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Kinetics of 1-Dehydrogenation of Steroids by *Septomyxa affinis*

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Summary

Mathematical models for the kinetics of 1-dehydrogenation of steroids by *Septomyxa affinis* are considered. A model providing for product inhibition fits experimental data best. Product inhibition was also demonstrated directly.

INTRODUCTION

1-Dehydrogenation is an important step in current manufacture of pharmaceutical corticosteroids, and use of *Septomyxa affinis* fermentation to conduct this reaction has been described.[1] The organism attacks a variety of different steroids, but at varying relative rates.[2] In the instance of 1-dehydrogenation as exemplified by oxidation of 11β,21-dihydroxy-4,17(20)-pregnadiene-3-one (dienediol) and to a lesser extent by oxidation of hydrocortisone, utilization of dissolved substrate initially approximates first-order kinetics, but reaction rate deviates from such kinetics as the reaction approaches termination. In addition, apparent first-order reaction rate constants calculated from initial phases of the reaction tend to vary with initial concentration of substrate. These facts indicate a higher order of complexity than simple first-order reaction, and a detailed investigation of kinetics in 1-dehydrogenation of dienediol and of hydrocortisone was undertaken.

The overall 1-dehydrogenation of a steroid by *S. affinis* is given by the reaction:

$$\text{Substrate} + {}^1/_2\,O_2 \xrightarrow{S.\ affinis} \text{Product} + H_2O$$

As concerned in this paper the reaction was carried out by induced, washed cells of the organism under conditions of excess oxygen, constant optimum pH, and constant temperature.

THEORETICAL

The kinetic model is postulated as follows. The enzyme catalyst, located on membranes of the cells, supplies a number of activity sites for the reaction. Substrate molecules, in solution, are activated by forming a complex with an enzyme activity site by an adsorption process. The adsorbed enzyme–substrate complex is then converted at a finite reaction rate to an enzyme–product complex. This step is considered to be rate-determining for the overall dehydrogenation reaction, since the overall reaction has been found to be complete at a certain finite time. The rate of this step is influenced by initial substrate concentration; initial product concentration; substrate and product concentrations at any specific instant during the reaction; and total amount of enzyme present. Following this conversion step, the enzyme–product complex undergoes a desorption step in which free product is formed and the enzyme sites are regenerated.

This simple reaction scheme will be complicated if complexes of higher order are formed. For example, an enzyme–substrate complex may interact with a product molecule to form an enzyme–substrate–product complex. Formation of such tertiary complexes would be dependent on structure of enzyme site; structure of substrate and product; and forces of interaction between the molecules.

Let S be substrate, P be product, and $E \cdot S$, $E \cdot P$, $E \cdot S_2$, $E \cdot P_2$, and $E \cdot S \cdot P$ be different types of enzyme complexes.

Scheme I.

$$E + S \rightleftharpoons E \cdot S \quad \text{(a)}$$

$$E \cdot S \rightleftharpoons E \cdot P \quad \text{(b)}$$

$$E \cdot P \rightleftharpoons E + P \quad \text{(c)}$$

Besides steps (a), (b), and (c), the following further interactions to form enzyme complexes are possible.

Scheme II. $E \cdot P + P \rightleftharpoons E \cdot P_2$ (d)

Scheme III. $E \cdot S + S \rightleftharpoons E \cdot S_3$ (e)

Scheme IV. $E \cdot S + P \rightleftharpoons E \cdot S \cdot P$ (f)

$E \cdot P + S \rightleftharpoons E \cdot S \cdot P$ (g)

First, let us consider the mathematical development of reaction scheme I, which only includes steps (a), (b), and (c). The rate v of the rate-determining step (b) is:

$$v = -d[\mathrm{S}]/dt = k_r[\mathrm{E}\cdot\mathrm{S}] \tag{1}$$

where k_r is the reaction rate constant.

