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Kinetics of the Hydrolysis of Phenylphosphate Catalyzed by Potato Phosphatase

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The hydrolysis of phenylphosphate catalyzed by potato phosphatase was investigated to a high degree of reaction, and the liberation of phenol was followed colorimetrically. The reaction was studied with different initial concentrations of enzyme and substrate and with and without phosphate added. It was found that the following chronometric integral can describe the reaction:

$$E \cdot t = [k_a + k_b\ (p + a)] \ln \frac{a}{a-x} - k_c \cdot x$$

where t is time, a initial substrate concentration, x phosphate concentration at the time t, p the concentration of phosphate added and k_a, k_b and k_c are constants. A reaction mechanism based on the chronometric integral found is suggested.

Potato phosphatase is a group-specific acid phosphatase present in potato juice. Pfankuch [1] found the optimum pH 5.8, and he showed that Mg^{++} had little or no inhibiting effect on the activity of the enzyme, whereas F^- was a strong inhibitor. The Michaelis constant of β-glycerophosphate was $K_s = 18$ mM, and strong inhibition was found on addition of phosphate. The dissociation constant, K_p, of the enzyme-phosphate complex was found to be 1.8 mM. Helferich and Stetter [2] found the optimum pH for the substrate phenylphosphate to be 5.2—5.3. At a low degree of reaction it was found that the reaction followed first-order kinetics. Sripathi *et al.*[3] found optimum pH 5.3 and $K_s = 10$ mM for β-glycerophosphate. For an enzyme preparation from sweet potato Kondo *et al.*[4,5] found optimum pH 5.3—6.0 and $K_s = 1.7$—2.0 mM for β-glycerophosphate, $K_s = 12$ mM for glucose-1-phosphate and $K_s = 0.6$ mM for phenylphosphate. Very active preparations of the potato phosphatase have also been prepared by Schramm and Flammersfeld [6].

The reaction is by all the investigations quoted above only examined to low degrees of reaction. In this investigation the reaction was studied to high degrees of hydrolysis of the substrate, and the influence of the inhibition of phosphate was examined. The reaction was followed by determining the phenol liberated during the hydrolysis.

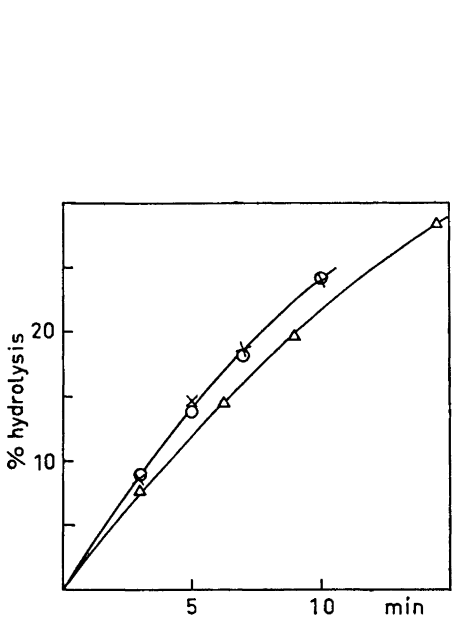

Fig. 1. Effect of Mg++ and phenol upon the rate of hydrolysis. ⊙——⊙ phenylphosphate concentration 4 mM, ×——× phenylphosphate concentration 4 mM and phenol concentration 6 mM, Δ——Δ phenylphosphate concentration 4 mM and concentration of Mg++ = 15 mM.

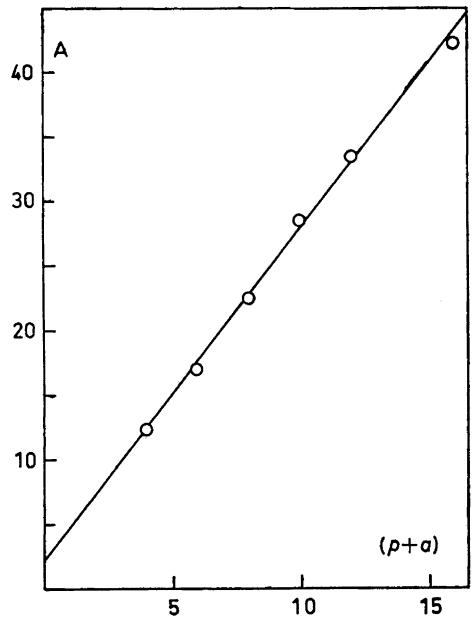

Fig. 2. Relation between the values of ($p_{\perp}$ + a) (abscissa) and the values of A (ordinate).

