar to those found in *Escherichia coli*. Oxidase and peroxidase systems involving formate and H_2 were demonstrated. Pyridine nucleotides are not involved in any of these systems. During the oxidation of formate by O_2, small amounts of H_2O_2 are formed.

Cytochromes of the *b* and *c* types are shown to be present in cells grown with formate as an energy source and either fumarate or nitrate as an electron acceptor. Some of the chemical characteristics of the cytochromes were determined. The protoheme of the cytochrome of the *b* type was isolated and identified. The cytochrome of the *c* type was partially reduced by ascorbate. Both cytochromes could be reduced by H_2 or formate and the cytochrome of the *b* type could be oxidized by fumarate suggesting a catalytic role for this cytochrome in fumarate reduction. Both cytochromes were partially reduced by succinate.

INTRODUCTION

The isolation and description of an anaerobic, cytochrome containing vibrio, *Vibrio succinogenes*, have been recently reported[1]. The organism gains energy for anaerobic growth by coupling the oxidation of H_2 or formate to the reduction of electron acceptors such as fumarate or nitrate. The organism is capable of limited growth with O_2 as an electron acceptor only if the O_2 concentration is kept below 4% in the gas phase. *V. succinogenes* contains cytochromes spectrally similar to those of the *b* and *c* types, and resting cells have been shown to reduce fumarate and nitrate with H_2 as the electron donor[1]. It was of interest to characterize the electron-transport systems in this organism in order to be able to outline the role of the cytochromes in these anaerobic oxidation–reduction reactions.

The present communication reports the partial characterization of the enzyme systems which oxidize formate and H_2 and reduce fumarate, NO_3^- and O_2. The

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cytochromes are also partially characterized, both with regard to some of their chemical characteristics and with regard to some of the substrate-linked oxidation–reduction reactions in which they are able to participate.

METHODS AND MATERIALS

The growth medium used in these studies has been previously described[1] and contained formate as an energy source, fumarate as an electron acceptor and thioglycolate as a reducing agent. For the studies in which the cytochrome content of the extracts was quantitatively determined; the medium was modified by increasing the level of formate to 0.6 % and K_2HPO_4 to 1 %. When KNO_3 was used as an electron acceptor, it was substituted for fumarate at a level of 0.15 %. Cells were obtained by inoculating 20 l of medium, which contained either fumarate or NO_3^- as an electron acceptor, with a 1-l culture which had been grown in an identical medium for 12 h. Attempts were made to keep the concentration of O_2 at a minimum by placing this medium in an almost completely filled carboy. After 13 h, the 20 l were centrifuged in a Sharples centrifuge and washed twice with 0.01 % β-mercaptoethanol. The cells were suspended in 20–40 ml of 0.01 % β-mercaptoethanol and exposed to sonic oscillation for 30–45 min in a 10-kc Raytheon sonic oscillator. During sonic oscillation, the cells were kept in a H_2 atmosphere at a temperature of 1–5°. The broken cells were centrifuged at 8000 × *g* for 20–30 min to remove remaining whole cells and some of the large debris. The supernatant extract was used in the experiments to be described unless otherwise mentioned.

H_2 and O_2 consumption and CO_2 evolution were assayed by conventional manometric methods. H_2O_2 was assayed by the method of PATRICK AND WAGNER[2] as modified by DOLIN[3]. Protein was determined by the method of LOWRY *et al.*[4].

Spectra were obtained with a Cary automatic-recording spectrophotometer. Anaerobic spectrophotometric experiments were conducted in cuvettes which had been fused with sidearms from Thunberg tubes and which were evacuated and flushed with helium at least twice to remove oxygen.

