Cyanide Resistance in *Achromobacter*

I. Induced Formation of Cytochrome $a_2$ and Its Role in Cyanide-Resistant Respiration

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ABSTRACT

ARIMA, KEI (University of Tokyo, Tokyo, Japan), AND TETUO OKA. Cyanide resistance in *Achromobacter*. I. Induced formation of cytochrome $a_2$ and its role in cyanide-resistant respiration. J. Bacteriol. 90:734-743, 1965.—By following the cytochrome concentrations during the growth cycle and under various conditions (aerobic, aerobic plus KCN, reduced aeration, anaerobic plus NaNO3 and KCN, anaerobic plus NaNO3) in *Achromobacter* strain D, a close relationship between the formation of cytochrome $a_1$ (and $a_2$) and the difficulty of oxygen utilization was demonstrated. Cytochrome $o$, which was the only oxidase found in aerobic log-phase cells, was present in bacterial cells grown under various conditions; the amount present had no relation to the degree of cyanide resistance. On the other hand, cytochrome $a_2$ (and $a_1$) was inducible, and a close relation was observed between the amount of cytochrome and resistance to cyanide. Spectrophotometric observations indicated that, among the cytochromes present in resistant cells, cytochrome $a_2$ could be oxidized most easily in the presence of cyanide and that cytochrome $b_1$ could be oxidized without the oxidation of cytochrome $a_1$. We concluded that cytochrome $a_2$ is a cyanide-resistant oxidase capable of catalyzing the oxidation of cytochromes in the presence of cyanide. Cytochrome $a_2$ is also resistant to azide, an inhibitor of cytochrome oxidase.

It was previously reported from this laboratory that a strain of *Achromobacter* was found to give adaptive growth and respiration in the presence of $10^{-3}$ m KCN (Mizushima and Arima, 1960a, b, c). Because of the increased content of each cytochrome, especially of cytochrome oxidases, it was postulated that cyanide-resistant respiration was caused by the increased amount of the oxidases (cytochromes $a_1$ and $a_2$) which were inhibited by cyanide. As the difference spectrum (reduced minus oxidized) obtained from cells grown in the presence of cyanide shows two distinct peaks at 595 and 625 mμ, it is necessary to determine which oxidase plays the main role in cyanide-resistant respiration. This report describes studies on the terminal electron-transport systems of this strain of *Achromobacter* under various growth conditions, and the role of cytochrome $a_2$ in cyanide-resistant respiration.

MATERIALS AND METHODS

Organism and growth media. A doubly auxotrophic mutant was induced by treatment of *Achromobacter* strain 7 (Mizushima and Arima, 1960a) with nitric acid according to the method of Kaudewitz (1959). This mutant (*Achromobacter* strain D), which requires isoleucine and adenine (or guanine) for growth, was used throughout this work.

Cells were cultivated at 30 C in four different ways: with vigorous aeration in a bouillon medium containing 0.5% meat extract, 1.5% peptone, 0.5% NaCl, and 0.5% K2HPO4, (pH 7.0) (referred to as the sensitive cells); with reduced aeration (one-tenth aeration); aerobically in the bouillon medium containing $10^{-3}$ m KCN (the resistant cells); and anaerobically in the bouillon medium containing 2.0% NaNO3 (nitrate-grown cells). Vigorous aeration was achieved by passing through the culture a volume of air per minute equal to the volume of the culture or by reciprocal shaking (120 oscillations per min) of 100 ml of the medium in 500-ml Sakaguchi flasks. Growth was measured turbidimetrically with a Kotaki nephelometer.

Preparation of cell-free extracts. Bacterial cells were harvested at the growth phase indicated, washed twice with 0.8% NaCl, and suspended in an appropriate volume of 0.1 m phosphate buffer, pH 7.4 (usually 50 mg (dry weight) of cells per ml); the cells were then exposed to sonic oscillation (Toyko Riko, 10 kc) for 10 min. The resultant turbid preparation was centrifuged at 10,000 × g for 10 min to remove cell debris and unbroken cells. The supernatant fluid was collected and used as crude cell-free preparation. For further fractionation,
Figure 1. Difference spectra of Achromobacter strain D grown under various conditions. (A) Cyanide-sensitive cells. (B) Cyanide-resistant cells. (C) Nitrate-grown cells. Solid lines, reduced minus oxidized difference spectra; dashed lines, reduced plus CO minus reduced difference spectra.

