Metabolism of Dihydro Streptomycin Sensitive, Resistant and Dependent *Vibrio comma.* (23581)

HANNAH FARKAS-HIMSLEY (Introduced by A. L. Olitzki)
Department of Bacteriology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

Previous research of Sevag(1), Rosanoff and Sevag(2) on drug sensitive microorganisms showed that development of resistance to various drugs is accompanied by changes is metabolism. It seemed, therefore, of interest to compare certain enzymatic activities of streptomycin sensitive, resistant and dependent strains of *Vibrio comma*. A quantitative comparison of the nitratase activity, oxygen uptake and reduction of neotetrazolium was undertaken

Materials and methods. V. comma (Ogawa strain), sensitive to dihydro streptomycin was used throughout. Resistant and dependent mutants were obtained from this sensitive strain as described by Olitzki and Olitzki(3). *Casein hydrolysate egg medium* (CHEM) (4) was used throughout to obtain abundant growth of all 3 strains used. *Non-proliferating suspensions* were prepared from bacteria which had been grown at 37°C for 18 hours on solid CHEM either with or without nitrate (0.3 g/liter medium). The cells were repeatedly washed with distilled water by centrifugation until no color reaction with Gries-Ilosvay reagent was observed in the supernatant fluid. Final bacterial suspension was adjusted to the required turbidity with the aid of a Unicam Spectrophotometer. *Cell free extracts* were prepared by disintegrating the bacterial suspension in a 9KC Raytheon sonic vibrator for 20 min. and removing the cell debris by centrifugation in Sorvall centrifuge at 10,000 rpm for 10 min. *Determination of nitrate reducing activity.* The reaction mixture for determination of nitrate reducing activity contained: potassium nitrate, 5 μmoles; mannitol or another H-donor, 50 μmoles; and Sorensen phosphate buffer (pH 7.8). The mixture was kept 15 min. in water bath at 37°C and then 1 ml of either cell suspension (0.5 mg N) or cell free extract (10 mg N) was added. The volume was made up of 2.5 ml with distilled water and samples removed at intervals for nitrate determination. *Nitrite determinations* were carried out by the method of Feigl(5). Enzymatic reaction was stopped by adding 1 ml of sulphanilic acid (1% sol. in 30% glacial acetic acid). Three minutes later 1 ml of alpha-naphthylamine (0.03% sol. in 30% glacial acetic acid) was added. The volume was made to 10 ml and the color intensity was measured in the Unicam spectrophotometer at 540 mμ. A standard curve was prepared with nitrite. O_2 uptake was measured in Warburg apparatus with air as the gas phase and 15% KOH in central cup. *Neotetrazolium reduction.* According to Kun and Abood(6) the reaction mixture contained: Sorensen phosphate buffer (pH 7.8), 100 μmoles; H-donor, 30 μmoles; dihydro streptomycin, 10 μg/ml; and neo-tetrazolium, 1 mg. This mixture was kept for 15 min. in water bath at 37°C, and then the bacteria (0.5 mg N dry weight) were added. The final volume was 2 ml. The reaction was stopped when desired by addition of 5 ml of isobutanol to the reaction mixture. After shaking and standing for 24 hours at room temperature, the isobutanol was removed. This extraction was repeated with further 5 ml of isobutanol. The color intensity of the combined extracts was determined at 490 mμ, in a Unicam spectrophotometer.

Results. Nitratase activity of non-proliferating cells grown in presence and absence of nitrate. The results obtained with the various strains are summarized in Fig. 1. Similar results were obtained whether glucose or mannitol served as the H donor. The sensitive strain reduces nitrate at a much greater rate when grown in the presence of nitrate. It was surprising to find that both resistant and dependent strains failed to reduce nitrate. The complete loss of activity of the nitratase system which accompanied the change to either a resistant or a dependent strain in vibrios, was not found in the case of dihydro streptomycin resistant *Escherichia*

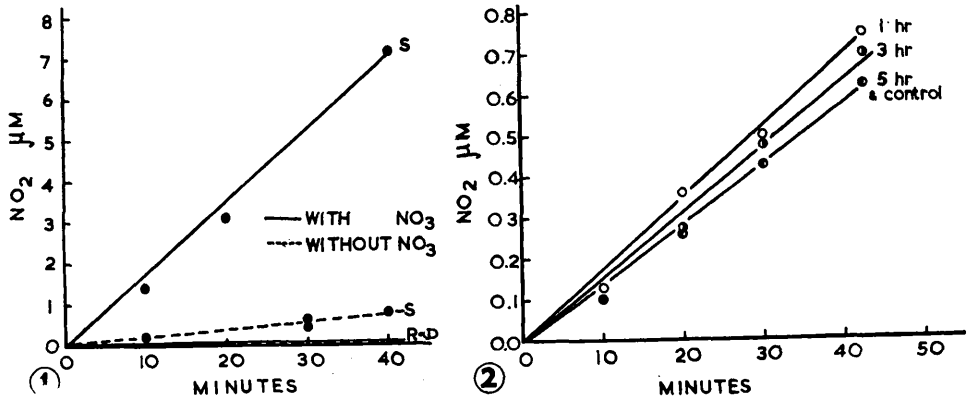

FIG. 1. Nitrate reduction by non-proliferating, dihydro streptomycin sensitive, dependent and resistant *Vibrio comma* cells. Grown in presence or absence of nitrate (0.3 g/l). Reaction mixture: H-donor, mannitol or glucose, 50 µmoles; phosphate buffer, 50 µmoles; KNO_3, 10 µmoles; total volume, 2.5 ml.