The method used for the extraction and characterization of the heme of the cytochrome of the *b* type was a combination and modification of the methods of BASFORD *et al.*[5] and RAWLINSON AND HALE[6] and is similar to the method described by MOSS[7]. Extracts of cells grown on fumarate or nitrate containing 76 mg of extract protein were lyophilized and the powder was quantitatively transferred to centrifuge tubes with 40 ml of cold acetone (0°). After centrifugation, the precipitated protein was washed with 40 ml of a cold solution of chloroform and methanol (2:1, v/v) and again with 40 ml of cold acetone. The protoheme was extracted from the washed protein by mixing with 40 ml of cold acetone containing 1 % of 2.4 *N* HCl. This extraction was repeated twice, and the pooled acid–acetone extract was concentrated under vacuum to a volume of about 20 ml. This concentrate was extracted first with 25 ml and then with 10 ml of acidic ether (1 vol. 1.5 *N* HCl, 4 vol. ether) to purify the ether-soluble protoheme. The ether layers were combined and washed first with 30 ml and then with 20 ml of 0.5 *N* HCl and finally with 20 ml of 3 % NaCl. The ether was removed under vacuum and the residue was suspended in 3.5 ml of pyridine and 3.5 ml of 0.2 *N* KOH. It was necessary to centrifuge some preparations at this point to remove a small amount of white precipitate which formed after the

addition of KOH. This acid–acetone extraction method may be useful in examining extracts of other microorganisms especially when it is desirable to detect a small amount of cytochrome *b* which is masked by the presence of a large amount of cytochrome *c*.

In order to examine the cytochrome content of the residue remaining after acid–acetone extraction, the protein was suspended in 15 ml of a solution of equal volumes of pyridine and 0.2 *N* KOH by grinding in a tissue homogenizer.

RESULTS

H_2 oxidation by methylene blue, benzyl viologen, fumarate and nitrate

Table I illustrates the rate of H_2 oxidation by cell-free extracts with various electron acceptors. The ability of methylene blue and benzyl viologen to serve as acceptors for the uptake of H_2 demonstrates the presence of the enzyme hydrogenase.

TABLE I

RATE OF H_2 AND FORMATE OXIDATION WITH VARIOUS ELECTRON ACCEPTORS

The H_2 oxidation rate is measured as μl H_2 uptake/min/mg protein at 37°. Warburg cups contained 47 m*M* K_2HPO_4 (pH 7.0), 0.15 mg β-mercaptoethanol, extract of fumarate-grown cells, and 6.3 m*M* methylene blue, fumarate or nitrate or 3.2 m*M* benzyl viologen in a total volume of 3.15 ml. The gas phase was H_2. The formate oxidation rate is measured as μl CO_2 produced/min/mg protein. Conditions as above except 3.3 m*M* formate, 111 m*M* K_2HPO_4 (pH 6.2) and a N_2 gas phase was used.

Acceptor	*H_2 oxidation rate*	*Formate oxidation rate*
None	0	0.0
Methylene blue	332	2.9
Benzyl viologen	17	1.5
Fumarate	77	1.5
Nitrate	0	0.0

The presence of another enzyme, fumarate reductase, and the ability of these extracts to couple the hydrogenase to the fumarate reductase is indicated by the use of fumarate as acceptor. Previous work[1] has shown that fumarate is quantitatively reduced to succinate in whole cells. Although in whole cells H_2 reduced NO_3^- to NH_3 (see ref. 1), attempts to prepare sonic extracts which could catalyze NO_3^- reduction with H_2 were unsuccessful. If catalytic quantities of benzyl viologen were added to these extracts, NO_3^- did serve as acceptor demonstrating that not the NO_3^- reductase but some intermediate site was destroyed during preparation of extracts. Apparently, benzyl viologen can substitute for this destroyed site. The addition of $FeSO_4$, Na_2MoO_4, menadione bisulfite, ATP or flavin mono- or dinucleotide did not restore the couple between H_2 and NO_3^-. H_2 did not reduce DPN or TPN. DPNH or TPNH were not oxidized by fumarate or NO_3^-.