EXPERIMENTAL

Enzyme. Potato juice was fractionated by means of acetone and tannin as described by Helferich and Stetter [2]. Aqueous solutions of the most active fractions were used for the experiments. A slight activity of phenoloxydase in the enzyme solutions was completely inhibited by 0.5 p.p.m. sodiumsulfide. This reagent had no influence on the phosphatase activity. The enzyme solutions were stored at 0°C with some toluene added.

Substrate. The substrates were composed of 5 ml freshly prepared disodiumphenylphosphate solutions (Merck) and 4 ml 0.2 M citrate buffer (pH = 5.3). Investigating the phosphate inhibition the quantity of phosphate wanted was added to the phenylphosphate solution as KH_2PO_4.

Phenol determination. The phenol liberated during the reaction was determined colorimetrically with 4-aminoantipyrene as described by Hockenhull *et al.*[7]. The extinction was measured at 510 mμ in a Beckman spectrophotometer in 1 cm cells. A 0.1 mM solution of crystalline phenol (Merck) in 0.1 M HCl was used as a phenol standard. This solution was checked by iodometric determination. By neutralisation with 0.1 M NaOH and dilution of this stock solution different solutions with known contents of phenol were prepared. With a content of between 5×10^{-5} and 27×10^{-5} millimole phenol a linear relation between extinction and phenol concentration was found. The relative error of the phenol determination of the digest is 1.5 %.

Table 1. $a = 4$ mM, E = 1, A = 12.28 min, B = −0.95 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	3.176	2.05	0.05	0.06
3	2.830	3.14	0.14	0.09
4	2.480	4.43	0.43	0.13
6	2.045	6.38	0.38	0.20
8	1.728	8.15	0.15	0.27
11	1.334	10.95	−0.05	0.41
14	1.048	13.65	−0.35	0.57
17	0.787	16.92	−0.08	0.80
21	0.544	21.22	0.22	1.22
25	0.421	24.25	−0.75	1.62
30	0.267	29.69	−0.31	2.63
35	0.178	34.59	−0.41	4.01
40	0.141	37.41	−2.59	5.10
46	0.075	45.10	−0.90	9.70
52	0.056	48.67	−3.33	13.04

Procedure. The substrate (9 ml) and the enzyme solution (1 ml) were preheated in a water thermostat at 20.0°C. The reaction was started by adding 1 ml of enzyme solution. The moment of half emptied pipette was defined as time zero. At different times 0.5 ml of the digest was withdrawn with a constriction pipette and poured into 2 % K_2CO_3 (1.5—4.5 ml) by means of which the enzyme reaction was stopped. The moment of half emptied pipette was defined as the time of the withdrawal. The phenol determination was performed on samples of this solution either directly or after suitable dilution. In all the experiments the concentrations of phenylphosphate and of added phosphate are given as millimoles of the substance concerned per litre of digest.

RESULTS

Optimum pH. Different digests with phenylphosphate concentration $a = 4$ mM and the same amount of enzyme but with varying pH (4.75—5.60) were examined. After the same time of reaction ($\alpha < 5$ %) a sample of the digest was withdrawn, and the degree of reaction at this time was chosen as a measure of the enzyme activity. Optimum was found at pH 5.3.

Effects of Mg^{++} and phenol. Fig. 1 shows the results of an experiment with $a = 4$ mM and the concentration of $Mg^{++} = 15$ mM. It is seen that Mg^{++} inhibits the reaction slightly. Fig. 1 shows too that addition of phenol (6 mM) had no effect on the rate of hydrolysis. These results are in agreement with the examinations by Pfankuch [1] and Helferich and Stetter [2].