FIG. 2. Effect of preincubation with dihydro streptomycin, 10^3 µg/ml, on reduction of nitrate by sensitive *V. comma*. Reaction mixture as in Fig. 1. Bacteria grown in absence of nitrate.

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Effect of preincubation with dihydro streptomycin on the nitratase activity of non-proliferating sensitive V. comma. Preincubation for 1 hour with dihydro streptomycin at concentrations of 10 to 10^4 µg/ml resulted in increased nitratase activity (Fig. 3). Preincubation with 10^5 µg/ml of dihydro streptomycin, however, had no effect on nitratase activity (Fig. 3). When preincubation of the sensitive strains with 10^3 µg/ml dihydro streptomycin was prolonged to 5 hours, only a slight reduction in observed nitratase activity, as compared to 1 hour preincubation, occurred (Fig. 2).

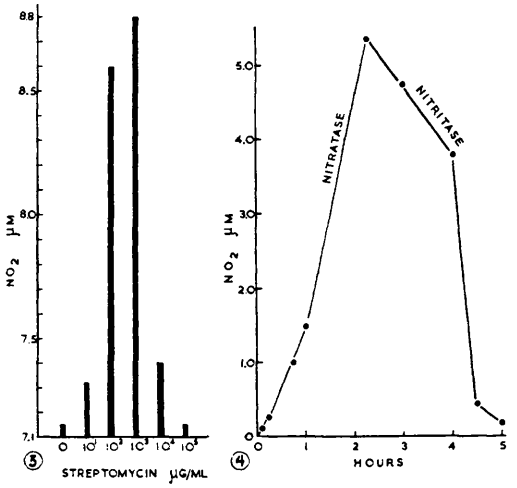

FIG. 3. Stimulation of nitrate reduction by sensitive non-proliferating *V. comma* in presence of dihydro streptomycin. Reaction mixture as in Fig. 1.; incubation 40 min.

FIG. 4. Nitrate and nitrite reduction by sensitive non-proliferating *V. comma* grown in absence of nitrate.

Nitrite reducing system in dihydro streptomycin sensitive, resistant and dependent V. comma. During determination of nitratase activity of the sensitive strain, the NO_2 content of the reaction mixture reached a maximum after 2 hours and subsequently decreased as seen in Fig. 4. The ammonia content of the reaction mixture increased as the NO_2 content fell. These observations indicate the presence in *V. comma* of a nitrite reducing enzyme system similar to those already demonstrated in other microorganisms (6-8). The resistant and dependent strains are unable to reduce nitrite.

Nitratase activity of cell free extracts of sensitive, resistant and dependent V. comma. To determine whether absence of nitratase activity was due to permeability changes in the cell membrane or whether the enzyme was affected, extracts of the 3 strains were tested for nitrate reducing activity. Cell free extracts from sensitive strains were very active but those from resistant and dependent strains were inactive. Addition of DPNH which, as previously demonstrated (8-10) is a necessary factor for the nitrate reducing system, enhanced the activity of CFE from sensitive bacteria by more than 100%, but failed to activate the CFEs from resistant or dependent

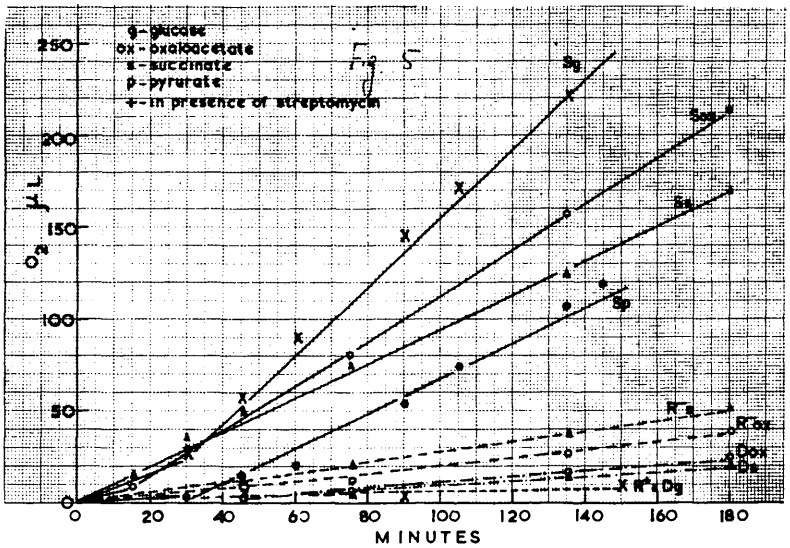

FIG. 5. Oxygen uptake by non-proliferating *V. comma* cells, sensitive—S; resistant—R; and dependent—D, to dihydro streptomycin in presence of various substrates. Substrates, 0.07 mM; dry wt cells, 0.1 mg; phosphate buffer (pH 7.8), 50 μmoles; total volume, 2 ml. All values corrected for endogenous respiration.

strains. In further experiments CFE from an active sensitive strain was added to CFE from a non-active dependent strain so that any cofactors necessary for enzymatic activity might be supplied. No additional activity was observed.

