INHIBITOR STUDIES OF DIPHTHERIAL SUCCINIC DEHYDROGENASE

NORMAN STRAUSS¹ AND GREGORY J. JANN

Department of Bacteriology, University of California at Los Angeles, California

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It had previously been found that the addition of ferrous ion to cultures of the diphtheria bacillus—above that necessary for optimal toxin production—resulted in the disappearance from the culture supernatant of a porphyrin, iron, and diphtheria toxin in the molar ratio of 4:4:1 (Pappenheimer, 1947). On the basis of this data, it was suggested that the toxin was a precursor of the protein moiety of an iron-containing respiratory enzyme. Further studies suggested that the enzyme involved was succinic dehydrogenase (Pappenheimer and Hendee, 1947). Subsequent work (Pappenheimer and Hendee, 1949) carried out with a preparation partially purified by differential centrifugation, revealed that the enzyme was either identical or closely bound to cytochrome b, that the latter was slowly auto-oxidizable, and that the presence of cytochrome c and the cytochrome oxidase was in doubt. It was also found that the enzyme would not reduce cytochrome c.

Although a direct attack on the question of whether or not the protein moiety of the enzyme arises from diphtheria toxin depends on the solubilization and further purification of the enzyme, it was felt by the authors that valuable data could be obtained by kinetic and inhibitor studies of a partially purified preparation obtained while attempting such a solubilization and purification. Specifically, this paper deals with the determination of the Michaelis constant (K_s) of the system, and the enzyme-inhibitor dissociation constants (K_i) for several substances found to be inhibitory.

MATERIALS AND METHODS

Cultures. A strain of *Corynebacterium diphtheriae* isolated from a typical case of diphtheria was used. Microscopic and colonial morphology, in conjunction with fermentation tests, showed it to be a gravis type. *In vivo* and *in vitro* tests

(King *et al.*, 1949) showed it to be toxigenic. The organism was grown at 37 C on Difco blood agar base containing 0.5 per cent sodium acetate. Maximum growth was obtained in 24 hr. The cells were harvested with 0.067 M phosphate buffer of pH 7.4, washed 3 times in fresh buffer, drained, and stored at 2 C until needed.

Preparation of extracts. The procedure was as follows: 1.5 g wet weight of cells were suspended in 10 ml of 0.067 M phosphate buffer of pH 7.4 (all operations were carried out in this buffer except where otherwise noted) and treated at 0 C (all operations were carried out at this temperature) in a 9,000-cycle Raytheon sonic oscillator, 115 v, for 40 min. The suspension was centrifuged at $2,000 \times G$ to remove fragments and the sediment was discarded. The supernatant fluid was found to have a QO_2^N of 1360. The supernatant fluid was centrifuged at $12,000 \times G$ in a Sorvall centrifuge, model SS-1, for 20 min. The sediment was discarded and the supernatant fluid was brought to 25 per cent ammonium sulfate saturation by slow addition of the solid salt. The resulting suspension was centrifuged at $12,000 \times G$ for 15 min. The supernatant fluid was discarded and the sediment resuspended in half the original volume of cold buffer. This suspension was then dialyzed against 3 changes of phosphate buffer for 36 hr. The resulting straw-colored preparation was stored at -15 C. The final preparation had a QO_2^N of 8360, representing a 6-fold purification. The activity recovered was 60 per cent. This preparation lost approximately 15 per cent of its activity over a 6-month period.

Manometric assay. Rates were followed by the usual Warburg technique. Each flask contained: enzyme, diluted so as to give suitable rates of oxygen uptake, 1.0 ml; sodium succinate of varying concentrations, 0.2 ml; 0.025 M methylene blue in the sidearm, 0.2 ml; 0.067 M phosphate buffer of pH 7.4, 1.5 ml; 0.032 M potassium cyanide, 0.1 ml; and inhibitor, when used, 0.1 or 0.2 ml. The volume in each flask was brought to

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TABLE 1
Results of K_s^* and $K_i^\dagger$ determinations by the
manometric method

Inhibitor	Inhibitor Concentration (Molarity)	$K_s \times 10^3$	K_i (M)
Fumarate	0.00	3.2	—
	3.13×10^{-3}	—	1.0×10^{-3}
	6.25×10^{-3}	—	1.4×10^{-3}
Fumarate	0.00	4.7	—
	3.13×10^{-3}	—	1.9×10^{-3}
	6.25×10^{-3}	—	2.9×10^{-3}
Malonate	0.00	4.0	—
	4.06×10^{-3}	—	3.7×10^{-3}
	8.12×10^{-3}	—	3.7×10^{-3}
Polymyxin B sulfate	0.00	4.5	—
	3.87×10^{-4}	—	7.8×10^{-4}
	7.74×10^{-4}	—	12.0×10^{-4}

* Enzyme-substrate dissociation constants.

† Enzyme-inhibitor dissociation constants.

3.2 ml with distilled water. All measurements were made at 37.4 C. The initial rate of uptake of oxygen was directly proportional to the enzyme concentration. The initial rate was independent of the methylene blue concentration used and of the shaking rate of the Warburg apparatus (108 strokes per min). The uptake of oxygen was linear for 9 to 12 min. Methylene blue was tipped in at zero time and readings taken every 3 min thereafter for 9 min. In all cases rates were calculated by the least square method. No oxygen uptake occurred in the absence of substrate, and such controls were routinely omitted. The pH value of each flask was checked following each experiment.

Spectrophotometric assay. Although it was found that the enzyme would reduce potassium ferrieyanide and 2,6-dichlorophenol indophenol in the presence of succinate, this method was not used because of a 50-per cent decrease in rate in 1 min.