Formate oxidation by methylene blue, benzyl viologen and fumarate

The rate of CO_2 evolution from formate in the presence of electron acceptors is also shown in Table I. A formic dehydrogenase, similar to the one found in *E. coli*

by PECK *et al.*[8], is indicated by the use of benzyl viologen and methylene blue as acceptors. A couple between the formic dehydrogenase and the fumarate reductase is demonstrated by the use of fumarate as acceptor. The lack of gas evolution in the absence of an electron acceptor demonstrates a lack of a hydrogenlyase system. Hydrogenlyase, therefore, is not required for the couple between formate and fumarate. DPN is not reduced with formate as substrate.

Oxidation of formate with O_2 as acceptor

Formate can also be oxidized by O_2 in the presence of these extracts. It has been observed that appreciable amounts of H_2O_2 are formed during this reaction although no stoichiometric relationship between H_2O_2 production and O_2 consumption could be obtained. In several experiments with different extracts, the amount of H_2O_2 which could be detected following the completion of O_2 consumption varied between 0.056 and 0.22 μmoles of H_2O_2 produced per μmole of O_2 consumed. These values for H_2O_2 may be somewhat low since reducing agents which can react with H_2O_2 such as β-mercaptoethanol were present in the Warburg cups. O_2 consumption and H_2O_2 production with formate as substrate will be more fully detailed in the following paper.

Since much less than 1 μmole of H_2O_2 was formed per μmole of O_2 consumed, the possibility was considered that a mechanism for disposal of H_2O_2, such as a peroxidative enzyme system, was present in extracts of this organism. Table II shows that a relatively weak system for the peroxidative oxidation of formate does exist in these extracts. It is probable that the rate of the H_2O_2-dependent CO_2 evolution from formate shown in Table II does not represent the maximum rate of the peroxidase system since separate studies have shown that the formic dehydrogenase, which is probably a required enzyme for the operation of the peroxidase system, is partially inhibited by the amounts of H_2O_2 added as substrate for this system. One further statement should be made with regard to the possible mechanism of the peroxidative decomposition of formate. Although the typical catalase which is found in most aerobic cells is known to catalyze this reaction[9], catalase cannot be demonstrated either in the cells[1] or in extracts of this organism. This latter fact is shown in Table II where no appreciable gas is evolved from H_2O_2 unless formate is also present. The gas evolved in the presence of both H_2O_2 and formate is completely absorbed by KOH.

TABLE II

PEROXIDATIVE OXIDATION OF FORMATE

Warburg cups contained 50 m*M* K_2HPO_4 (pH 6.0), 0.15 mg β-mercaptoethanol, 3.3 m*M* formate, 1.3 m*M* H_2O_2, 17.5 mg extract protein from fumarate-grown cells in a total volume of 3.0 ml. Formate and/or H_2O_2 were added from the sidearm to start the reaction. Reaction was measured at 37° with N_2 as gas phase. Boiled extract was prepared by heating for 10 min at 100°.

Additions	*CO_2 evolved (μl/20 min)*
Extract + formate	6
Extract + H_2O_2	9
Extract + formate + H_2O_2	60
Boiled extract + formate + H_2O_2	10

Oxidation of H_2 with O_2 as acceptor

The Knallgas reaction was also demonstrable in these extracts. When a reaction mixture, similar to the one used to assay formate oxidation described in Table I (without formate or electron acceptor), was placed in a Warburg cup and gassed with a mixture of 80% H_2 and 20% air, gas was taken up at the rate of 1.06 μl/min/mg extract protein. If H_2 alone, or air alone was the gas phase, no appreciable amount of gas was consumed. It was also possible to demonstrate the peroxidative oxidation of H_2. When a reaction mixture similar to that shown in Table II (minus formate) was equilibrated under H_2, no gas was consumed until 3.5 μmoles of H_2O_2 were added from the sidearm. H_2 was then consumed at the rate of 0.32 μl/min/mg extract protein. No gas was consumed if the extract was boiled for 10 min. A peroxidative oxidation of H_2 has been demonstrated in *Acetobacter peroxydans* (see refs. 10 and 11).

We were not able to demonstrate the oxidation of DPNH or TPNH by O_2 with these extracts.

