STUDIES ON THE LIPOLYTIC ENZYME ACTION

II. KINETICS OF LIVER ESTERASE ACTIVITY

by

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In a recent paper SCHØNHEYDER AND VOLQVARTZ[1] have found that the hydrolysis of rac. 1-caprylyl glycerol by pig pancreatic lipase may be explained by a reaction mechanism corresponding to a closed sequence consisting of three partial reactions. It had not previously been possible to put forward a reaction mechanism on the basis of which an equation could be derived that corresponded closely to the course of the hydrolysis process conditioned by pancreatic lipase. The same holds for liver esterase, although it was clear that pancreatic lipase and liver esterase differed from each other by the type of their kinetics. SOBOTKA AND GLICK[2] using mono-, di- and tributyrin describe the kinetics of liver esterase as a linear or o molecular reaction. They find a linear course of the reaction which is maintained even after 90% of the substrate has been hydrolyzed. Also KNAFFL-LENZ[3] has stated that the hydrolysis of ethyl butyrate by liver esterase is a zero-order reaction (alkaline reaction). PEIRCE[4] finds that the unimolecular reaction velocity constant decreases during the course of the reaction (ethyl butyrate).

On the basis of our investigations on the reaction kinetics of pancreatic lipase it was thought reasonable to take up the kinetics of liver esterase for renewed examination.

EXPERIMENTAL

Substrate. As mentioned previously[1] rac. 1-caprylyl glycerol seems to be a most suitable substrate for studying the kinetics of enzymatic ester hydrolysis in homogeneous aqueous solution. For this reason this substance was also used as substrate in the present investigation. The *enzyme material* used was obtained by extracting chopped, acetone and ether dried rabbit liver with 1% NaCl solution. The *continuous titration technique* and other procedures followed in this investigation, including the preparation of the substrate were identical with those described by SCHØNHEYDER AND VOLQVARTZ[1].

RESULTS AND DISCUSSION

Our preliminary experiments show that no significant difference exists between the liver esterase activity towards L(—)-1-caprylyl glycerol and rac. 1-caprylyl glycerol (Fig. 1). This makes it unlikely that there are any differences in the activity of liver esterase towards the enantiomorphic forms in rac. 1-caprylyl glycerol. The experiments recorded in Table I show that addition of caprylate ion before the enzyme is added causes a definite inhibition of the enzyme activity whereas glycerol is without inhibitive

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effect in the concentrations examined. It appears further from Table I that by standing for 6¾ hours at the experimental temperature (22° C) and at pH 7.1 the enzyme does not undergo any change in activity. In these experiments v_o (the initial velocity) has been determined graphically by means of the tangent to the first part of the hydrolysis curve.

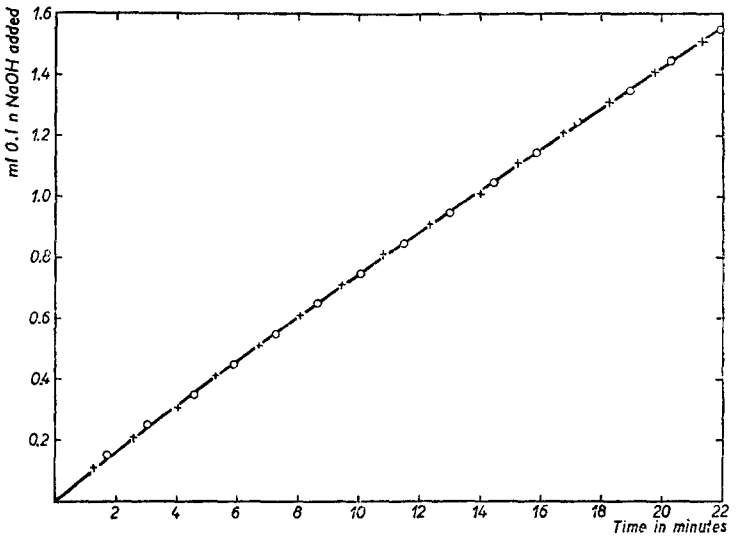

Fig. 1. Hydrolysis of L(—)-1-caprylyl glycerol (+) and rac. 1-caprylyl glycerol (o). 116.6 mg of the esters mentioned were dissolved in 400 ml 1% NaCl solution + 2 ml veronal buffer + 2 ml extract of rabbit liver esterase. For neutralization 0.1 *N* NaOH was used.