From (a) and (c), we get:

$$K_S = [\mathrm{E}\cdot\mathrm{S}]/[\mathrm{E}][\mathrm{S}] \tag{2}$$

$$K_D = [\mathrm{E}\cdot\mathrm{P}]/[\mathrm{E}][\mathrm{P}] \tag{3}$$

where K_S is the adsorption equilibrium constant and is equivalent to the inverse of the conventional Michaelis constant and K_D is the desorption equilibrium constant. Assuming that the total amount of enzyme is constant during the course of reaction, the total amount of enzyme E_0 is:

$$E_0 = [\mathrm{E}] + [\mathrm{E}\cdot\mathrm{S}] + [\mathrm{E}\cdot\mathrm{P}] \tag{4}$$

Substituting eqs. (2) and (3) into eq. (4) and rearranging gives:

$$[\mathrm{E}] = E_0/(1 + K_S[\mathrm{S}] + K_D[\mathrm{P}]) \tag{5}$$

Again, substituting eqs. (2) and (5) into eq. (1), we obtain the overall rate equation as follows:

$$-d[\mathrm{S}]/dt = k_rK_S[\mathrm{E}][\mathrm{S}] = k_rE_0K_S[\mathrm{S}]/(1 + K_S[\mathrm{S}] + K_D[\mathrm{P}]) \tag{6}$$

The time integral of eq. (6) is obtained by the conventional kinetic approach. Let a be the initial substrate concentration, P_0 be the initial product concentration, and x be the amount of substrate per unit volume reacted at time t. Therefore, at any given time during the course of reaction:

$$[\mathrm{S}] = a - x \tag{7}$$

$$[\mathrm{P}] = P_0 + x \tag{8}$$

Substituting eqs. (7) and (8) into eq. (6) and rearranging, we get:

$$k_rE_0\,(dt/dx) = 1/[K_S(a - x)] + 1 + [K_D(P_0 + x)]/[K_S(\mathrm{a} - x)] \tag{9}$$

Equation (9) is integrated between time zero and time t, giving:

$$k_r E_0 t = (1/K_S\,[1 + K_D\,(a + P_0)]\ln[a/(a - x)] + [1 - (K_D/K_S)]x \tag{10}$$

Equation (10) will reduce to the conventional Michaelis equation if the amount of enzyme interacted with product is small. In other words, if K_D is small in comparison with K_S, then:

$$k_r E_0 K_S t = \ln[a/(a - x)] + K_S x \tag{11}$$

This equation will describe a first-order reaction if either K_S is relatively small or at an early stage of reaction. In the latter case, x is a relatively small value.

Let us consider reaction scheme II by taking into account step (d):

$$K_P = [\mathrm{E \cdot P_2}]/[\mathrm{E \cdot P}][\mathrm{P}] \tag{12}$$

where K_P is the equilibrium constant. Then the total amount of enzyme in the system is:

$$E_0 = [\mathrm{E}] + [\mathrm{E \cdot S}] + [\mathrm{E \cdot P}] + [\mathrm{E \cdot P_2}] \tag{13}$$

Substituting eqs. (2), (3), and (12) into eq. (13) and rearranging, we get:

$$[\mathrm{E}] = E_0/(1 + K_S[\mathrm{S}] + K_D[\mathrm{P}] + K_P K_D[\mathrm{P}]^2) \tag{14}$$

Again, substituting equations (2) and (14) into equation (1), the overall rate equation according to reaction scheme II is obtained:

$$-d[\mathrm{S}]/dt = k_r E_0 K_S[\mathrm{S}]/(1 + K_S[\mathrm{S}] + K_D[\mathrm{P}] + K_P K_D[\mathrm{P}]^2 \tag{15}$$

The time integral of eq. (15) is:

$$k_r E_0 t = \frac{1}{K_S}\,[1 + K_D(P_0 + a + 2K_P P_0 a + K_P P_0^2 + K_P a^2)]$$

$$\ln\frac{a}{a - x} + \left[1 - \frac{K_D}{K_S}\,(1 + 2K_P P_0 + K_P a)\right]x - \frac{K_P K_D}{2K_S}\,x^2 \tag{16}$$

Equation (16) can be reduced to eq. (10) if K_P is very small. In this case only a small amount of enzyme forms an $\mathrm{E \cdot P_2}$ complex.

The proposed schemes III or IV will reduce to the same type of time integral equation as eq. (16). The only difference will be in the coefficients before each term.

EXPERIMENTAL

Enzyme

Water suspensions of washed, induced *Septomyxa affinis* cells, stored under refrigeration at approximately 10,000 DU/ml., were available.[2] The enzyme level desired in any experiment was attained by centrifuging the cells and resuspending them in tris buffer at the proper concentration.