The influence of varying substrate, phosphate and enzyme concentrations

The influence of the substrate concentration on the rate of reaction. Tables 1—4 show the results of four experiments performed at different substrate concentrations, a, but with the same enzyme concentration. The reactions did not follow zero-order, first-order or second-order kinetics, but the experi-

Table 2. $a = 6$ mM, E = 1, A = 17.00 min, B = −0.90 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	5.076	2.01	0.01	0.06
3	4.691	3.00	0.00	0.09
4	4.320	4.08	0.08	0.12
6	3.718	6.09	0.09	0.19
8	3.300	7.73	−0.27	0.25
11	2.630	10.99	−0.01	0.37
14	2.251	13.29	−0.71	0.47
17	1.757	17.06	0.06	0.67
21	1.452	20.03	−0.97	0.86
25	1.051	25.16	0.16	1.27
30	0.828	29.01	−0.99	1.66
35	0.540	36.02	1.02	2.65
41	0.425	39.98	−1.02	3.42
47	0.228	50.40	3.40	6.53
54	0.202	52.43	−1.57	7.40
62	0.086	66.84	4.84	17.61
80	0.029	85.27	5.27	52.61

mental results were fitted by the following rate equation consisting of a sum of a first-order and a zero-order term:

$$t = \mathrm{A} \cdot \ln \frac{a}{a-x} + \mathrm{B} \cdot x \qquad (1)$$

where t is time, a initial substrate concentration, x phosphate concentration at the time t and A and B are constants.

Table 3. $a = 8$ mM, E = 1, A = 22.55 min, B = −0.95 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	6.987	2.09	0.09	0.06
3	6.602	3.01	0.01	0.09
4	6.228	3.97	−0.03	0.12
6	5.482	6.13	0.13	0.19
8	4.906	8.09	0.09	0.26
11	4.259	10.66	−0.34	0.35
14	3.597	13.84	−0.16	0.48
17	3.136	16.50	−0.50	0.59
21	2.512	20.91	−0.09	0.82
25	2.058	24.98	−0.02	1.06
30	1.574	30.56	0.56	1.47
35	1.222	35.92	0.92	1.97
41	0.842	43.97	−2.97	2.98
47	0.704	47.86	0.86	3.61
54	0.461	57.18	3.18	5.64
62	0.358	62.80	0.80	7.33
70	0.211	74.56	4.56	12.59
100	0.147	82.66	−17.34	18.21

Table 4. a = 10 mM, E = 1, A = 28.50 min, B = −0.90 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	8.962	2.19	0.19	0.06
3	8.593	3.06	0.06	0.09
4	8.177	4.10	0.10	0.12
5	7.812	5.06	0.06	0.15
6	7.332	6.45	0.45	0.19
8	6.764	8.23	0.23	0.25
10	6.196	10.22	0.22	0.31
12	5.824	11.65	−0.35	0.36
15	5.064	14.96	−0.04	0.48
18	4.592	17.32	−0.68	0.58
21	3.880	21.47	0.47	0.76
24	3.532	23.83	−0.17	0.87
28	2.968	28.28	0.28	1.11
33	2.488	32.88	−0.12	1.39
39	2.056	37.93	−1.07	1.76
46	1.508	46.27	0.27	2.52
55	1.188	52.78	−3.22	3.29
70	0.624	70.63	0.63	6.55

The values of A and B were determined graphically by the method described by Schønheyder and Volqvartz [8]. The values of A and B at different substrate concentrations are shown in Table 9. The enzyme concentration in experiments 1—4 is arbitrarily fixed as 1. In Tables 1—4 corresponding values of experimental t values and t values calculated by eqn. (1) are shown.

By differentiation of eqn. (1) is found:

Table 5. a = 8 mM, p = 4 mM, E = 1, A = 33.50 min, B = −0.95 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	7.307	2.38	0.38	0.06
3	7.036	3.38	0.38	0.08
4	6.792	4.34	0.34	0.11
6	6.328	6.26	0.26	0.16
8	5.869	8.36	0.36	0.21
11	5.328	11.09	0.09	0.29
14	4.746	14.41	0.41	0.39
17	4.259	17.56	0.56	0.49
21	3.792	21.02	0.02	0.62
25	3.318	25.03	0.03	0.78
30	2.842	29.77	−0.23	0.99
35	2.445	34.43	−0.57	1.22
41	1.990	40.90	−0.10	1.60
47.5	1.645	46.95	−0.55	2.03
54	1.344	53.44	−0.56	2.58
62	1.005	62.85	0.85	3.60
70	0.838	68.79	−1.21	4.40
80	0.669	76.17	−3.83	5.61
100	0.454	88.95	−11.05	8.46

