

## Inhibitory Activities of Lansoprazole against Respiration in *Helicobacter pylori*

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**Lansoprazole and its derivative AG-1789 dose-dependently inhibited cellular respiration by an endogenous substrate and decreased the ATP level in *Helicobacter pylori* cells. The inhibitory action of lansoprazole and AG-1789 against respiration was specific to substrates such as pyruvate and  $\alpha$ -ketoglutarate and similar to the inhibitory action of rotenone, which is an inhibitor for the mitochondrial respiratory chain. Growth inhibition by lansoprazole and AG-1789 as well as by rotenone was augmented at high oxygen concentrations under atmospheric conditions. Since the 50% inhibitory concentrations of these compounds for the respiration were close to their MICs for *H. pylori* growth, the growth inhibition might be due to respiratory inhibition by these compounds.**

*Helicobacter pylori*, a gram-negative bacterium that colonizes the human gastric mucosa and damages epithelial cells by association and cytotoxin release (5), is the principal cause of gastritis and peptic ulcer and an etiological factor in gastric carcinoma (25–27).

Lansoprazole is an antiulcer benzimidazole proton pump inhibitor (PPI) like omeprazole which acts on gastric ( $H^+/K^+$ ) ATPase of parietal cells (9). Iwahi et al. reported that lansoprazole and its analogs inhibit the growth of *H. pylori* at concentrations of several micrograms per milliliter (13). This inhibition appeared to be specific to *H. pylori* since the growth of more than 27 other bacterial species was not affected by lansoprazole even at 100  $\mu\text{g/ml}$  (13). Since analogs which do not have the activity of a PPI also act against *H. pylori* (13), the chemical moieties corresponding to the bactericidal action are suggested to be different from those in PPIs. One investigator also reported effective bactericidal activity of PPIs in *H. pylori* in vitro (28). From a study in vivo, it has been suggested that the increase in gastric pH due to the PPI makes the antibiotic more active against the organism by inhibiting gastric juice volume and thereby increasing the concentration of antibiotic. Therefore, lansoprazole as well as omeprazole has been used in combination with an antibiotic (amoxicillin or clarithromycin) to eradicate *H. pylori* (14).

Whether PPIs are in fact directly bactericidal is still controversial. Since lansoprazole acts only against *H. pylori*, elucidating the mechanism of its bactericidal action will facilitate the development of drugs to treat *H. pylori* infections. Previously, it was reported that lansoprazole or omeprazole inhibited the activity of urease in cell extract as well as in whole cells of *H. pylori* at concentrations which are inhibiting to *H. pylori* growth (20). *H. pylori* has extremely large amounts of urease, and its activity may help the bacterium to survive in the acidic environments of the stomach. The urease inhibition of PPIs, how-

ever, was not related to the growth inhibition of lansoprazole since the growth of a urease-deficient strain was also inhibited by lansoprazole (21). In addition, an analog of lansoprazole, AG-1789, which is not a PPI but inhibits *H. pylori* growth, did not inhibit the urease activity. These results suggested that the chemical moiety corresponding to the bactericidal action is different from that corresponding to the urease inhibition.

The bactericidal action of lansoprazole or omeprazole against *H. pylori* has been studied previously. Belli et al. reported that omeprazole did not affect the activity of F-type ATPase in the membrane fraction of *H. pylori* at concentrations which are inhibitory for cell growth, indicating no role for the F-type ATPase in the bactericidal action of PPIs (4). The P-type ATPase is reported to be inhibited by lansoprazole (64  $\mu\text{g/ml}$ ); however, this inhibition is effective in only an acidic environment (pH 4.0) (17). Nakao et al. reported that concentrations of lansoprazole close to the MIC (3 to 13  $\mu\text{g/ml}$ ) for *H. pylori* growth inhibited the motility of *H. pylori* within minutes (24).

*H. pylori* is a microaerophilic bacterium exhibiting a strict respiratory form of metabolism and oxidizing organic acids as an energy source (15). In this report, we describe the inhibitory effect of lansoprazole and AG-1789 on the respiratory system in *H. pylori* and discuss the possible involvement of the inhibition in the bactericidal action of PPIs.