Succinic dehydrogenase activity of extracts

Since the reduction of fumarate indicated the presence of a fumarate reductase, it was of interest to see whether succinic dehydrogenase activity could be demonstrated. Attempts to demonstrate O_2 uptake in the presence of succinate and phenazine methosulfate or the reduction of ferricyanide by succinate were unsuccessful. These extracts were, however, able to rapidly reduce nitro-blue tetrazolium and neo-tetrazolium salts with succinate as substrate. Since the primary function of this enzyme in this organism is to reduce fumarate, and since we were unable to demonstrate succinate oxidation by the usual acceptors such as phenazine methosulfate and ferricyanide, this enzyme would appear to be similar to the "anaerobic" type of succinic dehydrogenase such as the one found in *Micrococcus lactilyticus* by PECK *et al.*[12].

Localization of enzymes

In an attempt to determine whether the hydrogenase and the enzyme system which carries out the reduction of fumarate by H_2 were located on heavy particles, an extract was centrifuged at 144000 $\times$ *g* for 1 h and the pellet was suspended in a volume of 0.01 % β-mercaptoethanol equal to the volume of extract from which it had been obtained. The pellet and the supernatant solution contained about equal amounts of hydrogenase activity as assayed with methylene blue under conditions similar to those described in Table I. The pellet contained more than 3 times the activity of the enzyme system which coupled H_2 to fumarate, as the supernatant solution, suggesting that the enzyme system responsible for fumarate reduction is mostly located on large particles. This latter system is also located on large particles in *E. coli* (see ref. 13).

Cytochrome content of extracts of fumarate- and nitrate-grown cells

Fig. 1 illustrates spectra of extracts of cells grown on fumarate or NO_3^- which were obtained by placing equal amounts of aerated extract in two cuvettes and reducing the sample cuvette with a few crystals of hydrosulfite, using the other cuvette as a blank. A separate experiment showed that without the addition of hydrosulfite, the cytochromes were in the oxidized form since no reduced cytochrome

peaks were visible when the spectrum was recorded without hydrosulfite, using a cuvette containing H_2O as the blank. As seen in Fig. 1, extracts of fumarate-grown cells exhibit peaks in the visible region at 552 and 523 mμ, indicative of a cytochrome of the *c* type. Shoulders on these peaks at approx. 560 and 529 mμ indicate a cytochrome of the *b* type with spectral properties similar to the cytochrome b_1 of *Escherichia coli*. The large peak in the Soret region is undoubtedly a composite of the peaks due to both cytochromes *b* and *c*. The presence of additional cytochromes cannot be detected in these spectra. In contrast to the extract of fumarate-grown cells, the extract of nitrate-grown cells exhibits greater absorption in the regions typical of cytochrome *c*, but does not exhibit obvious shoulders in the regions typical of cytochrome *b*. Using the extinction coefficients suggested by CHANCE[11] for mammalian cytochromes, we calculated that there were 1.6 μmoles of cytochrome *c* and 1.1 μmoles of cytochrome *b* per gram protein in the extract of fumarate-grown cells and 3.9 μmoles of cytochrome *c* per gram protein in the extract of nitrate-grown cells.

The inability to establish the presence of cytochrome *b* in extracts of nitrate-grown cells was of special interest for two reasons. Experiments reported below demonstrate that reduced cytochrome *b* is actively oxidized by fumarate in the presence of extracts of fumarate-grown cells. It has also been found that resting-cell suspensions and extracts of cells grown on nitrate actively reduce fumarate with H_2. These observations suggested that a more critical test for the presence or absence of cytochrome *b* in extracts of nitrate-grown cells should be applied in order to assess the role of cytochrome *b* in electron transport to fumarate in *V. succinogenes*. Therefore, extracts of fumarate- and nitrate-grown cells were extracted with acid–acetone by the procedure described under METHODS AND MATERIALS in order to extract any protoheme, the typical ether-soluble prosthetic group of cytochrome *b*. This procedure would not be expected to extract any heme of the type associated with cytochrome *c*.