Dehydrogenation Procedure

A 100-ml. portion of cell suspension at the desired enzyme potency in Tris phosphate buffer (0.1*M* in Tris) was contained in a 500-ml. large-mouth Erlenmeyer flask on a magnetic stirrer at room temperature (27°C.). Dienediol oxidations were run at pH 7.0, and hydrocortisone oxidation at pH 7.5. These pH values are optimal for the individual reactions.[2] At zero time the reaction was begun by slowly adding substrate as a feed solution in 2:1 acetone–propylene glycol. The reaction mixture was stirred at a rate so that adequate oxygen for reaction was provided and oxygen supply was not limiting. At suitable intervals an aliquot of the reaction mixture was withdrawn and diluted, if necessary, to contain 150–250 μg. of steroid/ml. A 3-ml. aliquot was extracted with 5 ml. of redistilled methylene chloride, and in the case of hydrocortisone oxidation the extraction was repeated. Extracts were dried and read in 3A alcohol at 243 and 268 mμ. Steroid content was calculated from the absorptivities of dienediol and its 1-dehydro analog[2] and of hydrocortisone and prednisolone (for hydrocortisone, $a_{243} = 44.4$, $a_{268} = 5.40$; for prednisolone, $a_{243} = 41.1$, $a_{268} = 20.2$). Results were checked by quantitative papergram assays.[2]

Enzyme potency in the reaction mixture was checked by 1-dehydrogenase assay[2] before reaction and at termination of the experiment to insure that the potency remained unchanged during the course of reaction. Constant potency was found in all cases for the data reported here.

RESULTS

Dienediol

Kinetic data on the dehydrogenation of dienediol at three different initial dienediol concentrations and a common enzyme potency (1000 DU/ml.) are given in Table I. A conventional first-order kinetics

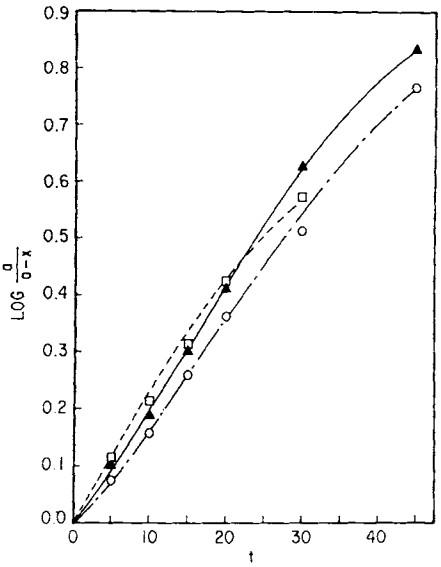

Fig. 1. A plot of log $a/(a - x)$ vs. t based on first-order kinetics: (O) data of expt. 1, $a = 0.620$ mmole/l.; (▲) data of expt. 2, $a = 0.472$ mmole/l.; (□) data of expt. 3, $a = 0.280$ mmole/l.

plot, log $a/(a - x)$ versus t, is shown in Figure 1. The experimental points fail to conform to elementary first-order kinetics on two counts. First, the slopes of the straight-line portion of the curves vary with initial substrate concentration; and second, the curves deviate from straight lines at late stages of the reaction. Such behavior is also inconsistent with Michaelis' theory.[3]

In Figure 2 the data are plotted as t/x versus $(1/x)$ log $[a/(a - x)]$ in accordance with eq. (10), which considers product inhibition. To conform to this equation, the plot should yield straight lines with a common intercept. The slopes of the lines should be dependent on initial substrate concentration, and would also be dependent on initial product concentration if any product had been present at zero time. The data thus plotted conform well to these requirements. The curves are straight lines with a common negative intercept, and the slopes vary with initial substrate concentration. This indicates that accumulating reaction product inhibits the reaction.