### MATERIALS AND METHODS

**Materials.** Lansoprazole and its analog AG-1789 were donated by the Pharmaceutical Research Division of Takeda Chemical Industries, Ltd., Osaka, Japan. Chemical structures of lansoprazole and its analog AG-1789 were reported elsewhere (13). Rotenone and rifampin were purchased from Sigma Chemical Co., St. Louis, Mo. Penicillin and erythromycin were purchased from Banyu Pharmaceutical Co., Ltd., and Wako Pure Chemicals Co., Osaka, Japan, respectively. Other inhibitors and substrates for respiration were purchased from Wako.

The strain of *H. pylori* used in the present study, NCTC 11637, was cultured in *Brucella* broth (Becton Dickinson, Cockeysville, Md.) containing 5% horse serum under a microaerobic atmosphere, produced with the use of a pack BBL CampyPak (Becton Dickinson) with gentle shaking at 37°C for 20 h as described previously (21).

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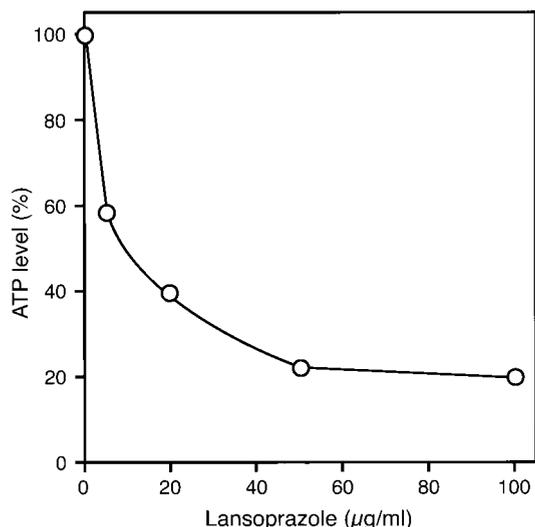


FIG. 1. Effect of lansoprazole on cellular ATP level in *H. pylori*. *H. pylori* cells ( $10^8$  per ml) were incubated with various amounts of lansoprazole for 30 min at  $37^\circ\text{C}$ . The cellular ATP level was assayed as described in Materials and Methods. The percentage of control was determined by the following equation: percentage = (ATP levels of cells incubated with lansoprazole) (ATP levels of cells incubated without lansoprazole) $^{-1}$  (100). Each point represents the mean value derived from two experiments.

**Assay of respiratory activity with inhibitors.** Respiration of whole cells ( $10^8$ /ml) was monitored polarographically with a Clark-type oxygen electrode (YSI Inc., Yellow Springs, Ohio) in a semiclosed cell containing 10 mM HEPES buffer (pH 7.0) and 0.9% NaCl at  $37^\circ\text{C}$  as described previously (23). Various kinds of substrates and inhibitors were added into an oxygen electrode vessel with a syringe. Respiratory activity (oxygen uptake per minute) was determined from polarographic traces of the oxygen electrode.

**Assay of cellular ATP level.** The level of ATP in *H. pylori* cells was assayed using a luciferin-luciferase-based bioluminescence assay kit, Lucifer LU plus (Kikkoman Corporation, Chiba, Japan), as described previously (11).

**Determination of MICs under different growth conditions.** The MICs of lansoprazole, AG-1789, rotenone, and antibiotics for growth of *H. pylori* were determined as described previously (21). Briefly, these inhibitors, which were dissolved in 100% dimethyl sulfoxide or phosphate-buffered saline and diluted 10- and 100-fold with phosphate-buffered saline, were put into 24-well tissue culture plates (Corning Glass Works, Corning, N.Y.). One milliliter of *Brucella* agar (Becton Dickinson) containing 5% horse serum was added to each well. *H. pylori* cells were cultured in a liquid medium with shaking at  $37^\circ\text{C}$  as described above, and then  $10^5$  cells in the logarithmic phase were inoculated into each well. After a 3- or 4-day incubation in a microaerobic atmosphere described above or in 10%  $\text{CO}_2$  incubator, the MICs were defined as the inhibitor concentrations (in micrograms per milliliter) which produced 90% growth inhibition.