The supernatant fluid was again centrifuged at 105,000 × g for 1 hr in a Spinco ultracentrifuge (model L), and the reddish precipitate was collected (particulate fraction).

Oxygen uptake. The activity of the succinoxidase system was measured by conventional manometric techniques and by use of a rotatory platinum electrode (Yanagimoto Manufacturing Co., Kyoto, Japan). Inhibition by cyanide was measured manometrically with ROH equilibrated with cyanide in the center well, according to the method of Krebs (1935). The activity of the reduced nicotinamide adenine dinucleotide (NADH) oxidase system was measured spectrophotometrically as described by Mizushima and Arima (1960a).

 Succinic dehydrogenase activity. Succinic dehydrogenase was measured manometrically at 30 C with phenazine methosulfate as an acceptor or spectrophotometrically with 2,6-dichlorophenol-indophenol by the method of Guiditta and Singer (1959).

Measurement of nitrate and nitrite reductase
activities of cell-free preparations. Nitrate and nitrite reductase activities were measured by the increase or decrease of nitrite in the reaction mixture. The reaction was stopped by the addition of 10% cold perchloric acid. After separating the precipitate by centrifugation, nitrate was measured by adding to the supernatant fraction 0.5 ml of 1% sulfanilamide and 0.5 ml of 0.02% N-(1-naphthyl)-ethylene diamine. Absorbancy at 540 μm was measured in a Hitachi EPU-2 spectrophotometer.

Nitric oxide formation by resting cells. Evolution of nitric oxide from nitrite was measured manually with nitrogen as the gas phase, according to the method of Najjar and Allen (1954).

Spectrophotometric measurements. The difference spectra of cell-free preparations were recorded with a Cary recording spectrophotometer (model 14), with the use of 0.1 to 0.2 optical-density slide wire. A cuvette with a 1-cm light path was used. The width of the slit was maintained under the limit of 0.3 μm. Physiological reduction and oxidation of cytochromes were produced by adding to the cuvette a few milligrams of sodium succinate and bubbling with air or by adding a few milligrams of KNO3 or NaNO2. Oxidation by nitrate or nitrite was measured in a Thunberg-type cuvette with nitrogen as the gas phase. The cytochromes were also reduced chemically by adding to the cuvette a few milligrams of Na2S2O4 and were oxidized by K3Fe(CN)6.

Spectra were measured by use of the difference-spectra methods described by Chance (1954). Absorption spectra were measured against a similar cell-free preparation containing the oxidized cytochromes.

Table 1. Cytochrome contents of Achromobacter grown under various conditions

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Generation time</th>
<th>Maximal turbidity</th>
<th>Change in optical densitya of cytochrome</th>
<th>KCN concenb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>b1 (560-540)</td>
<td>a1 (595-610)</td>
<td>a2 (625-650)</td>
</tr>
<tr>
<td>Aerobic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log phase (sensitive cells)</td>
<td>0.5</td>
<td>300</td>
<td>0.0144</td>
<td>±d</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>1,200</td>
<td>0.0164</td>
<td>0.0010</td>
<td>0.0037</td>
</tr>
<tr>
<td>Reduced aeration (one-tenth aeration)</td>
<td>2-3</td>
<td>180</td>
<td>0.0255</td>
<td>0.0015</td>
</tr>
<tr>
<td>Aerobic plus 10^-2 M KCN (resistant cells)</td>
<td>1</td>
<td>600</td>
<td>0.0260</td>
<td>0.0020</td>
</tr>
<tr>
<td>Anaerobic plus 2% NO3^- (nitrate-grown cells)</td>
<td>2-3</td>
<td>200</td>
<td>0.0365</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

a The change in optical density, at the wavelengths (millimicrons) indicated in parentheses, was measured from the difference spectrum (Na2S2O4 reduced minus oxidized) per 10 mg of protein per ml per cm of light path.

b Calculated from the CO difference spectrum.

c Concentration of cyanide required for 50% inhibition of respiration. Experimental conditions were the same as in Table 4.

d May or may not be present.

The nomenclature of the cytochromes was that used previously (Mizushima and Arima, 1953c). The definition of cytochrome o was that of Castor and Chance (1959). Cytochrome o was measured from the peak at 572 μm to the trough at 554 μm, or from the peak at 418 μm to the trough at 432 μm in the CO difference spectra. Cytochrome α1 was measured from the peak at 416 μm to the trough at 610 μm in the reduced-oxidized spectra, but this estimation inevitably allows some error. Cytochrome α2 was measured from its maximum at 625 μm to the trough at 650 μm in the reduced-oxidized spectra.