Table 6. $a = 8$ mM, $p = 8$ mM, E = 1, A = 42.3 min, B = −1.1 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	7.468	2.31	0.31	0.05
3	7.248	3.36	0.36	0.08
4	7.040	4.34	0.34	0.10
6	6.681	6.16	0.16	0.15
8	6.333	8.05	0.05	0.19
11	5.856	10.84	−0.16	0.27
14	5.299	14.46	0.46	0.37
17	4.893	17.37	0.37	0.45
21	4.470	20.74	−0.26	0.56
25	3.971	25.21	0.21	0.71
30	3.462	30.44	0.44	0.91
35	3.040	35.48	0.48	1.12
41	2.554	42.29	1.29	1.44
47	2.198	48.27	1.27	1.77
54	1.789	56.53	2.53	2.31
62	1.504	63.54	1.54	2.85
80	0.880	85.54	5.54	5.25
100	0.586	102.40	2.40	8.15

$\Delta t = (A/(a-x) + B) \cdot \Delta x$ where Δx is the deviation of x; this deviation varies during the reaction. The relative error of the phenol determination $\Delta x/x = 0.015$, which gives $\Delta t_{calc} = (|A|/|a-x| + |B|) \cdot 0.015 \cdot x$. Δt_{calc} of all the experimental times are calculated and shown together with $\Delta t_{expt} = (t_{calc} - t_{expt})$ in the tables. The deviation of t_{expt} is not taken into account as it is only of importance for small values of t.

Table 7. $a = 8$ mM, E = 0.5, A = 47.5 min, B = −1.95 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	7.470	2.23	0.23	0.07
3	7.239	3.27	0.27	0.10
4	7.030	4.25	0.25	0.13
6	6.646	6.18	0.18	0.18
8	6.322	7.90	−0.10	0.24
11.5	5.680	11.73	0.23	0.36
14	5.312	14.21	0.21	0.44
18	4.816	17.90	−0.10	0.56
21	4.506	20.45	−0.55	0.65
25	4.086	24.28	−0.72	0.80
30	3.635	28.96	−1.04	0.98
35	3.251	33.51	−1.49	1.18
41	2.806	39.63	−0.37	1.47
47	2.355	47.08	0.08	1.87
54.5	2.026	53.59	−0.91	2.28
62	1.680	61.81	−0.19	2.86
70	1.504	66.71	−3.29	3.27
81.5	1.120	79.97	−1.53	4.58
100	0.861	91.96	−8.04	6.12

Table 8. a = 8 mM, E = 0.25, A = 93.00 min, B = −3.95 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
3	7.558	3.54	0.54	0.11
4	7.449	4.46	0.46	0.14
6	7.204	6.56	0.56	0.20
8	7.013	8.35	0.35	0.25
11	6.715	11.15	0.15	0.34
14	6.476	13.62	−0.38	0.42
17.5	6.168	16.96	−0.54	0.52
21	5.898	20.03	−0.97	0.62
25	5.501	24.95	−0.05	0.78
30	5.149	29.75	−0.25	0.94
35	4.816	34.60	−0.40	1.11
41	4.400	41.37	0.37	1.35
47	4.051	47.70	0.70	1.59
54	3.725	54.20	0.20	1.85
62.5	3.354	62.49	0.01	2.22
70.5	2.973	72.20	1.70	2.66
80	2.723	79.40	−0.60	3.02
100	2.202	97.08	−2.92	4.02

The influence of phosphate concentration on the rate of reaction. Experiment 3 with phenylphosphate concentration a = 8 mM was repeated with phosphate of a concentration p = 4 mM and p = 8 mM added to the digest. Enzyme concentration as in experiment 3, E = 1. The results are shown in Tables 5 and 6. t_{calc} has been calculated by eqn. (1). The experiments show that phosphate has a strong inhibiting effect on the hydrolysis.

The influence of enzyme concentration on the rate of reaction. With constant substrate concentration, a = 8 mM, the enzyme concentration was varied. Tables 7 and 8 show the experimental results with E = 0.5 and E = 0.25. t_{calc} has been calculated by eqn. (1).