The resulting spectrum of the pyridine hemochromogen of the acid–acetone extractable heme of the nitrate-grown cells is shown in Fig. 2 and is compared to the

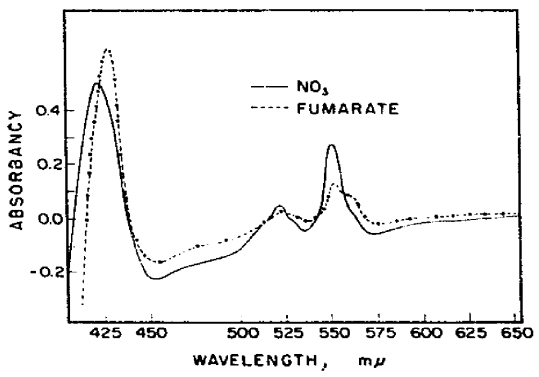

Fig. 1. Difference spectra of extracts of fumarate-grown and NO_3^--grown cells. Cuvettes contained 4.3 mg of fumarate or NO_3^- extract protein per ml and 71 m*M* K_2HPO_4 (pH 7.1) in a total volume of 3 ml. The extracts were reduced with a few crystals of sodium hydrosulfite.

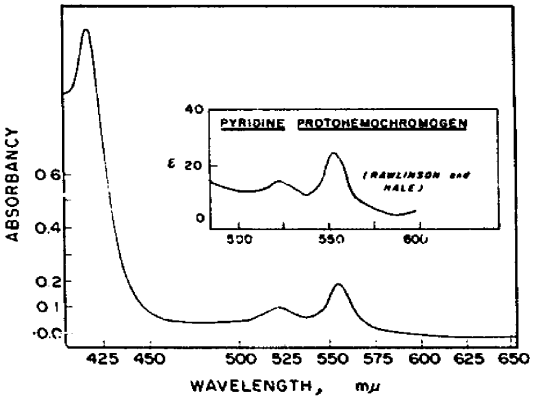

Fig. 2. The pyridine hemochromogen of the acid–acetone extractable pigment. These spectra were obtained by extracting the heme from 76 mg of extract protein from cells grown on NO_3^- and dissolving the heme in 7.0 ml of pyridine plus KOH as described in METHODS AND MATERIALS. The hemochromogen was reduced with a few crystals of hydrosulfite.

pyridine hemochromogen of protoheme which was isolated from the cytochrome *b* of *Corynebacterium diptheriae* by RAWLINSON AND HALE[6]. All spectra have peaks at 556 and 524 mμ in the visible region. An identical spectrum was obtained when an equal amount of extract protein from fumarate-grown cells was analyzed.

The cytochrome content of the protein residue remaining after acid–acetone extraction was examined by suspending the residue in pyridine and KOH as described under METHODS AND MATERIALS and recording the difference spectra with the sample cuvette reduced with a few crystals of hydrosulfite. The resulting spectra of the extracts of fumarate- and nitrate-grown cells are shown in Fig. 3. The spectra resemble those usually found for the pyridine hemochromogen of a cytochrome of the *c* type, with peaks in the visible at 549 and 514 mμ. It will be noted that the extract of nitrate-grown cells contains about 3 times as much of this pyridine hemochromogen as the extract of fumarate-grown cells. Applying the extinction coefficients suggested by BASFORD *et al.*[5] for the pyridine hemochromogens to the data in Figs. 2 and 3,

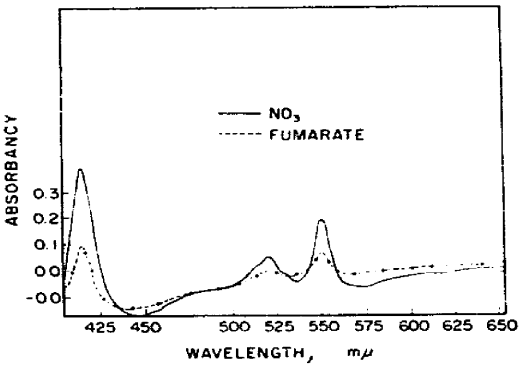

Fig. 3. Pyridine hemochromogen difference spectra of residues after acetone extraction. The residues remaining after extracting 76 mg of extract protein from cells grown on fumarate or NO_3^- with acid–acetone were suspended in 15 ml pyridine plus KOH as described in METHODS AND MATERIALS. One 3-ml cuvette was used as blank and the other was reduced with a few crystals of hydrosulfite.