Carbon monoxide spectra were obtained by bubbling the gas into a cell-free preparation reduced with Na2S2O4 and measuring the spectra against a reduced preparation. The bubbling was repeated until saturation occurred. Neutralized KCN (10^-2 M) was added to the indicated concentrations.

Protein. Protein was measured by the Folin method, as modified by Hagihara (1956).

Biochemical reagents. NADH2 (Na2), phenazine methosulfate, and 2-N-9-nonyl-hydroxyquinoline-N-oxide (HQNO) were purchased from Sigma Chemical Co., St. Louis, Mo.

Results

Cytochrome systems of cells grown under various conditions. The bacterium was cultivated in four different ways. Spectrophotometric analysis showed that the main respiratory pigments of cells grown with vigorous aeration were cytochrome b1 and cytochrome o (Fig. 1A and Table
The same bacterium grown aerobically in the presence of $10^{-3}$ M KCN formed an electron-transport system consisting of cytochrome $b_1$, cytochrome $a_1$, cytochrome $a_2$, and cytochrome $o$ (Fig. 1B). These resistant cells produced about twice as much cytochrome $b_1$ and more than 10 times as much cytochromes $a_1$ and $a_2$, but the content of cytochrome $o$ was lower in aerobic cells.

When the bacterium was grown with limited aeration (one-tenth of vigorous aeration), the cytochrome pattern developed was the same as those of the resistant cells, as reported previously (Mizushima, Oka, and Arima, 1960).

Cells grown in deep standing culture with nitrate contained three times as much cytochrome $b_1$ and a few times as much cytochromes $a_1$ and $a_2$ as the sensitive cells (Fig. 1C). The large content of the cytochrome $b_1$ is thought to be related to the high level of activity of the nitrate reduc-
Changes in the cytochrome contents with changes of the growth conditions. The changes in cytochrome content during the growth of a vigorously aerated culture are shown in Fig. 2. The aerobic culture in the logarithmic phase showed a prominent cytochrome $b_1$ (500 m$\mu$) maximum in the $\alpha$ region and a prominent Soret maximum at 430 m$\mu$. On entering the stationary phase, the formation of cytochromes $a_1$ and $a_2$ was induced, and finally the spectrum became nearly the same as that of the resistant cells. No significant change in cytochrome $a$ content was observed during the change from one growth phase to another.

The changes in the spectrum after the addition of $10^{-3}$ M KCN to log-phase aerobic cells are shown in Fig. 3. The content of cytochromes $a_1$ and $a_2$ was increased with the recovery of growth, which occurred about 2 hr after the addition of cyanide. The cytochrome $a$ content either did not change or decreased a little (Table 2).

When cells grown in the presence of $10^{-3}$ M KCN were transferred into cyanide-free medium and allowed to grow aerobically, the change in "degree of resistance to cyanide" was rather complex (Mizushima and Arima, 1960a; Oka and Arima, 1965), and the change in the spectrum was also complex (Fig. 4). Nonetheless, as the growth went on, the peaks of cytochromes $a_1$ and $a_2$ faded away and the content of cytochrome $b_1$ doubled once (3$^\dagger$), and three times (3$^\ddagger$) in the cyanide-free medium.

Table 3. Various respiratory activities of the cyanide-sensitive and cyanide-resistant cells

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Sensitive</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d_{50}$</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td>$d_{10}$</td>
<td>210</td>
<td>140</td>
</tr>
</tbody>
</table>

* Results are expressed as $d_{50}$ [microliters of O$_2$ per milligram (dry weight) of cells per hour], except for NADH$_2$ oxidase activity which is expressed as change in optical density at 340 m$\mu$ per milligram of nitrogen per minute.

† 2,6-Dichlorophenol-indophenol.

‡ Phenazine methosulfate.

Table 4. Effects of various inhibitors on the oxidation of succinate by intact cells

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conditions</th>
<th>$pH$</th>
<th>Per cent inhibition of respiration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCN</td>
<td>$5 \times 10^{-3}$ M</td>
<td>7.4</td>
<td>100</td>
</tr>
<tr>
<td>NaN$_3$</td>
<td>$2.5 \times 10^{-4}$ M</td>
<td>5.2</td>
<td>88</td>
</tr>
<tr>
<td>BAL†</td>
<td>$2 \times 10^{-3}$ M</td>
<td>7.4</td>
<td>60</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>5-50 $\mu$g/ml</td>
<td>7.4</td>
<td>0</td>
</tr>
<tr>
<td>HQNO</td>
<td>50 $\mu$g/ml</td>
<td>7.4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Succinate oxidation by intact cells was measured with a Warburg respirometer. Each Warburg vessel contained about $4 \times 10^8$ cells, 1.6 ml of 0.1 M phosphate buffer (pH 7.4), and 0.2 ml of an inhibitor or the buffer in the main compartment; 0.2 ml of 0.2 M succinate was in the side arm; and 0.2 ml of 20% KOH was in the center well. The total volume was 2.2 ml.