Nitrogen determinations. Nitrogen was determined by the micro-Kjeldahl method.

Chemicals. The disodium salt of succinic acid was obtained from the Eastman Chemical Co. Fumaric acid was obtained from the same company as 98 per cent pure, but it was further purified by sublimation at 205 C. Malonic acid

was obtained from the Delta Chemical Works. Neomycin, polymyxin B sulfate, bacitracin, penicillin G, and streptomycin sulfate were all obtained from Nutritional Biochemicals Corp. Aureomycin and diphtheria antitoxin were obtained from the Lederle Labs. Chloromycetin was obtained from Parke, Davis and Co. All solutions were adjusted to pH 7.4 prior to use.

RESULTS AND DISCUSSION

Approximations of the enzyme-substrate dissociation constant were obtained by the Lineweaver and Burk (1934) method. This method is based on two major assumptions (Michaelis and Menten, 1913): first, that the substrate is present in much higher concentration than enzyme, and second, that the rate-limiting step is the breakdown of the enzyme-substrate complex into product and free enzyme, so that the steady state concentrations of free enzyme, substrate, and enzyme-substrate complex approach equilibrium. The extent of the approximation will depend on how small the rate constant of the rate-limiting step is with respect to k_2 (Briggs and Haldane, 1925).

The values of K_s obtained were:

3.5×10^{-3} M, 3.6×10^{-3} M, 4.5×10^{-3} M, 3.2×10^{-3} M, 4.7×10^{-3} M, 4.4×10^{-3} M, 5.2×10^{-3} M, 4.0×10^{-3} M, 4.5×10^{-3} M.

Figure 1. Lineweaver-Burk plot of fumarate inhibition. Straight lines were calculated statistically.

Figure 2. Lineweaver-Burk plot of malonate inhibition. Straight lines were calculated statistically.

TABLE 2

The effects of various antibiotics on succinic dehydrogenase activity

Inhibitor	Inhibitor Concentration	Activity	Deviation From Control
	(mg/ml)	($\mu\text{LO}_2/\text{hr}$)	%
Bacitracin.....	.194	452	-0.44
Neomycin.....	.467	453	-0.22
Penicillin G.....	.468	453	-0.22
Polymyxin B sulfate*..	.375	367	-19.2
Control.....	—	454	—

* Concentrations of Na_2SO_4 as high as 0.03 M had no inhibitory effect.

The average value is 4.2×10^{-3} M with a mean deviation from the mean of 12.6 per cent. The spread of the values must at present be attributed to experimental error. These determinations were carried out on the same enzyme preparation over a period of about 3 weeks.

Enzyme-inhibitor dissociation constants were calculated using the value of the K_s obtained in each inhibitor experiment, as shown in table 1. It can be seen that fumarate is a moderately strong competitive inhibitor (figure 1) of the reaction, its affinity for the enzyme being of the same order of magnitude as that of succinate.

TABLE 3

The effect of various antibiotics and of diphtheria antitoxin on succinic dehydrogenase

Inhibitor	Inhibitor Concentration	Activity	Deviation From Control
	(mg/ml)	($\mu\text{LO}_2/\text{hr}$)	%
Streptomycin sulfate.....	0.313	399	+3.9
Aureomycin.....	0.157	383	-0.26
Chloromycetin.....	0.157	377	-1.8
Diphtheria antitoxin.....	1 unit/ml	373	-2.9
Control.....	—	384	—

Figure 3. Lineweaver-Burk plot of polymyxin B sulfate inhibition. Straight lines were calculated statistically, O no inhibitor; O, 2.6×10^{-4} M inhibitor; O, 7.7×10^{-4} M inhibitor.

Such a product inhibition is not surprising, being often encountered with other systems. Malonate was also tested as an inhibitor. The results are shown in table 1 and in figure 2. It can be seen that malonate is a strong competitive inhibitor of the reaction.

Of the antibiotics tested only polymyxin B sulfate showed activity. The results are shown in tables 2 and 3. A plot of $1/V$ versus $1/S$ (figure 3) revealed a family of straight lines having different intercepts but essentially the same slope. A possible explanation of this type of in-

hibition involves a system in which only the enzyme-substrate complex is attacked (uncompetitive inhibition), as opposed to a non-competitive inhibition in which both free enzyme and enzyme-substrate complex are attacked. The expression obtained, then, from a straightforward Lineweaver-Burk derivation (Ebersole *et al.*, 1944) is:

$$1/V = [1 + (I)/K_i]/V_{\max} + [K_s/V_{\max}]1/(S) \quad (1)$$

where V is the initial reaction velocity, $V_{\max}$ is the maximum velocity (S) is the substrate concentration; (I) the inhibitor concentration; and K_s and K_i are enzyme-substrate and enzyme-inhibitor dissociation constants, respectively. Inhibitor dissociation constants were calculated from equation (1) assuming the molecular weight of polymyxin B sulfate to be 1459 (Hausmann and Craig, 1954).

It is to be emphasized that values of K_s , and values of K_i for each inhibitor can be accepted only as orders of magnitude, and not as final values, since the assay method is relatively crude due to the deviation from linearity of initial reaction rates after 9 min.

SUMMARY

Diphtherial succinic dehydrogenase has been purified 6-fold by simple ammonium sulfate fractionation. This partially purified particulate enzyme has been used for kinetic and inhibitor studies. The Michaelis constant of the system was found to be of the order of 4×10^{-3} M. It was found that fumarate and malonate inhibit the enzyme competitively. Evidence presented

indicates that polymyxin B sulfate inhibits the enzyme uncompetitively.

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