TABLE I

DEPENDENCE OF THE INITIAL VELOCITY OF THE HYDROLYSIS OF RAC. 1-CAPRYLYL GLYCEROL ON ADDED CAPRYLATE AND GLYCEROL

The reaction mixture in each experiment consisted of 0.6819 m.eqv. ester + 400 ml 1% NaCl + 2 ml veronal buffer + 2 ml enzyme extract + varying amounts of sodium caprylate or glycerol, made up with 1% NaCl to 409 ml. The enzyme extract, adjusted to pH 7.10, was divided into two portions, A and B. A was kept at the experimental temp. 22° and used for exp. a—c. B was kept at 0° and used for exp. d—i.

Exp. no.	Time	Sodium caprylate* m.eqv.	Glycerol* m.eqv.	v_o/10 mins ml 0.100 *N* NaOH
a	10^{45}	0	0	0.450
b	14^{45}	0	0	0.440
c	17^{00}	0	0	0.450
d		0.347	0	0.360
e		0.695	0	0.284
f		1.390	0	0.192
g		3.474	0	0.113
h		0	1.369	0.455
i		0	3.423	0.445

* added to the mixture before addition of enzyme.

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TABLE II

HYDROLYSIS OF RAC. 1-CAPRYLYL GLYCEROL (EXP. 6)

ENZYME: RABBIT LIVER ESTERASE. a = 0.4581 m.eqv.

t min	x m.eqv.	a %	$\frac{x}{t}10^4$	$\frac{1}{t}\ln\frac{a}{a-x}10^4$	$\frac{1}{t}(35.6\ln\frac{a}{a-x}+53.7x)$
1.85	0.0141	3.1	76.2	168.1	1.005
2.58	0.0191	4.2	74.0	164.3	0.984
3.30	0.0241	5.3	73.0	163.3	0.973
4.59	0.0342	7.5	74.5	168.6	1.000
5.90	0.0442	9.7	74.9	171.7	1.014
7.33	0.0543	11.9	74.1	172.2	1.011
8.78	0.0643	14.0	73.2	172.0	1.006
10.30	0.0744	16.2	72.2	171.9	1.000
11.65	0.0844	18.4	72.4	174.7	1.011
13.08	0.0945	20.6	72.2	176.5	1.017
14.68	0.1045	22.8	71.2	176.3	1.007
16.25	0.1146	25.0	70.5	177.1	1.009
17.80	0.1246	27.2	70.0	178.5	1.011
19.50	0.1347	29.4	69.1	178.6	1.007
22.93	0.1548	33.8	67.5	179.8	1.003
24.45	0.1648	36.0	67.4	182.3	1.011
26.44	0.1749	38.2	66.2	181.8	1.003
28.15	0.1849	40.4	65.7	183.6	1.006
30.35	0.1950	42.6	64.3	182.7	0.995
32.32	0.2050	44.8	63.4	183.5	0.994
34.10	0.2151	47.0	63.1	185.9	1.001
36.20	0.2251	49.1	62.2	186.7	0.999
38.41	0.2352	51.3	61.2	187.5	0.997
40.61	0.2452	53.5	60.4	188.6	0.996
42.83	0.2553	55.7	59.6	190.3	0.997
45.19	0.2653	57.9	58.7	191.5	0.997
47.70	0.2754	60.1	57.7	192.7	0.996
50.22	0.2854	62.3	56.8	194.2	0.997
53.02	0.2955	64.5	55.7	195.3	0.995
55.69	0.3055	66.7	54.9	197.4	0.997
58.30	0.3156	68.9	54.1	200.3	1.004
62.00	0.3256	71.1	52.5	200.1	0.994
64.40	0.3357	73.3	52.1	204.9	1.010
68.62	0.3457	75.5	50.4	204.7	0.999
72.20	0.3558	77.7	49.3	207.6	1.004
76.28	0.3658	79.9	48.0	210.0	1.005
81.65	0.3759	82.1	46.0	210.4	0.996
84.30	0.3809	83.2	45.2	211.2	0.995
87.23	0.3859	84.2	44.2	211.8	0.992
98.98	0.4060	88.6	41.0	219.6	1.002
104.05	0.4110	89.7	39.5	218.6	0.990
106.40	0.4161	90.8	39.1	224.5	1.009
112.60	0.4211	91.9	37.4	223.4	0.996
118.70	0.4261	93.0	35.9	224.2	0.991
124.10	0.4311	94.1	34.7	228.1	0.999
132.00	0.4362	95.2	33.0	230.3	0.998
138.75	0.4412	96.3	31.8	237.8	1.017
168.40	0.4512	98.5	26.8	249.1	1.031