there is 0.51 μmole of cytochrome *b* and 0.84 μmole of cytochrome *c* per gram protein in the extract of fumarate-grown cells and 0.55 μmole of cytochrome *b* and 2.4 μmoles of cytochrome *c* per gram protein of the extract of nitrate-grown cells. Thus, the extract of nitrate-grown cells contains approximately the same amount of cytochrome *b* as the extract of fumarate-grown cells.

Other properties of the cytochromes

The cytochrome pigments are not adsorbed on the NH_4^+ form of IRC-50 resin by procedures which have been used to adsorb cytochrome *c* prepared from heart muscle. The extracts of cells grown on fumarate were examined for the presence of any carbon monoxide-binding pigments by reducing the extract in two cuvettes with a few crystals of hydrosulfite and bubbling CO slowly through one cuvette for 1 min. When difference spectra were recorded using the cuvette without CO as the blank, no peaks were detectable. This result was interpreted to indicate that no appreciable amounts of CO-binding pigments such as cytochromes a_1, a_2, a_3, or *o* were present in this extract.

An attempt was made to determine whether or not the cytochromes were associated with particles in these extracts. A suspension of cells was disrupted by sonic oscillation for 15 min and the extract was then centrifuged at 8000 × *g* for 15 min to remove remaining whole cells and the largest debris. A spectrum was then recorded

using a cuvette containing H_2O as a blank. This extract was then centrifuged at 144000 × *g* for 1 h in the Spinco ultracentrifuge and a spectrum of the supernatant solution was recorded. It was found that centrifugation at 144000 × *g* had sedimented a little more than half of the absorption due to the *b* and *c* peaks. The pellet after this centrifugation was of a deep red color. These results indicate that at least half of the cytochromes of the *b* and *c* type are associated with heavy particles. Whether the cytochromes remaining in the supernatant solution are truly soluble, or are merely associated with smaller particles which cannot be sedimented at 144000 × *g* for 1 h, is a question which cannot be answered at this time.

Extraction of cytochrome c from acetone-dried cells

If fumarate-grown cells were acetone-dried according to the procedure suggested by GUNSALUS[15] and were then allowed to stand at 4° in 0.5% K_2HPO_4 (pH 7.0) for 4 h, the supernatant solution following centrifugation for 2 h at 15000 × *g* exhibited absorption maxima at approx. 552, 523 and 418 mμ, indicating that cytochrome *c* had been at least partially extracted from the acetone-dried cells by this procedure. This preparation of cytochrome *c* was shown to be free from cytochrome *b* since no protoheme could be extracted from this preparation by the acid–acetone extraction procedure described previously.

None of this preparation of cytochrome *c* was precipitated by centrifugation at 144000 × *g* for 3 h in the Spinco ultracentrifuge. The cytochrome was not adsorbed on the NH_4^+ form of IRC 50. If 20 μmoles of ascorbate were substituted for hydrosulfite as reducing agent for this preparation, the positions of the reduced peaks were identical, but the total absorption at the peaks was only 35–40% of that obtained with hydrosulfite. The spectra were determined in anaerobic cuvettes with ascorbate added from the sidearm to prevent any reoxidation of reduced cytochrome by O_2. Under these conditions, ascorbate gave nearly complete reduction of mammalian cytochrome *c*. The fact that only partial reduction is obtained with the vibrio cytochrome *c* would indicate either that the vibrio cytochrome *c* does not have as high an O-R potential as mammalian cytochrome *c*, or that there are more than one cytochrome *c* pigments in this preparation which differ in O-R potential, but not in absorption spectra. We have not investigated either of these possibilities any further. If all of the cytochrome *c* of this organism were of a similar low O-R potential as that found for the cytochrome c_3 of *Desulfovibrio desulfuricans* (see ref. 16), one would expect no reduction by ascorbate.