## RESULTS AND DISCUSSION

At first, we examined the ATP level in *H. pylori* cells when  $10^8$  cells per ml were incubated with various amounts of lansoprazole for 30 min at  $37^\circ\text{C}$ . The ATP level of cells incubated with lansoprazole decreased dose-dependently (Fig. 1). The concentration causing a 50% decrease was approximately 10  $\mu\text{g}/\text{ml}$ . This value was close to the MIC, 3 to 13  $\mu\text{g}/\text{ml}$ , for *H. pylori* growth (13). As shown in Fig. 2, when KCN was added instead of lansoprazole, the ATP level decreased dose-dependently in parallel to the reduction of the respiration by succinate used as a substrate. Thus, the reduction in ATP level evoked by lansoprazole was suggested to be due to an inhibition of ATP synthesis caused by respiratory inhibition. Since

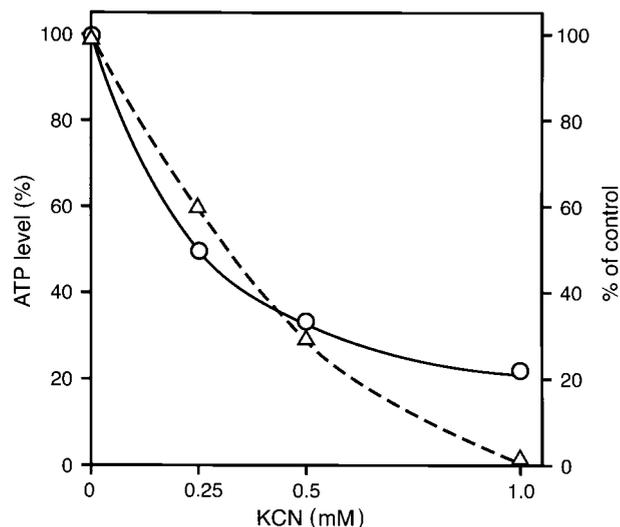


FIG. 2. Effect of KCN on cellular respiration and ATP level in *H. pylori*. *H. pylori* cells ( $10^8$  per ml) were incubated with various amounts of KCN for 10 min at  $37^\circ\text{C}$ . The cellular ATP level (solid line) was determined as described for Fig. 1 except that KCN was used instead of lansoprazole. Respiratory activity by succinate used as the substrate was determined from polarographic traces with or without various amounts of KCN by the oxygen electrode. The percentage of control in the respiration (dotted line) was determined using the following equation: percentage = (respiratory activity with KCN) (respiratory activity without KCN) $^{-1}$  (100). Each point represents the mean value derived from two experiments.

about 20% of the total cellular ATP level still remained even when respiratory activity disappeared completely at  $10^{-3}$  M KCN (Fig. 2), the remaining ATP level under high amounts of lansoprazole may not be linked to the respiratory activity.

The inhibitory effect of lansoprazole on cellular respiration in *H. pylori* was examined. Respiration of whole cells ( $10^8$  cells/ml) with or without various amounts of lansoprazole was monitored polarographically. Lansoprazole dose-dependently inhibited the cellular respiration of endogenous substrate. An analog of lansoprazole, AG-1789, which is not a PPI but shows bactericidal activity with a MIC of 0.8  $\mu\text{g}/\text{ml}$  (13), also inhibited the respiration dose-dependently (Fig. 3a). The 50% inhibitory concentrations were 13 and 0.3  $\mu\text{g}/\text{ml}$  for lansoprazole and AG-1789, respectively (Fig. 3b). These values were close to the corresponding MICs for *H. pylori* growth as described previously (13). As shown in Fig. 3b, about 90% of total cellular respiration was lost in the presence of high concentrations of lansoprazole or AG-1789, and then remaining respiration (about 10%) by the endogenous substrate was not inhibited by  $10^{-3}$  M KCN (data not shown). Previously, we reported that *H. pylori* has a *cbb*<sub>3</sub>-type cytochrome *c* oxidase which is highly sensitive to KCN (50% inhibitory concentration, 4  $\mu\text{M}$ ) (22), and this oxidase functions in the respiratory chain of *H. pylori* as a terminal oxidase (1, 22). Thus, the KCN-insensitive respiration may be due to  $\text{O}_2$  uptake not via *cbb*<sub>3</sub>-type cytochrome *c* oxidase but via quinol oxidase (1, 15), soluble NADPH oxidase, or cytochrome *c* peroxidase (5).