TABLE I
Kinetics of 1-Dehydrogenation of Dienediol at Three Substrate Levels
(Enzyme Potency = 1000 DU/ml.; Level of Substrate Solvent = 1%)

Expt. no.	a, mmole/l.	t, min.	$a - x$ (diene-diol), mmole/l.	x (triene-diol), mmole/l.	$\log \frac{a}{a-x}$	$\frac{1}{x} \log \frac{a}{a-x}$	t/x
1	0.620	0	0.620	0.000	0.000	—	—
		5	0.522	0.098	0.076	0.78	50
		10	0.432	0.188	0.157	0.85	52
		15	0.345	0.275	0.254	0.93	54
		20	0.270	0.350	0.361	1.03	57
		30	0.188	0.432	0.518	1.20	70
		45	0.107	0.513	0.713	1.49	88
		60	0.062	0.558	1.000	1.79	108
		120	0.036	0.584	—	—	
2	0.472	0	0.472	0.000	0.000	—	—
		5	0.371	0.101	0.104	1.03	50
		10	0.303	0.169	0.192	1.13	59
		15	0.230	0.242	0.301	1.24	62
		20	0.182	0.290	0.414	1.43	69
		30	0.110	0.362	0.630	1.70	84
		45	0.069	0.403	0.834	2.07	112
		60	0.045	0.427	1.000	2.39	133
		120	0.020	0.452	—	—	—
3	0.280	0	0.280	0.000	0.000	—	—
		5	0.219	0.061	0.107	1.75	82
		10	0.172	0.108	0.212	1.96	93
		15	0.135	0.145	0.316	2.18	104
		20	0.107	0.173	0.418	2.42	116
		30	0.074	0.206	0.578	2.80	135
		60	0.024	0.256	—	—	

For a plot of the type shown in Figure 2 the following relationships exist:

$$m = (2.3/k_r E_0)\ [(1/K_S) + (K_D/K_S)\ a] \tag{17}$$

$$I = (1/k_r E_0)[1 - (K_D/K_S)] \tag{18}$$

where m is the slope and I is the intercept.

In eq. (17), let:

$$m' = (m/2.3) = (1/k_r E_0)\ [(1/K_S) + (K_D/K_S)a] \tag{19}$$

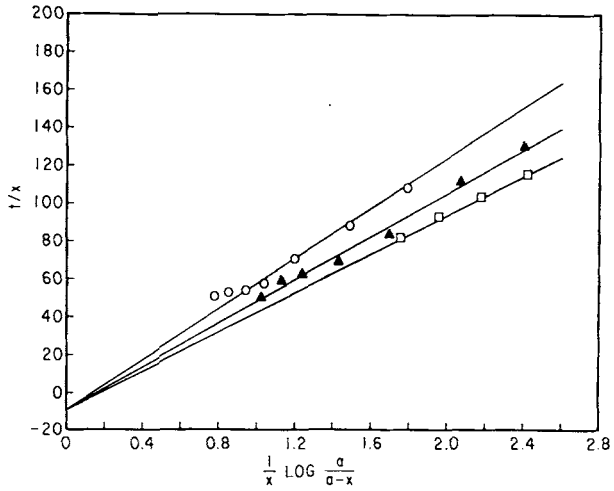

Fig. 2. A plot of t/x vs. $(1/x)$ log $[a/(a - x)]$ based on product inhibition mechanism: (O) data of expt. 1, a = 0.620 mmole/l.; (▲) data of expt. 2, a = 0.472 mmole/l.; (□) data of expt. 3, a = 0.280 mmole/l.

Dividing eq. (19) by eq. (18) and rearranging gives:

$$m'/I = (K_D/K_S)[a + (m'/I)] + (1/K_S) \tag{20}$$

Accordingly, a plot of m'/I versus $[a + (m'/I)]$ should give a straight line whose slope is equal to K_D/K_S and whose intercept is $1/K_S$. Table II shows the calculations, and Figure 3 determines the values of K_S and K_D as:

$$K_S = 1.67 \text{ l./mmole}$$

$$K_D = 2.54 \text{ l./mmole}$$

TABLE II
Calculated Values of m' and $m'/I + a$ from Data of Table I

Expt. no.	a, mmole/l.	I	m'	$\frac{m'}{I} + a$
1	0.620	−10	29.4	−2.32
2	0.472	−10	25.0	−2.03
3	0.280	−10	20.1	−1.73