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The hydrolysis of rac. 1-caprylyl glycerol was in some experiments followed almost to completion. An examination of the reaction curves showed immediately that the equation

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

gives a very good agreement with the experimental data over the whole reaction course. A and B are constants. An example elucidating this fact is presented in Table II (liver

TABLE

HYDROLYSIS OF RAC. 1-CAPRYLYL GLYCEROL BY LIVER ESTERASE AT VARYIN

Exp. 7^{I}		a = 0.2290 m.eqv.	Exp. 7^{II}		a = 0.9162 m.eqv.
t min	x m.eqv.	$\frac{40.13\,\Delta \ln + 48.75\,x}{t}$	t min	x m.eqv.	$\frac{87.99\,\Delta \ln + 48.75\,x}{t}$
6.60	0.0266	0.986	6.67	0.052	(1.151)
7.75	0.0317	0.970	10.13	0.072	1.056
10.36	0.0417	0.975	13.08	0.092	1.055
12.91	0.0518	0.993	16.33	0.112	1.026
15.66	0.0618	0.998	19.50	0.132	1.032
18.75	0.0719	0.994	22.68	0.152	1.034
21.81	0.0819	0.997	25.90	0.172	1.030
25.02	0.0920	1.004	29.30	0.192	1.026
28.63	0.1020	1.000	32.75	0.212	1.023
32.45	0.1121	1.000	36.37	0.232	1.018
36.73	0.1221	0.995	39.90	0.252	1.017
41.05	0.1322	0.999	43.35	0.272	1.021
46.15	0.1422	0.994	46.78	0.292	1.026
51.42	0.1523	0.998	50.82	0.312	1.020
57.85	0.1623	0.992	54.85	0.332	1.017
64.76	0.1724	0.996	58.54	0.352	1.022
73.50	0.1824	0.990	62.27	0.372	1.027
83.79	0.1925	0.991	66.66	0.392	1.024
95.80	0.2025	1.006	70.98	0.412	1.023
			75.30	0.432	1.025
		average 0.994	79.63	0.452	1.028
			84.50	0.472	1.026
		$a_{max.}$ = 88.4%	89.35	0.492	1.027
			94.50	0.512	1.026
			99.50	0.532	1.029
			105.00	0.552	1.029
			110.66	0.572	1.031
			116.42	0.592	1.033
			122.42	0.612	1.036
			129.25	0.632	1.035
			136.56	0.652	1.034
			144.55	0.672	1.031
					average 1.029
					$a_{max.}$ = 73.7 %

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exp. 6). In this experiment 0.4581 m.eqv. ester was dissolved in 400 ml 1% NaCl + 2 ml veronal buffer. 3 ml enzyme extract were added. The hydrolysis was followed to 98.5% cleavage. It appears from Table II, columns 4 and 5 that the process is neither a zero-order nor a first-order reaction, but is satisfied by equation (1), in which A and B were computed graphically. (1) is analogous with the well known equation formulated by HENRI AND MICHAELIS

$$Vt = kEt = K_m \ln \frac{a}{a - x} + x, \qquad (2)$$

III

INITIAL SUBSTRATE CONCENTRATIONS (EXP. 7). $t = A' \ln \frac{a}{a-x} + Bx$.