In *H. pylori*, the respiratory chain has not been fully characterized despite recent efforts (2, 6, 7, 15). Although *H. pylori* does not have a complete tricarboxylic acid cycle (29), organic

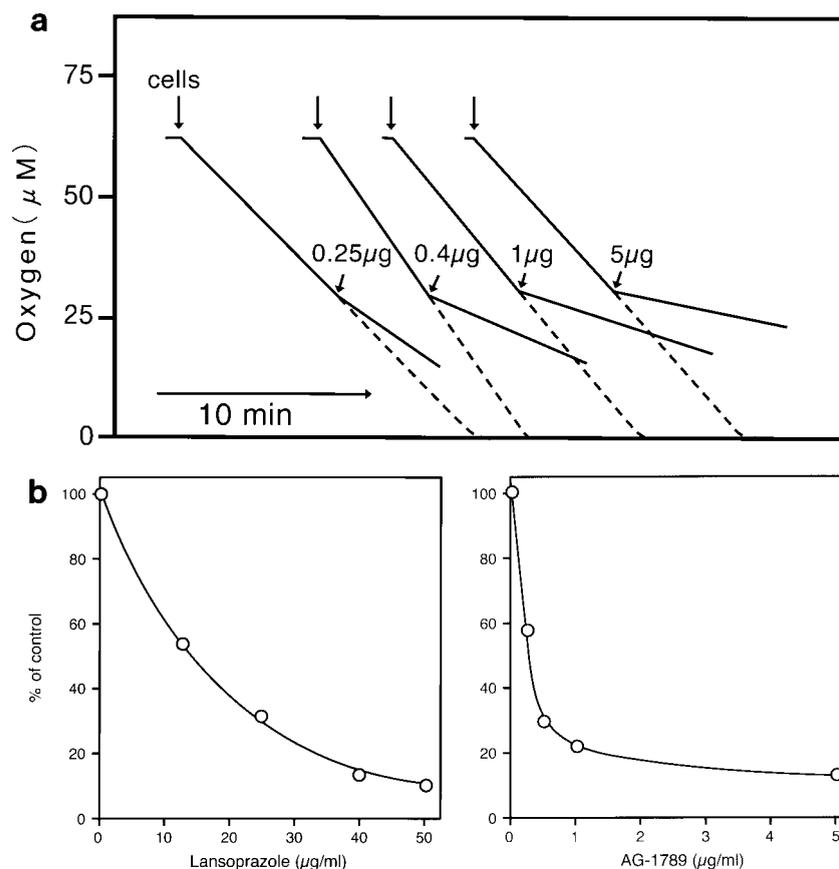


FIG. 3. Effect of lansoprazole and AG-1789 on cellular respiration of *H. pylori*. (a) At the indicated times (arrows), an amount of AG-1789 was added into an oxygen electrode vessel to give a final concentration of 0.25, 0.4, 1.0, or 5.0  $\mu\text{g/ml}$ . Dotted lines represent control experiments without AG-1789. (b) Respiratory activity was determined as described for Fig. 2, and the percentage of control was determined using the following equation: percentage = (respiratory activity with lansoprazole or AG-1789) / (respiratory activity without lansoprazole or AG-1789)  $\times$  100. Each point represents the mean value derived from two experiments.

acids such as pyruvate, succinate,  $\alpha$ -ketoglutarate, and isocitrate are metabolized by the *H. pylori* cells and used as respiratory substrates (15). The present study showed that the oxygen uptake of whole cells of *H. pylori* increased upon addition of pyruvate, succinate,  $\alpha$ -ketoglutarate, and DL-lactate after a decrease in the respiration of endogenous substrate as shown by the dotted lines in Fig. 4. Figure 4a shows the effect of rotenone, which is a potent inhibitor for NADH-quinone oxidoreductase, complex I in the respiratory chain, on the cellular respiration of various substrates. A low concentration (4  $\mu\text{M}$ , 1.6  $\mu\text{g/ml}$ ) of rotenone inhibited the respiration of pyruvate and  $\alpha$ -ketoglutarate. That of succinate and DL-lactate was little inhibited (Fig. 4a), although we could not define the inhibitory effect of rotenone on isocitrate respiration because of its very low respiration-inducing activity. Thus, there appeared to be different respiratory pathways between pyruvate or  $\alpha$ -ketoglutarate and succinate or DL-lactate in *H. pylori*. The presence of complex I in *H. pylori* was suspected since NADH-quinone oxidoreductase lacked an NADH-binding domain as determined from genome sequence analysis of *H. pylori* (10). Chen et al. reported that NADH dehydrogenase activity in the membrane fraction of *H. pylori* was not inhibited by rotenone (7). However, our results showing that cellular respiration was sen-