Enzymic reduction and oxidation of the cytochromes

Fig. 4 shows a difference spectrum obtained upon reduction of extracts with H_2 in anaerobic cuvettes. This spectrum is similar to the spectrum obtained by reduction with hydrosulfite both with regard to position and height of peaks. Fig. 4 also demonstrates that the addition of fumarate from the sidearm of the anaerobic cuvette to the extract reduced with H_2 causes the peaks due to reduced cytochrome *b* to almost completely disappear leaving a spectrum in which only the reduced cytochrome *c* is evident. The addition of H_2O instead of fumarate from the siderarm does not cause any spectral change. The fact that cytochrome *b* can be reduced by H_2 and oxidized by fumarate suggests a possible catalytic role of cytochrome *b* as an electron carrier between the hydrogenase and the fumarate reductase.

A similar spectrum to that obtained with H_2 can also be obtained if 10 μmoles of formate are added as reductant. When the anaerobic cuvettes in which the cytochromes have been reduced by H_2 or a small amount of formate are aerated, the peaks due to reduced cytochromes disappear, indicating that aeration causes an oxidation of the reduced cytochromes.

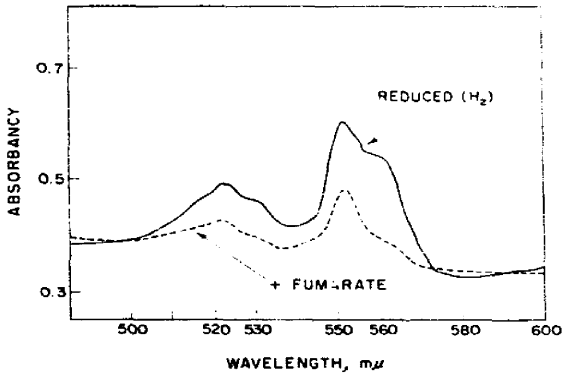

Fig. 4. Difference spectra of cytochromes reduced with H_2 and oxidized with fumarate. Cuvettes contained 91 mM K_2HPO_4 (pH 7.2), 1.2 ml of extract from fumarate-grown cells, and H_2O to 3.3 ml. The cuvette was a modified Thunberg cuvette with a sidearm. H_2 gas was bubbled slowly through the cuvette for 10 min. The sidearm (containing 0.3 ml of 0.02 M fumarate) was then connected, and the cuvette was evacuated, filled with H_2 and evacuated again. The gassing and evacuation process was repeated. Spectra were then recorded with an open cuvette with an identical reaction mixture as a blank. If helium was used instead of H_2 to gas the cuvettes, the cytochromes were not reduced. After recording the reduced spectrum, the sidearm containing the fumarate was tipped, and the spectrum rerecorded.

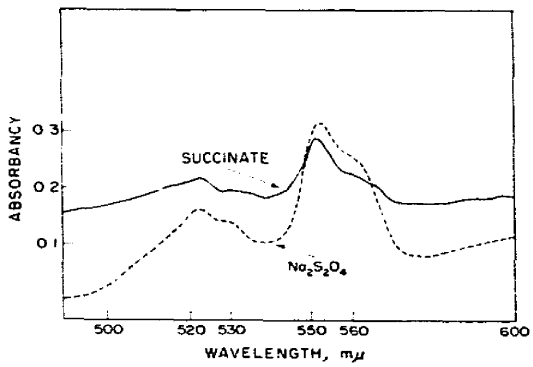

Fig. 5. Reduction of cytochromes by succinate and hydrosulfite. Reaction mixtures and the blank are the same as in Fig. 4. 6 μmoles of succinate were added from sidearm of cuvette which had been previously evacuated and regassed with helium three times. A few crystals of hydrosulfite were added to obtain the hydrosulfite-reduced spectrum.