The variation of A and B with varying a, p and E. Table 9 shows that B is a constant, when a and p vary. Experiments 1—6 give an average value $B_{av} = -k_c = -0.96$ min/mM (E = 1). The A values change when a and p vary. In experiments 1—6 the values of A are found to fit a linear function of $(p + a)$, see Fig. 2. By the method of the least squares is found when E = 1:

Table 9.

Expt. No.	a mM	p mM	E	A min	B min/mM
1	4	0	1	12.28	−0.95
2	6	0	1	17.00	−0.90
3	8	0	1	22.55	−0.95
4	10	0	1	28.50	−0.90
5	8	4	1	33.50	−0.95
6	8	8	1	42.30	−1.10
7	8	0	0.5	47.50	−1.95
8	8	0	0.25	93.00	−3.95

$$A = 2.18 + 2.56\ (p + a) \tag{2}$$

Experiments 3, 7 and 8 show that A and B are inversely proportional to the enzyme concentration: $A = A'/E$ and $B = B'/E$. The empirical equation (1) then becomes:

$$E \cdot t = [k_a + k_b\ (p + a)] \cdot \ln \frac{a}{a - x} - k_c \cdot x \tag{3}$$

where $k_a = 2.18$, $k_b = 2.56$ and $k_c = 0.96$ when $E = 1$.

REACTION MECHANISM

The simplest reaction schemes that might describe the experimental results will be the following four where X_{1-4} are different enzyme forms, S phenylphosphate, P phosphate and F phenol:

1)

$$X_1 + S \rightleftharpoons X_2 \qquad (\pm 1)$$
$$X_2 \rightarrow X_1 + P + F \qquad (+ 2)$$
$$X_1 + P \rightleftharpoons X_3 \qquad (\pm 3)$$

2)

$$X_1 + S \rightleftharpoons X_2 + P \qquad (\pm 1)$$
$$X_2 \rightarrow X_1 + F \qquad (+ 2)$$

3)

$$X_1 + S \rightleftharpoons X_2 \qquad (\pm 1)$$
$$X_2 \rightarrow X_1 + P + F \qquad (+ 2)$$
$$X_2 + P \rightleftharpoons X_3 \qquad (\pm 3)$$

4)

$$X_1 + S \rightleftharpoons X_2 \qquad (\pm 1)$$
$$X_2 \rightarrow X_1 + P + F \qquad (+ 2)$$
$$X_2 + P \rightleftharpoons X_3 \qquad (\pm 3)$$
$$X_1 + P \rightleftharpoons X_4 \qquad (\pm 4)$$

The reaction with one molecule water, which is necessary in the hydrolysis, is omitted in the schemes, as it cannot be detected in the reaction kinetics, perhaps this reaction is involved in the step (+ 2). Scheme 1) represents competitive inhibition, scheme 3) uncompetitive and scheme 4) noncompetitive inhibition.

The reaction schemes were then treated mathematically using the steady state method as described by Christiansen [9,10]. Calling the steady state rate v and the reaction probabilities for the steps indicated w_i, we have from reaction scheme 1):

$$v = w_1 \cdot x_1 - w_{-1} \cdot x_2$$
$$v = w_2 \cdot x_2$$
$$w_3 \cdot x_1 = w_{-3} \cdot x_3$$

(x_{1-3} are the concentrations of the enzyme forms indicated). Solutions of the equations yield:

$$x_1 = v(w_2 + w_{-1})/w_1 \cdot w_2$$
$$x_2 = v/w_2$$
$$x_3 = v \cdot w_3(w_2 + w_{-1})/w_1 \cdot w_2 \cdot w_{-3}$$