sitive to rotenone suggested the presence of rotenone-sensitive complex I-like structure in the respiratory chain of *H. pylori*. In *H. pylori*, pyruvate and  $\alpha$ -ketoglutarate are dehydrogenated by different dehydrogenase systems from those in the usual proteobacteria producing NADH, which is the major electron donor in the respiratory chain. Two-step systems generating NADPH seem to be present in *H. pylori*; pyruvate and  $\alpha$ -ketoglutarate may be oxidized with flavodoxin (15), and reduced flavodoxin reduces NADP. NADPH-menaquinone oxidoreductase, corresponding to complex I, oxidizes NADPH, while succinate directly reduces menaquinones. Menaquinones may be then oxidized by the cytochrome *bc<sub>1</sub>* complex of the respiratory chain, since 6  $\mu\text{M}$  antimycin A inhibited cellular respiration by succinate as well as by pyruvate or  $\alpha$ -ketoglutarate (data not shown). Other investigators also reported that antimycin A inhibited succinate-cytochrome *c* reductase or inhibited cellular respiration by succinate (3, 7). From these facts, it was suggested that an enzyme system corresponding to complex III is present in *H. pylori*. Concerning the terminal oxidase in the respiratory chain of *H. pylori*, the *cbb<sub>3</sub>*-type cytochrome *c* oxidase functioned as described in our previous study (22).

To elucidate which step in the respiratory chain of *H. pylori* is inhibited by lansoprazole, the inhibitory action was examined