Oxygen uptake in the presence of various substrates was compared for all 3 strains. Here again striking differences were observed as seen in Fig. 5. The sensitive strains showed considerably higher rates of O_2 uptake with all substrates tested as compared to resistant and dependent strains. As for sensitive bacteria, in the presence of glucose or pyruvate, a lag period of 30 min. was observed. In the presence of oxalacetate this lag period was shorter and absent in presence of succinate.

Rate of tetrazolium reduction in presence of various substrates; effect of dihydro streptomycin. The rates of neo-tetrazolium reduction by all 3 strains were compared in the presence and absence of dihydro streptomycin. The differences are recorded in Fig. 6. The sensitive strain was very active in the presence of all substrates when tested after 45 or 105 minutes, as compared to resistant and dependent strains, which were inactive. It is of interest to note that the presence of dihydro streptomycin stimulates activity of the sensitive strain except in the presence of malate, where a retarding effect was observed. When the resistant or dependent strains were further incubated for 20 hours, slight activity could be demonstrated. It was thus demonstrated that contrary to

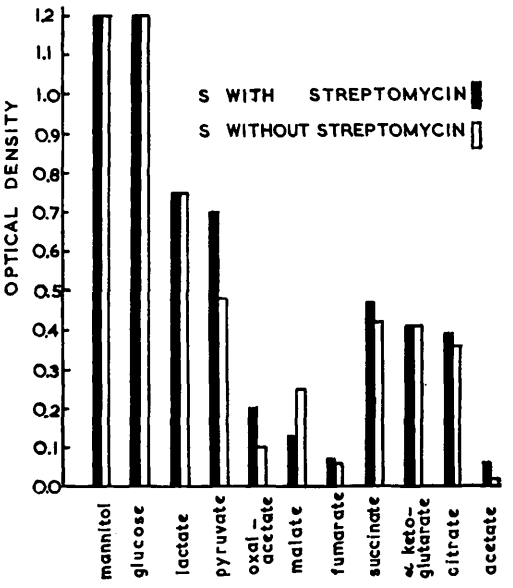

FIG. 6. Reduction of neo-tetrazolium by sensitive *V. comma* in presence of various substrates. For reaction mixture see Methods. Reaction, 105 min.

observations with sensitive bacteria, dihydro streptomycin has a retarding effect on reduction of neo-tetrazolium by resistant or dependent strains except in the case of the resistant strain in the presence of pyruvate.

Discussion. The results presented indicate that development of dependence or resistance to dihydro streptomycin in *V. comma* is accompanied by far reaching changes in the enzyme systems studied. Thus, a loss of nitratase and nitritase activity by non-proliferating *V. comma* cells, and of nitratase activity of their extracts, accompanied the development of resistance or dependence to dihydro streptomycin.

The inability of antibiotics to inhibit certain enzymatic activities of a sensitive strain, as in the case of nitratase activity and tetrazolium reduction in presence of various substrates, has also been observed with other antibiotics and microorganisms(11-14).

Differences in rate of reduction of neo-tetrazolium were observed when the 3 strains were tested in the presence of various substrates. The development of resistance or dependence was accompanied by a decrease in the neo-tetrazolium reduction rate.

In the sensitive strain the presence of the antibiotic has in fact a stimulating effect on neo-tetrazolium reduction in the presence of various substrates (except malate). Stimulation here observed is in sharp contrast to the complete inhibition (except for pyruvate) by the drug, observed in resistant and dependent strains.

Stimulation of certain enzymatic activities in a sensitive strain, in presence of the antibiotic, has also been reported by Roote and Polglase(15). Stimulation of an enzymatic reaction in an intact cell by an antibiotic, with respect to both nitrate and tetrazolium reducing activity, may result in disorganization of normal metabolic pathways.

Summary. 1) Nitrate reductase is inactive in non-proliferating dihydro streptomycin resistant and dependent *V. comma* cells and their extracts, as compared to the sensitive parent strain. Nitratase activity was found in resting cells of the sensitive strain but not in resistant and dependent mutants. Dihydro streptomycin did not inhibit but rather stimulated the nitratase activity of the sensitive strain. 2) Oxygen uptake in the presence of any of the following substrates was strongly inhibited in resistant and dependent mutant as compared to the sensitive strain: glucose, pyruvate, succinate, malate, oxaloacetate, α-ketoglutarate and fumarate. 3) Striking differences in rate of reduction of neo-tetrazolium were also observed in the presence of various substrates when the 3 strains were compared. The same pattern of high activity in the sensitive strain and practically, no activity in the dependent and resistant strains was always prevalent. Here too, the presence of antibiotic resulted in stimulation of activity of the sensitive strain in the presence of most substrates tested.

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