The reduction of cytochromes by succinate and hydrosulfite is shown in Fig. 5. It can be seen that succinate gives only about 30–40 % reduction of cytochrome *b* and 50–60 % reduction of cytochrome *c* as compared with hydrosulfite. The addition of 40 μmoles of succinate does not increase the amount of cytochrome reduction. The reason for this incomplete reduction by succinate is not apparent, but the fact that the cytochrome *c* is more fully reduced than the cytochrome *b* suggests that the *c* is of a higher O-R potential than the *b*. The fact that both cytochromes are somewhat reduced by succinate suggests that neither cytochrome has an O-R potential far below that of the fumarate–succinate system.

We have not been able to demonstrate the reduction of cytochromes by DPNH under anaerobic conditions.

DISCUSSION

From the characteristics of the formic dehydrogenase and the hydrogenase which have been described, it would appear that these two enzymes are similar to enzymes

which have been previously found in *Escherichia coli* (see ref. 8) and certain other microorganisms[17]. Although the reduction of fumarate by H_2 has been previously reported in *E. coli* (see ref. 18), the reduction of fumarate by formate has apparently not been previously demonstrated in a microorganism which does not contain the formic hydrogenlyase system. Indeed, it has been postulated that in *E. coli* this latter reaction is mediated by the hydrogenlyase system[19].

Pyridine nucleotides would appear to play no part in the enzyme systems oxidizing formate or H_2 or reducing fumarate, cytochromes or O_2 since we have not been able to demonstrate either their reduction or oxidation by these substrates.

Although this organism has been designated as an anaerobe due to its inability to grow in air and because it exhibits maximum growth under anaerobic conditions in the presence of an electron acceptor such as fumarate, its cytochrome components appear to be similar to those usually found in aerobic forms of life. The cytochrome *b* of this organism has the same prosthetic group as the cytochrome *b* of mammalian tissue. The O-R potential of the cytochromes *b* and *c* would appear to be close to the potential of these cytochromes in mammalian tissues. There is no doubt that the majority of the cytochrome *c* pigment of this organism is of a much higher O-R potential than the low potential cytochrome c_3 of the anaerobe *D. desulfuricans* (see ref. 16). No appreciable amounts of cytochromes of the a_1, a_2, a_3, or *o* type which are usually associated with oxidase activity could be detected either in difference spectra or by analysis for CO-binding pigments. However, the possibility cannot be excluded that the methods used for the detection of these pigments were not sufficiently sensitive.

With regard to the physiological role of the cytochromes in fumarate reduction, the work presented in this paper indicates that cytochrome *b* may be involved as an electron carrier between the hydrogenase and the fumarate reductase since the cytochrome is reduced by H_2 or formate and oxidized by fumarate. Further evidence will be presented in the following paper which is also consistent with the suggestion that cytochrome *b* is a quantitatively important carrier in fumarate reduction in this organism. Cytochrome *c*, or at least the majority of the cytochrome pigment absorbing in the region typical of cytochrome *c*, appears not to be involved in fumarate reduction since it is not appreciably oxidized by fumarate.

Little can be said regarding the role of the cytochromes in NO_3^- reduction since we were unable to prepare cell-free extracts which could reduce NO_3^-. The fact that cells grown with NO_3^- as acceptor have a much higher cytochrome *c* content than cells grown on fumarate might reflect a physiological role for cytochrome *c* in NO_3^- reduction such as has been suggested for the cytochrome *c* of *Achromobacter fischeri* (see ref. 20).

The ability of these extracts to oxidize formate and H_2 with O_2 as terminal acceptor, the formation of an appreciable amount of H_2O_2 during the oxidation of formate and the presence of a peroxidative mechanism for the oxidation of formate and H_2 suggest that the mechanism for the oxidation of formate (and perhaps also H_2) may be a combination of an oxidase system which forms H_2O_2, and a peroxidase system which destroys H_2O_2. Further evidence of the existence of this oxidase–peroxidase system, for a role of the cytochromes in this system and for the importance of this system in the respiration of this organism, will be presented in the following paper.

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