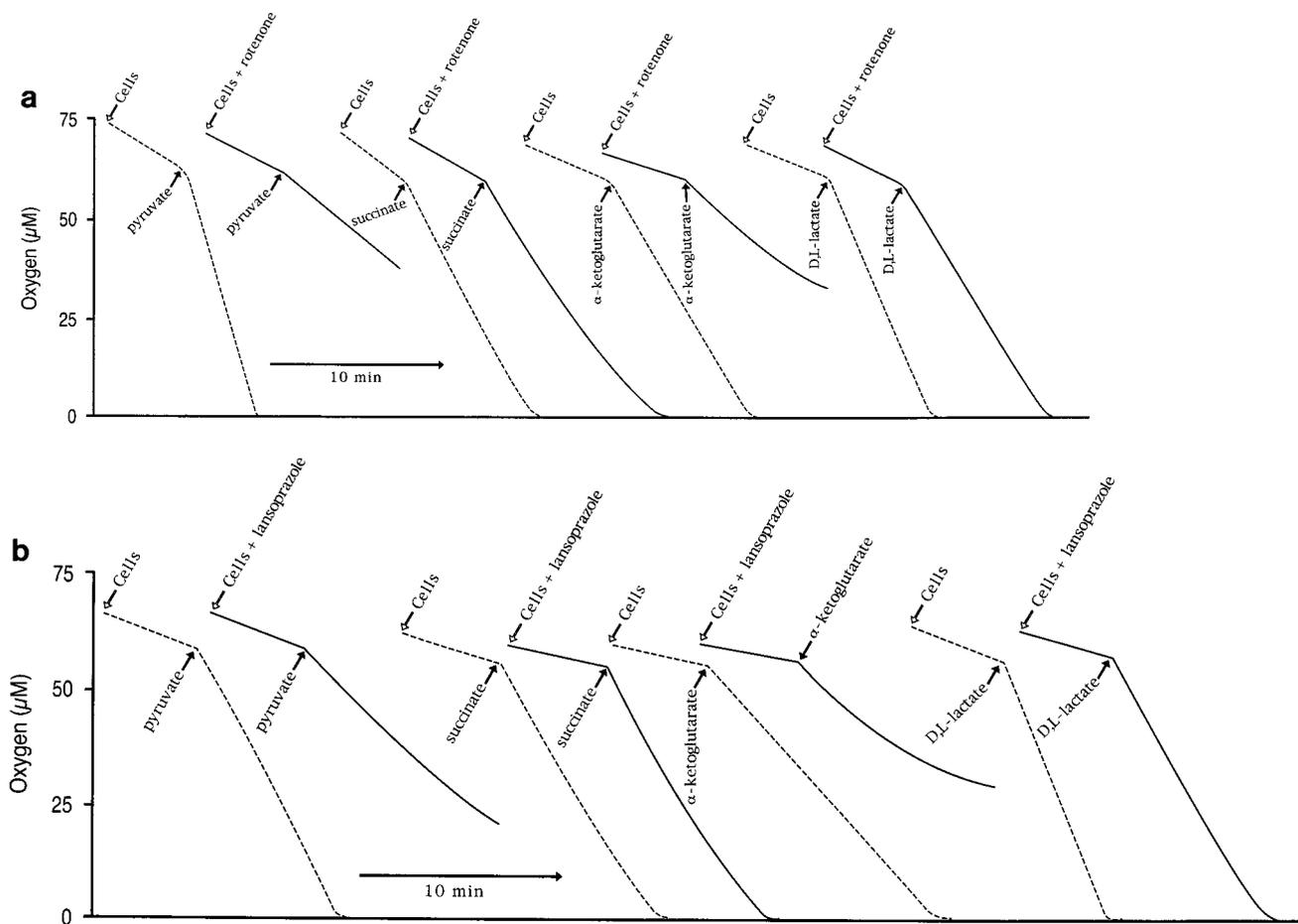


FIG. 4. (a) and (b). Effect of rotenone and lansoprazole on cellular respiration by various kinds of substrates in *H. pylori*. *H. pylori* cells with or without rotenone (1.6  $\mu\text{g/ml}$ ) (a) or lansoprazole (50  $\mu\text{g/ml}$ ) (b) were added to an oxygen electrode vessel. After 3 min, 5 mM pyruvate, succinate,  $\alpha$ -ketoglutarate, or DL-lactate was added. Dotted lines represent control experiments without rotenone or lansoprazole.

using various substrates. When lansoprazole (50  $\mu\text{g/ml}$ ) was added to the reaction mixture, it inhibited the respiration of pyruvate and  $\alpha$ -ketoglutarate (Fig. 4b). On the contrary, lansoprazole little inhibited the respiration of succinate and DL-lactate as also shown. The respiration of ascorbate plus *N,N,N',N'*-tetramethyl *p*-phenylenediamine, which was used as the substrate for terminal oxidase activity, was not inhibited by lansoprazole (data not shown). These inhibitory patterns were similar to those for rotenone (Fig. 4a) and suggest that a main inhibitory target of lansoprazole is the NADPH-quinone oxidoreductase system corresponding to complex I as in the case of rotenone, although it may inhibit the keto acid-dependent NADPH reduction.

In *H. pylori*, pyruvate seems to be the main substrate for energy production (18). Pyruvate was decarboxylated and dehydrogenated by  $\text{O}_2$ -labile pyruvate-flavodoxin oxidoreductase, and then a pair of hydrogen atoms were transferred to NADP by flavodoxin-NADP oxidoreductase (12).  $\alpha$ -Ketoglutarate was metabolized in a similar manner to pyruvate except that instead of pyruvate-flavodoxin oxidoreductase,  $\alpha$ -ketoglutarate-ferredoxin oxidoreductase, which is also highly sensitive to oxygen, was involved (12). These findings led us to examine the effect of lansoprazole on oxygen-labile pyruvate-flavodoxin

oxidoreductase. After the destruction of *H. pylori* cells as described previously (22), the soluble fraction was subjected to an assay for pyruvate-flavodoxin oxidoreductase activity according to the method of the previous work (12). Lansoprazole did not inhibit this oxidoreductase (data not shown).

We prepared subcellular fractions from disrupted *H. pylori* cells by sonication and sonicated supernatant; the soluble and membrane fractions were obtained as described previously (22). Oxidative activities of NADH or NADPH were assayed polarographically with a Clark-type oxygen electrode. As shown in Table 1, the activity of NADH oxidation in the membrane fraction was almost the same as that of NADPH, and neither of the activities in the soluble or membrane fractions was inhibited by  $5 \times 10^{-5}$  M rotenone or by lansoprazole (50  $\mu\text{g/ml}$ ) (data not shown). Similarly, the activity of NADH dehydrogenase in the membrane fraction is reportedly not inhibited by rotenone (7). Since the level of NADH or NADPH oxidative activity in the soluble fraction was extremely high (Table 1), the activity in the membrane fraction may be contaminated by the soluble fraction. There is a possibility that the NADPH oxidative activity in the membrane fraction is inactivated on cell destruction, so that the real activity dependent on NADPH-menaquinone oxidoreductase has not been detected.

