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Effect of Difluoromalonic Acid Di-Amide on Oxidation of Organic Acids by *Pseudomonas aeruginosa.* (28380)

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There have apparently been no studies on the effects of fluoromalonates on bacteria. Chari-Bitron(1) describes the action of monofluoromalonate in animals. He showed that it was decarboxylated to fluoroacetate and thus caused citrate accumulation. The succinic dehydrogenase and oxalacetic decarboxylase were only slightly inhibited by the compound. The following is a study of the action of difluoromalonate on washed suspensions of a strain of *Pseudomonas aeruginosa.* The oxidation of a number of organic acids including succinate, fumarate, and malate was inhibited by the compound. But when the cells were broken no inhibition, even with high concentrations, was found. It would appear that difluoromalonate is a non-specific inhibitor of the transport of these acids. Monofluoromalonate is much less effective and tetrafluorosuccinate and hexafluoroglutarate are inactive in the concentrations used.

Experimental. Pseudomonas aeruginosa was grown for 24 hours at 34° in Bacto nutrient broth, centrifuged and washed twice with water. The cells were taken up in 14 ml of 0.05 M Na-K phosphate buffer pH 7.8 and 0.5 ml was used in each Warburg vessel which had a final fluid of 2.0 ml. The fluorinated compounds, obtained from the Hynes Chemical Co. of Durham, were used as the di-amides. Some experiments done with the sodium salt of difluoromalonic acid showed that it had the same effects as the amide. Cells were broken by placing them in a 10 KC Raytheon sonic vibrator in the cold for 2 minutes. Unbroken cells were centrifuged down and 0.75 ml of the supernate was added to a mixture of the substrate, 0.7×10^{-3} NaCN and 1.0 ml of 0.46×10^{-4} M sodium 2,6-dichloroindophenol in a final volume of 3.0 ml. The rate of dye reduction was measured in a spectrophotometer at 600 mμ.

Difluoromalonamide (DFMA) was added to the cells before the substrate or 60 minutes after the substrate when the oxidation rate was linear. As shown in Table I for succinate and fumarate the inhibition percentages obtained are the same for both conditions which indicates that DFMA does not specifically inhibit possible permease induction. The same is true for benzoate oxidation which involves enzyme induction. In both cases a 70% inhibition occurred. (The inhibition percentages are the average of 4 readings taken during the 60 minutes following a 30 minute initial period except when enzymes are induced. Then they are the mean for 4 readings taken when linear oxidation rate is obtained.) After the first hour the inhibition percentage decreases partly because the control oxidation rate falls off and partly because some deamidation of DFMA occurs and the ammonium ion stimulates the oxidation rate of most of the substrates.

The inhibition percentage by DFMA is in-

TABLE I. Effect of 100 μg/ml of DFMA Added to the Cells (A) 15 Minutes Before or (B) 60 Minutes After 500 μg/ml of Succinic or Fumaric Acid on Oxidation of these Substrates.

Substrate	A	B
Succinate	70	68
Fumarate	64	60

TABLE II. Effect of 100 μg/ml of DFMA on Oxidation of 100, 200, and 400 μg Malonic Acid. Phosphate buffer pH 7.8.

Malonate (μg)	Inhibition (%)
100	69
200	67
400	71

dependent of substrate concentration. This is shown in Table II for malonate which is oxidized by this organism. Malonic acid concentration varied from 100 to 400 μg and DFMA concentration was kept constant at 200 μg. No difference in inhibition percentage was obtained. Pre-incubation of the cells with 20 μg of either mono or DFMA for 60 minutes failed to influence the subsequent oxidation of malonate. Malonate is oxidized after a short latent period and this is not eliminated by the fluoro compounds.

Inhibition by DFMA varies with the number of cells. It is difficult to keep the number exactly constant from experiment to experiment. The following figures were taken from the same experiment and are therefore comparable. The cells were preincubated for 60′ with 50 μg of the substrate subsequently to be added. This was done to eliminate differences in latent periods from whatever cause. One-half mg of each substrate was then added along with DFMA. The inhibition percentages were as follows: succinate 84%, itaconate 81%, malate 70%, fumarate 79%. These figures illustrate the non-specificity of the inhibition.

TABLE III. Effect of Various Fluorinated Compounds and of Malonic Acid on Reduction of 1.0 ml 0.46×10^{-4} M, 2,6-Dichloroindophenol by 1.0 mg Succinate in Presence of 0.75 ml of Sonicate and 0.7×10^{-3} NaCN. Total volume, 3.0 ml. Phosphate buffer pH 7.8. Figures represent decrease in light absorption in arbitrary units after 5 min. incubation at 25°.

	Compound added	Decrease in absorption
.....	.....	19
Succinate	.....	222
"	300 μg DFMA	235
"	300 " difluoromalonic acid	228
"	540 " tetrafluorosuccinic "	230
"	660 " hexafluoroglutaric "	223
"	300 " monofluoromalonamide	232
"	300 " malonic acid	126

Table III shows that none of the fluorinated compounds inhibit the succinic dehydrogenase in the sonicate of the cells. Malonate inhibits which indicates that no interfering substances were present.

Discussion. The inhibition by DFMA is specific in the sense that the monofluoro compound and fluorosuccinate and fluoroglutarate are essentially inactive in comparable concentrations. Since the di-amide is as active as the acid the free carboxyl group is not essential for activity and it is difficult to conceive of a possible mechanism of action. Chelation is ruled out since calcium and ferrous ions, both of which affect substrate oxidation in this organism, do not reverse the DFMA inhibition.

Summary. Difluoromalonic acid and its di-amide inhibit the oxidation of a number of organic acids by a strain of *Pseudomonas aeruginosa* although no inhibition could be demonstrated in sonicates of the cells. The di-amides of monofluoromalonic, tetrafluorosuccinic and hexafluoroglutaric acids are essentially inactive.

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