† 2,3-Dimercapto-propanol.
decreased to half, whereas the content of cytochrome o remained at nearly the same level (Table 2).

Nature of the electron transport system of sensitive and resistant cells. As described above, cyanide-resistant cells contain twice as much cytochrome b₁, more than 10 times as much cytochromes a₁ and a₂, and a little less cytochrome o than do aerobic cells in the log phase of growth. It was previously reported that there was no difference in total flavine content (Mizushima and Arima, 1960a).

In Table 3, various respiratory activities of intact cells and cell-free preparations are shown. Though the resistant cells contained many cytochromes, the activity of the succinoxidase system was two-thirds of that of aerobic cells. This may be the reflection of the low succinate dehydrogenase activity of the resistant cells.

Effects of various respiratory inhibitors are shown in Table 4. The inhibition caused by each reagent was measured under its most effective condition, and the concentration shown gave the maximal difference between per cent inhibition of the sensitive and the resistant cells. It is of interest that the resistant cells were also resistant to azide, but not to carbon monoxide. They were also resistant to 2,3-dimercapto-propanol (BAL). Similar results were obtained in the cell-free preparations, except that BAL stimulated rather than inhibited the oxidation of succinate. The data cited above suggest that the electron-transport system of the sensitive cells is very different from that of the resistant cells.

Identification of the oxidase active in resistant respiration. As shown in the next paper (Oka and Arima, 1965), the concentration of cyanide which causes 50\% inhibition of the respiration of resistant cells was about 200 times as much as that producing the same effect in the sensitive cells. To identify the oxidase which could catalyze the oxidation of cytochromes in the presence of 10⁻³ M KCN, the oxidation-reduction state of cytochromes in cell-free preparations from the resistant cells was studied.

Figure 5 shows the changes in the oxidation-reduction state of cytochromes after the addition of succinate to the air-oxidized preparation. When the reduction occurred in the absence of cyanide, cytochromes b₁, a₁, and a₂ were rapidly reduced (Fig. 5A). The peaks of cytochromes a₁ and a₂ vanished more rapidly than that of cytochrome b₁. When the preparation was exposed to mild aeration, cytochromes b₁, a₁, and a₂ were oxidized to the original level.

When the reduction proceeded in the presence of 10⁻² M KCN, the peaks of cytochromes b₁ and a₁ disappeared more rapidly than that of cyto-
chromosome $a_2$. This indicates that there is a crossover point (Chance and Williams, 1956) between cytochromes $b_1$ and $a_2$ or between cytochromes $a_1$ and $a_2$. On mild aeration, cytochrome $a_2$ was oxidized to one-half of the original level, and cytochromes $b_1$ and $a_1$ were oxidized to a lesser degree. On successive vigorous aeration, cytochromes $b_1$, $a_1$, and $a_2$ were oxidized nearly to the original level (Fig. 5B).

When the reduction took place in the presence of $5 \times 10^{-3}$ M KCN, the peaks of cytochromes $a_1$ and $b_1$ disappeared more quickly than that of cytochrome $a_2$, which was not completely reduced (Fig. 5C). Only cytochrome $a_2$ was oxidized by mild aeration. With successive vigorous aeration, cytochrome $b_1$ was oxidized, but cytochrome $a_1$ remained reduced. The oxidation of cytochrome $a_2$ was nearly complete, whereas that of cytochrome $b_1$ was not complete, in the presence of $5 \times 10^{-3}$ M KCN. The fact that the oxidation of cytochrome $b_1$ occurred without the oxidation of cytochrome $a_1$ indicates that there is at least one pathway of electron flow from cytochrome $b_1$ to oxygen that does not pass through cytochrome $a_1$. This pathway is resistant to cyanide and consists of cytochromes $b_1$ and $a_2$.