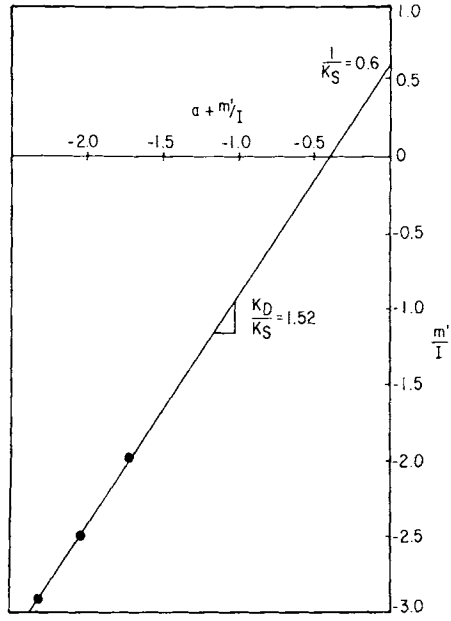

Fig. 3. Determination of K_S and K_D.

Substituting these values into eq. (10), a working kinetic equation for dienediol dehydrogenation is:

$$k_r E_0 t = 2.3\ [0.60 + 1.52\ (a + P_0)]\ \log\ [a/(a - x)] - 0.52x \quad (21)$$

The value of $k_r E_0$ as calculated from the slopes and intercepts of Figure 2 according to eqs. (18) and (19) is 0.052 mmole/l min. at an enzyme potency of 1000 DU/ml.

Product inhibition of dienediol dehydrogenation was also demonstrated directly as shown in Figure 4. The data are from an experiment in which (a) the product was added at zero time at about one-half of the initial substrate concentration, and (b) no product was added. When plotted according to eq. (10), the data followed closely the theoretical lines calculated from eq. (21).

The relationship between $k_r E_0$ and total enzyme in the system is given in Figure 5. These experiments were run at the same initial substrate concentration and different enzyme concentrations. As expected, a straight line relation is found.

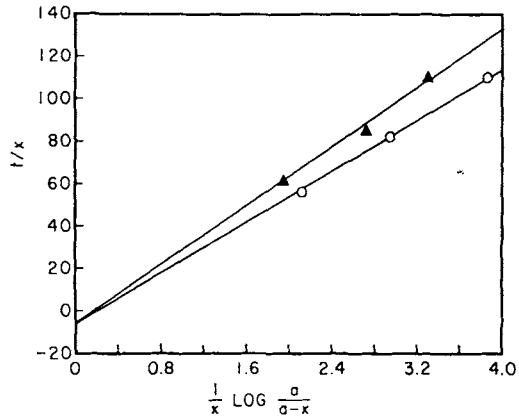

Fig. 4. Direct demonstration of product inhibition for 1-dehydrogenation of dienediol, level of substrate solvent = 1%, enzyme level = 1600 DU/ml.: (O) data of expt. 4, a = 0.312 mmole/l., P_0 = 0. (▲) data of expt. 5, a = 0.312 mmole/l. P_o = 0.143 mmole/l.; (—•—), theoretical line calculated from eq. (21).

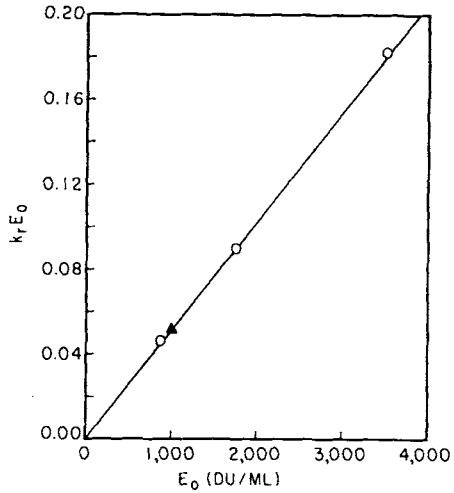

Fig. 5. Relationship between reactivity and enzyme potency, level of substrate solvent = 1%: (O) data of expts. 6, 7, and 8; (▲) data of expts. 1, 2, and 3.