The total enzyme concentration is $E = x_1 + x_2 + x_3$. Introducing the values of x_1, x_2, x_3 and $v = \frac{dx}{dt}$ and the values of w_i: $w_1 = k_1 \cdot (a—x)$, $w_{-1} = k_{-1}$, $w_2 = k_2$, $w_3 = k_3 \cdot (p + x)$ and $w_{-3} = k_{-3}$ where a is the initial substrate concentration, x the phosphate concentration at the time t and p the initial phosphate concentration, we find:

$$E \cdot dt/dx = (k_2 + k_{-1})/k_1 \cdot k_2 \cdot (a—x) + 1/k_2 + k_3 \cdot (k_2 + k_{-1})\,(p + x)/k_1 \cdot k_2 \cdot k_{-3} \cdot (a-x)$$

which by integration yields

1)

$$E \cdot t = \frac{k_2 + k_{-1}}{k_1 \cdot k_2} \cdot \left[1 + \frac{k_3}{k_{-3}}(p + a)\right] \cdot \ln \frac{a}{a—x} + \frac{k_2 + k_{-1}}{k_1 \cdot k_2}\left[\frac{k_1}{k_{-1} + k_2} - \frac{k_3}{k_{-3}}\right] \cdot x \quad (4)$$

which the has same form as the empirically found chronometric integral, eqn. (3).

When reaction scheme 2) is treated in the same manner, we find the following chronometric integral:

2)

$$E \cdot t = \frac{1}{k_1} \cdot \left[1 + \frac{k_{-1}}{k_2}(p + a)\right] \cdot \ln \frac{a}{a—x} + \frac{1}{k_2} \cdot \left[1— \frac{k_{-1}}{k_1}\right] \cdot x \quad (5)$$

which has also the same form as the empirically found chronometric integral, eqn. (3).

Scheme 3) gives the following chronometric integral:

3)

$$E \cdot t = \frac{k_2 + k_{-1}}{k_1 \cdot k_2} \ln \frac{a}{a—x} + \frac{1}{k_2}\left[1 + \frac{k_3}{k_{-3}} \cdot p\right] \cdot x + \frac{k_3}{2 \cdot k_2 \cdot k_{-3}} \cdot x^2$$

and 4) a similar integral. As both of these integrals are different from eqn. (3), the experimental facts cannot be satisfied by the schemes 3) and 4).

Comparing the chronometric integral of reaction scheme 2), eqn. (5), and the empirically found eqn. (3), it is found that:

$$k_{-1}/k_2 = k_b/k_a = 1.167$$

From this equation and reaction scheme 2) we then find, if the rate of reaction (— 1) is v_{-1} and the rate of reaction (+ 2) is v_2:

$$v_{-1}/v_2 = k_{-1} \cdot x/k_2 = 1.167 \cdot x \quad (6)$$

where x is the concentration of phosphate at the time considered. Eqn. (6) shows how many times X_2 will fall back after reaction (—1) compared with reaction (+ 2). By adding radioactive phosphate to the digest it should be

expected that the phosphate of the unhydrolyzed phenylphosphate and the radioactive phosphate were exchanged. Axelrod [11] has shown that citrus phosphatase cannot catalyze the transfer of inorganic phosphate to alcohols.

It was now investigated whether potato phosphatase would catalyze the exchange of phosphate. Radioactive phosphate, $NaH_2{}^{32}PO_4$, representing 10^9 counts per min per mM total phosphate, was added to 50 ml 20 mM phenylphosphate with buffer. Enzyme was added. The reaction was stopped, when 50 % of the substrate was hydrolyzed, and not hydrolyzed phenylphosphate was isolated from the digest by extraction with chloroform and several precipitations as described by Axelrod [11]. The isolated phenylphosphate showed 10^4 counts per min per mM phosphate. From eqn. (6) and the rate of hydrolysis is found that the isolated phenylphosphate should have shown about 10^8 counts per min per mM phosphate. This shows that reaction scheme 2) is not valid. Of the four reaction schemes proposed only scheme 1) can explain all the experimental results.

Introducing Michaelis constant, $K_s = \frac{k_2 + k_{-1}}{k_1}$, and the dissociation constant of the enzyme-phosphate complex, $K_p = k_{-3}/k_3$, in eqn. (4), it is found that:

$$E \cdot t = \frac{1}{k_2}\left[K_s + \frac{K_s}{K_p}(p + a)\right] \cdot \ln \frac{a}{a-x} + \frac{1}{k_2}\left[1 - \frac{K_s}{K_p}\right] \cdot x$$

Comparing this equation with the experimental eqn. (3), it is seen that with the enzyme and substrate here examined the values of K_s and K_p are found to be:

$$K_s = 1.36 \text{ mM and } K_p = 0.85 \text{ mM}.$$

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