Exp. 7III		a = 1.3742 m.eqv.	Exp. 7IV		a = 1.8323 m.eqv.
t min	x m.eqv.	$\frac{119.88\,\Delta \ln + 48.75\,x}{t}$	t min	x m.eqv.	$\frac{151.78\,\Delta \ln + 48.75\,x}{t}$
4.95	0.038	1.059	6.00	0.045	0.995
6.48	0.048	1.022	9.98	0.075	1.018
9.15	0.068	1.030	13.99	0.105	1.008
11.75	0.088	1.042	18.29	0.135	0.996
14.56	0.108	1.037	22.58	0.165	0.991
17.48	0.128	1.027	26.80	0.195	0.993
20.34	0.148	1.027	31.00	0.225	0.995
24.65	0.178	1.027	35.37	0.255	0.995
27.52	0.198	1.029	39.94	0.285	0.991
30.43	0.218	1.031	44.15	0.315	0.995
35.00	0.248	1.028	48.75	0.345	0.994
41.26	0.288	1.023	53.61	0.375	0.990
44.45	0.308	1.021	58.19	0.405	0.992
47.64	0.328	1.021	62.80	0.435	0.993
50.82	0.348	1.022	67.45	0.465	0.995
54.07	0.368	1.022	72.42	0.495	0.993
57.35	0.388	1.023	77.50	0.525	0.992
60.70	0.408	1.023	82.52	0.555	0.992
64.00	0.428	1.025	87.54	0.585	0.993
67.43	0.448	1.025	92.82	0.615	0.992
70.70	0.468	1.029	98.15	0.645	0.992
74.65	0.488	1.023	103.45	0.675	0.993
78.18	0.508	1.024	109.25	0.705	0.994
81.72	0.528	1.026	114.65	0.735	0.991
85.45	0.548	1.026	120.40	0.765	0.991
89.36	0.568	1.025	126.38	0.795	0.990
93.28	0.588	1.015	132.48	0.825	0.989
97.18	0.608	1.025			
101.18	0.628	1.026			average 0.994
105.22	0.648	1.026			
109.58	0.668	1.025			$a_{max.}$ = 45.0%
113.90	0.688	1.025			
118.37	0.708	1.025			
122.75	0.728	1.026			
127.53	0.748	1.024			
132.60	0.768	1.022			
		average 1.027			
		$a_{max.}$ = 55.9%			

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which has been deduced on the assumption that in the catalyzed reaction an intermediary compound of enzyme and substrate is formed, which is in equilibrium with substrate and enzyme. K_m = the enzyme-substrate equilibrium constant, k = velocity constant of the breakup of the intermediary complex. When the enzyme concentration is kept constant and put equal to 1 then A and B in (1) are equal to K_m/k and $1/k$ in (2). The problem whether the hydrolysis may be explained on the assumptions which form the basis of equation (2) can be examined by varying the initial substrate concentration at constant enzyme concentration.

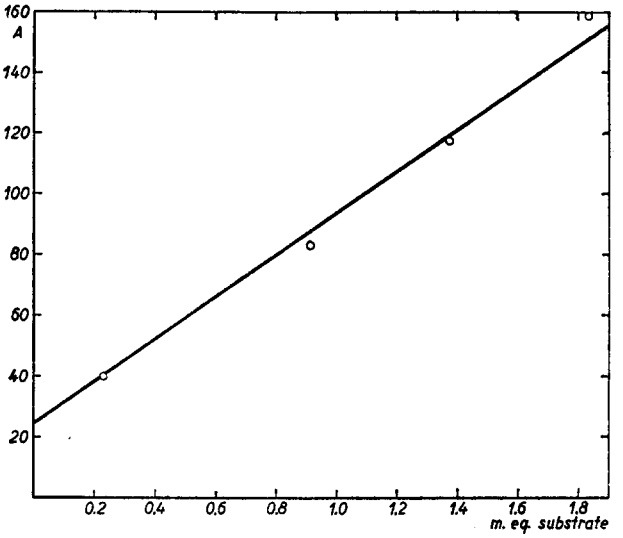

Fig. 2. Determination of $1/k_1$ and k_{-1}/k_1k_2 on the basis of A values computed in exp. 7.