Hydrocortisone to Prednisolone

Table III presents data on three hydrocortisone oxidations. The first run is on hydrocortisone alone at 1.40 mmole/l. and the second

and third runs are with prednisolone added at 1.39 and 2.78 mmole/l., respectively at zero time. Data from each run, plotted in Figure 6 as log $[a/(a - x)]$ versus t, yield a straight line to termination of the reaction. However, the slopes vary with initial product concentration. Hence the data do not conform to first-order rate kinetics, and product inhibition is again implicated.

It should be noted that eq. (10) can be reduced to an apparent first-order reaction equation, with slope dependent on initial substrate or initial product concentration, if $K_S = K_D$:

$$k_r E_0 t = 2.3\ [(1/K_S) + (a + P_0)] \log [a/(a - x)] \qquad (22)$$

TABLE III

Kinetic Data Indicating Product Inhibition in the 1-Dehydrogenation of Hydrocortisone to Prednisolone

(Enzyme Level = 9000 DU/ml.; Level of Substrate Solvent = 2.5%)

Expt. no.	a, mmole/l.	P_0, mmole/l.	t, hr.	$a - x$ (hydrocortisone), mmole/l.	x (prednisolone), mmole/l.	$\log \frac{a}{a - x}$
9	1.4	0	0	1.40	0.000	0.000
			0.5	1.07	0.330	0.116
			1.0	0.925	0.475	0.180
			1.5	0.760	0.660	0.265
			2.0	0.637	0.763	0.342
			3.0	0.443	0.957	0.500
			4.0	0.267	1.133	0.720
			6.0	0.078	1.322	—
10	1.4	1.39	0	1.40	0.000	0.000
			0.5	1.18	0.280	0.074
			1.0	0.945	0.455	0.170
			1.5	0.835	0.565	0.225
			2.0	0.721	0.679	0.335
			3.0	0.492	0.908	0.455
			4.0	0.315	1.085	0.640
			6.0	0.091	1.309	—
11	1.4	2.78	0	1.40	0.000	0.000
			0.5	1.15	0.250	0.086
			1.0	0.968	0.432	0.160
			1.5	0.850	0.550	0.215
			2.0	0.746	0.754	0.273
			3.0	0.640	0.760	0.343
			4.0	0.440	0.960	0.510
			5.0	0.334	1.066	0.624
			6.0	0.232	1.168	—

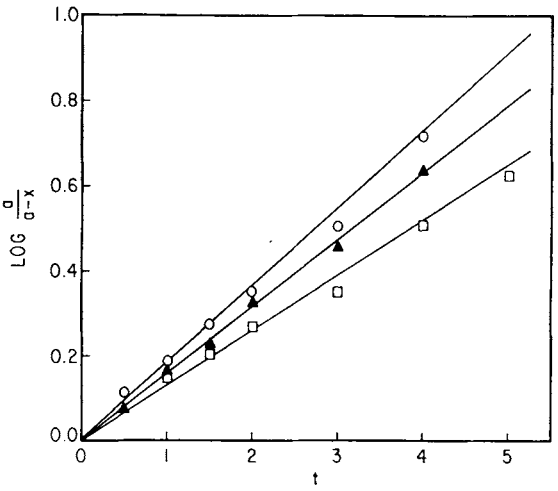

Fig. 6. Direct demonstration of product inhibition for 1-dehydrogenation of hydrocortisone: (O) data of expt. 9, a = 1.4 mmole/l., P_0 = 0; (▲) data of expt. 10, a = 1.4 mmole/l., P_0 = 1.39 mmole/l.; (□) data of expt. 11, a = 1.4 mmole/l., P_0 = 2.78 mmole/l.

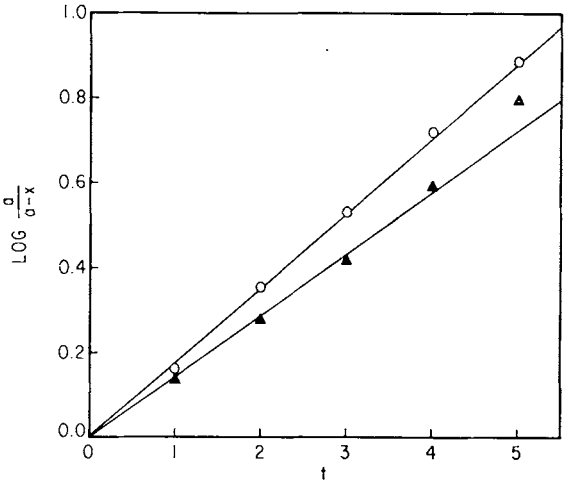

Fig. 7. Experimental results for oxidation of hydrocortisone at different initial substrate concentrations, enzyme level = 10,000 DU/ml., level of substrate solvent = 3%: (O) data of expt. 12, a = 1.39 mmole/l.; (▲) data of expt. 13, a = 4.05 mmole/l.