These data show that cytochrome $a_2$ is oxidized most easily in the presence of cyanide and that there is no more resistant bypath of electron flow from cytochrome $b_1$ to oxygen than that through cytochrome $a_2$. Therefore, we concluded that cytochrome $a_2$ is an oxidase relatively insensitive to cyanide and that it can mediate the oxidation of the cytochrome system in Achromobacter strain D in a high concentration of cyanide. Other possibilities that may explain the mechanism of cyanide-resistant oxygen uptake, for example, autoxidation of cytochrome $b_1$ or participation of cytochromes $a$ and $a_1$, are excluded.

Oxidation of cytochrome $a_2$ by nitrite. When nitrite was added to the cell-free preparation from the resistant cells, very slow oxidation of cytochrome $a_2$ occurred anaerobically. On the other hand, cytochrome $a_2$ was rapidly oxidized by the addition of nitrite, and this oxidation was not

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**Fig. 6.** Anaerobic oxidation of cytochrome $a_2$ with nitrite and nitrate. A 0.1-ml amount of 0.3 M NaNO$_3$ or KNO$_3$ was added to the crude cell-free preparation (from the resistant cells) from the side arm of a Thunberg-type cuvette with nitrogen as the gas phase. Total volume, 3.0 ml; pH, 7.4; temperature, 20°C. Oxidation with nitrite in the absence (○) and in the presence (■) of HQNO (10 μg/ml). Oxidation with nitrite in the absence (○) and in the presence (■) of HQNO (10 μg/ml).

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**Fig. 7.** Effect of HQNO on the reduction of nitrate by cell-free preparation from resistant cells. The reduction was conducted in a Thunberg tube with nitrogen as the gas phase (30°C). Reaction mixture contained 40 μmoles of sodium succinate and 60 μmoles of KNO$_3$ in 0.06 M phosphate buffer (pH 7.4).

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**Fig. 8.** Oxidation of cytochrome $b_1$ with nitrate. A 2-mg amount of KNO$_3$ was added anaerobically to the crude cell-free preparation from nitrate-grown cells. Cytochrome $b_1$ was reduced with endogenous substrate prior to the addition of nitrate. Conditions were the same as for Fig. 8.
inhibited by HQNO (Fig. 6). As the oxidation of cytochrome $a_2$ by nitrate was completely inhibited by HQNO, it was assumed that the oxidation of cytochrome $a_2$ on addition of nitrate was due to nitrite, which was the product of nitrate reductase. In fact, the cell-free preparation from the resistant cells contained the activity of the nitrate reductase system which was inhibited by HQNO (Fig. 7), but this preparation did not contain the activity of the nitrite reductase system. In addition, cells grown on nitrate did not form NO from NO$_2^-$. Thus far, we have not obtained any physiological explanation for the oxidation of cytochrome $a_2$ by nitrite, but it is of interest that cytochrome $a_2$, which functions as a cytochrome oxidase, also has some relation to the reduction of nitrite. We will discuss this point later.

Nature of the electron-transport system of nitrate-grown cells. The difference spectrum of nitrate-grown cells is shown in Fig. 1C. These cells had an electron-transport system consisting of cytochromes $b_1$, $a_1$, $a_2$, and $o$, and showed more cyanide-resistant respiration than the sensitive cells. On the anaerobic addition of nitrate, the cytochrome $b_1$ of nitrate-grown cells was rapidly oxidized to the level attained by oxidation with air (Fig. 8). The activity of the nitrate reductase system of nitrate-grown cells was inhibited by HQNO and was more sensitive to cyanide than was the respiration of the resistant cells.

Figure 9 shows the inhibition by nitrate and nitrite of oxygen consumption in the cell-free preparation from nitrate-grown cells. The inhibition by the limited amount of nitrate was soon ended, and the rate of oxygen consumption recovered to nearly the original level; the mode of inhibition by nitrite was quite different. The low concentration of nitrite did not inhibit oxygen consumption immediately after addition, but it gradually became effective with the decrease of the oxygen concentration and finally caused complete inhibition. The higher the concentration of nitrite was, the higher was the level of oxygen at which the consumption stopped. Moreover, the inhibition remained in effect. These facts suggest that nitrite acts as a competitive inhibitor of the cytochrome oxidase for oxygen, and that nitrite is not utilized as an electron acceptor in this bacterium.