TABLE 1. Oxidative activities of NADH or NADPH in subcellular fractions of *H. pylori*<sup>a</sup>

Cellular fraction	NADH results		NADPH results	
	Specific <sup>b</sup>	Total <sup>c</sup>	Specific <sup>b</sup>	Total <sup>c</sup>
Sonicated supernatant	29.6	6,861	28.0	6,500
Soluble fraction	43.6	6,838	41.4	6,468
Membrane fraction	2.1	155	2.0	148

<sup>a</sup> Subcellular fractions from *H. pylori* cells (8 g of wet weight) disrupted by sonication were obtained, and oxidative activities of NADH or NADPH were assayed polarographically with a Clark-type oxygen electrode at 37°C as described previously (23). Finally, 2.5 mM NADH or NADPH was added into an oxygen electrode vessel containing 10 mM KCl, 10 mM HEPES buffer (pH 7.2), and the subcellular fractions (0.1 to 1.0 mg of protein).

<sup>b</sup> Nanomoles of O<sub>2</sub> uptake/minute/milligram of protein.

<sup>c</sup> Nanomoles of O<sub>2</sub> uptake/minute.

The MIC of lansoprazole and its analog, AG-1789, for *H. pylori* was low at high concentrations of oxygen. Table 2 shows the MICs of lansoprazole and AG-1789 obtained under the different atmospheric conditions of the 10% CO<sub>2</sub> incubator (20% O<sub>2</sub>) and BBL CampyPak (about 12% CO<sub>2</sub> and 8% O<sub>2</sub>). MICs of both compounds were lower for 20% oxygen than for 8% oxygen (Table 2). Similar results have been reported by Midolo et al. (19), who observed that the oxygen concentration influenced the activities of lansoprazole and omeprazole against *H. pylori*. The MICs of lansoprazole for strain NCTC 11637 were 1 µg/ml in 20% oxygen, 16 µg/ml in 10% oxygen, and 64 µg/ml in 1% oxygen. Thus, the inhibitory effects of lansoprazole and AG-1789 on *H. pylori* growth were augmented by the high concentrations of oxygen under atmospheric conditions.

Table 2 also shows the MICs of rotenone. Growth of *H. pylori* was inhibited by a low dose of rotenone, and the inhibition was augmented by a high concentration of oxygen as in the case of lansoprazole or AG-1789. In contrast to MICs of lansoprazole, AG-1789, and rotenone, MICs of antibiotics such as penicillin, erythromycin, and rifampin were not affected by oxygen concentrations (Table 2).

Although oxygen is required for growth, anaerobic energy metabolism such as anaerobic respiration with fumarate as the terminal electron acceptor (1, 16) or anaerobic fermentation (8, 15) was suggested to occur. Thus, the energy metabolism may differ for cells grown under microaerophilic conditions and dependent on a more anaerobic energy metabolism and for those grown in 20% oxygen and requiring a more aerobic respiration.

Since lansoprazole, AG-1789, and rotenone inhibited aero-

bic respiratory activity in the present study, the increase in the oxygen sensitivity of these compounds against *H. pylori* growth may be related to a change(s) in chemical structure by oxidation of these inhibitors and/or altered energy metabolism induced by high oxygen atmosphere. In the present study, however, the inhibitory activity of neither lansoprazole nor rotenone was detected in a cell-free system of *H. pylori*. The targets for lansoprazole remain to be defined.

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TABLE 2. Effect of oxygen on inhibition by lansoprazole, AG-1789, rotenone, and antibiotics of *H. pylori* growth<sup>a</sup>

Compound	MIC (µg/ml) in	
	8% O <sub>2</sub>	20% O <sub>2</sub>
Lansoprazole	3.1	0.8
AG-1789	1.6	0.4
Rotenone	2.0	0.5
Penicillin <sup>b</sup>	0.025	0.025
Erythromycin	0.125	0.125
Rifampin	0.02	0.02

<sup>a</sup> MICs were determined as described in Materials and Methods.

<sup>b</sup> Results for penicillin are given in arbitrary units per milliliter.

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