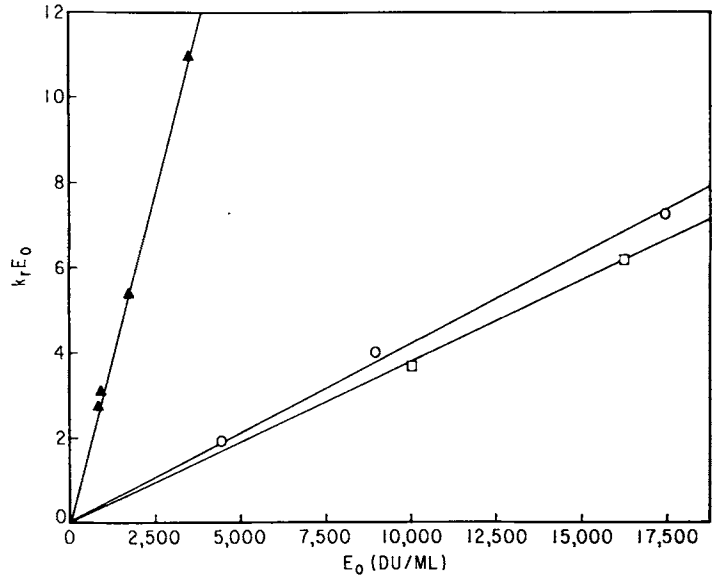

Fig. 8. Relationship between reactivity and enzyme potency: (O) hydrocortisone with 2% substrate solvent; (□) hydrocortisone with 3% substrate solvent; (▲) dienediol with 1% substrate solvent.

The slope of a line relating log $[a/(a - x)]$ to t is

$$m = \frac{k_r E_0}{2.3\ [(1/K_S) + (a + P_0)]} \tag{23}$$

The values of K_S and K_D were calculated from this relationship and the data in Figure 6 to be:

$$K_S = K_D = 0.15 \text{ l./mmole}$$

Thus, a working equation for hydrocortisone oxidation is:

$$k_r E_0 t = 2.3\ [6.7 + (a + P_0)] \log\ [a/(a-x)] \tag{24}$$

Experimental data obtained when different initial substrate concentrations were oxidized at the same enzyme potency conformed well to eq. (23) as shown in Figure 7.

The relationship between enzyme potency, which is determined by dienediol oxidation in the routine assay, and $k_r E_0$ for hydrocortisone

is given in Figure 8. The ratio of reaction rate constants on dienediol and on hydrocortisone is about 7. The moderate effect of substrate solvent concentration (2% compared to 3%) on reactivity is also shown.

DISCUSSION

Kinetic data in 1-dehydrogenation of dienediol and of hydrocortisone by cells of *Septomyxa affinis* can be explained by a mechanism which includes product inhibition. The reaction rate and equilibrium constants for oxidation of these two steroids are summarized in Table IV.

TABLE IV
Constants for Dienediol and Hydrocortisone Dehydrogenation

Substrate	K_S, l./mmole	K_D, l./mmole	k_rE_0, mmole/l. hr.[a]
Dienediol	1.67	2.54	7.7
Hydrocortisone	0.15	0.15	1.1

[a] At 2500 DU/ml. enzyme potency.

The much slower reaction rate on hydrocortisone as compared to dienediol is attributed to two factors. First, the reaction rate constant on hydrocortisone is one-seventh that on dienediol at the same enzyme potency. Second, the adsorption equilibrium constant, K_S, of dienediol is about eleven times larger than that of hydrocortisone. Thus, the relative rapidity of dienediol dehydrogenation is due to an intrinsically faster reaction rate, aided by a higher degree of enzyme–substrate complex formation. These factors outweigh the more serious product inhibition effect present in dienediol oxidation.

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