**DISCUSSION**

The above data suggest that the formation and function of cytochrome $a_2$ is closely related to the environment in which the utilization of oxygen is limited. The induced formation of cytochrome $a_2$ occurs on addition of potassium cyanide to the aerobic culture, during the transition from log to stationary phase, under limited aeration, or in the anaerobic culture with nitrate; the resultant cells, containing cytochrome $a_2$, in various concentrations, are more or less resistant to cyanide. In this connection, the work of Castor and Chance (1959) is very interesting to us. They reported that in *Escherichia coli* the oxidase activity of cytochrome $a_2$ appeared only in the stationary phase and that, for the respiration inhibited by CO, cytochrome $a_2$ activity predominated over cytochrome $o$ activity.

There are two well-known mechanisms that control the formation of cytochromes in microorganisms; one is "oxygen adaptation" (formation of cytochromes in response to oxygen as in yeast; Ephrussi and Slonimski, 1950), and the other is "oxygen suppression" as in *Pseudomonas* (Lenhoff, Nicholas, and Kaplan, 1956) and in dentiflarying bacteria (Verhooven and Takeda, 1956). Our data suggest that oxygen tension determines not only the amount of cytochromes formed but also the nature of the terminal electron-transport system. This information may help to explain the fact that the main difference between the bacterial and the mammalian cytochrome systems seems to be the presence of several oxidases in some bacteria in contrast to a single oxidase in mammalian tissue (Smith, 1961). In Fig. 10, the electron-transport systems of *Achromobacter* strain D grown under various conditions and the electron flow in them are summarized.

Cytochrome $a_2$ is a very interesting cytochrome in many respects. First of all, its function as a cytochrome oxidase was doubted (Smith, 1954), because there was no correlation between cytochrome $a_2$ content and the respiratory activity.
of bacteria (Tissieres, 1951, 1952; Moss, 1952). However, Castor and Chance (1859) concluded that cytochrome a2 was a true oxidase and that it was capable of catalyzing a large part of the respiration in the bacteria in which it was found. We also reported previously that there was a parallelism between cytochrome a2 content and cyanide-resistant respiratory activity, and we proposed that cytochrome a2 was the cytochrome oxidase in this *Achromobacter* strain D (Mizushima et al., 1960). In the present paper, we have shown that cytochrome a2 is an oxidase relatively insensitive to cyanide and that it plays the main part in cyanide-resistant respiration in *Achromobacter* strain D.

The purest cytochrome a2 thus far obtained is that of *P. aeruginosa* (Horio et al., 1961; Yamanaka and Okunuki, 1963). This cytochrome a2 in its crystalline form has two prosthetic groups (HEME a2 and c-type heme) and has two activities, a cytochrome oxidase activity and a nitrite reductase activity. As it is produced only in the presence of nitrate, physiologically it is a nitrite reductase. In contrast, the cytochrome a2 of *Achromobacter* strain D, which is also oxidized by nitrite, is formed and acts as a cytochrome oxidase which catalyzes the oxidation of cytochromes in the presence of cyanide or under low oxygen tension. In addition, Castor and Chance (1959) reported that in *E. coli* B, *Aerobacter aerogenes*, and *Proteus vulgaris* the oxidase activity of cytochrome a2 was found only in the stationary phase; this appearance in the stationary phase might be related to changes in oxygen concentration during growth of the cultures. Thus, it is of interest that these cytochromes, which are commonly called cytochrome a2 from the position of their a peaks, have some relation to anaerobic or nearly anaerobic energy metabolism.

As seen in Fig. 1B this bacterium contains another a-type cytochrome, cytochrome a1. In some bacteria, cytochrome a1 can function as a cytochrome oxidase (Castor and Chance, 1955), and in *Haemophilus* species cytochrome a1 is reported to have some relation to nitrate reduction (White and Smith, 1962; Williams (1961), on the other hand, suggested the possibility that cytochrome a1 functions similarly to cytochrome c. We found that one strain of *Pseudomonas pseudomallei*, which was able to grow in the presence of 2 X 10⁻² M arsenite (Arima and Beppu, 1964), contained cytochromes b1, a1, and a2 without cytochrome o. This bacterium was very suitable for studying the role of cytochrome a1 occurring with cytochrome a2. We found that cytochrome a1, like cytochrome a2, acted as an oxidase in this bacterium, though it was more sensitive to cyanide than was cytochrome a2 (unpublished data). As the relation of cytochrome a1 to nitrate reduction was not found in this strain of *Achromobacter*, it may be concluded that the resistant cells contained three different kinds of oxidase, cytochromes a1, a2, and o.

We may summarize the electron flow and the inhibition sites of some respiratory inhibitors in the cyanide-resistant cells as shown in Fig. 11.

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**LITERATURE CITED**


CASTOR, L., AND B. CHANCE. 1955. Photochemical