To this end measurements in four different substrate concentrations were performed (liver exp. 7^{I-IV}). The substrate was dissolved in 400 ml 1% NaCl solution + 2 ml veronal buffer. 2 ml enzyme were used for each experiment. In Table III are rendered the experimental data. In columns 3 and 4 (Table IV) are given the A and B values determined graphically. Column 5 contains the initial velocity (v_0/10 min) calculated from the equation $v_0 = \frac{a}{A + aB}$, which is obtained by differentiation of (1).

TABLE IV

EFFECT OF SUBSTRATE CONCENTRATION UPON A, B AND v_0 IN LIVER EXPERIMENT 7

Exp. no.	a m.eqv.	A	B	v_0/10 min $= \frac{10a}{A + aB}$	(v_0/a) 10^2	$\overline{B}$	A'
I	0.2290	40	50	0.0445	1.944	48.75	40.13
II	0.9162	83	50	0.0711	0.776	48.75	87.99
III	1.3743	117.5	50	0.0738	0.537	48.75	119.88
IV	1.8323	159	45	0.0759	0.414	48.75	151.78

It is seen in Table IV that A increases with increasing substrate concentration, whereas B is almost constant. The fact that $A = K_m/k$ varies with the substrate concentration is contrary to the assumptions which form the basis of equation (2).

When a/v_0 is plotted as ordinate with a as abscissa the points are found to lie on a straight line in good agreement with MICHAELIS' theory. K_m = 0.1911 and ke = V = 0.00842 were evaluated from the plot. Hence K_m/k = 22.7 and $1/k$ = 118.8. When t in the separate experiments is calculated by means of these "constants" and x and a, only a moderately good agreement between calculated and observed t values is obtained in the beginning of the experiment. At higher degrees of decomposition there is an increasing and considerable discrepancy. This reaction mechanism may therefore be ruled out.

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The first publication[1] in this series contains an examination of different possibilities explaining the kinetics of pancreatic lipase hydrolysis. Among other things the following two-step reaction mechanism was discussed:

$$A + OH^- + X_1 \rightleftharpoons X_2 + C^- \quad (\pm 1)$$
$$X_2 \rightarrow X_1 + G \quad (+2)$$

X_1 and X_2 are different forms of the enzyme, A = rac. 1-caprylyl glycerol, C^- = caprylate, G = glycerol. Treating this mechanism by the method of stationarity, CHRISTIANSEN[5], the following differential equation was obtained:

$$E\frac{dt}{dx} = \frac{1}{k_2} + \frac{1}{k_1}\frac{1}{a-x} + \frac{k_{-1}}{k_1 k_2}\frac{x}{a-x} \tag{3}$$

which on integration gives

$$Et = \frac{1}{k_1}\left(1 + \frac{k_{-1}}{k_2}a\right)\ln\frac{a}{a-x} + \frac{1}{k_2}\left(1 - \frac{k_{-1}}{k_1}\right)x \tag{4}$$

which is analogous with equation (1),

$$A = \frac{1}{k_1}\left(1 + \frac{k_{-1}}{k_2}a\right) \tag{5}$$

and

$$B = \frac{1}{k_2}\left(1 - \frac{k_{-1}}{k_1}\right) \tag{6}$$

B is positive when $k_1 > k_{-1}$.

(4) does not fit the experimental data for the hydrolysis by pancreatic lipase but agrees well with the data obtained for the hydrolysis of the same substrate by liver esterase. This appears from Fig. 2 and Tables III and IV. It is seen that at constant enzyme concentration A increases linearly with a whereas B is constant in agreement with (5) and (6). (5) is the equation of a straight line with the ordinate intercept $1/k_1$ and the slope $\beta = k_{-1}/k_1 k_2$. The slope of the line and the ordinate intercept (Fig. 2) were computed by the method of least sum of squares. The results were $\beta = 69.60$ and $k_1 = 0.04135$; $k_{-1}/k_2 = 2.88$. By entering $\bar{B} = 48.75$ in (4) one obtains $k_2 = 0.00845$ and $k_{-1} = 0.02432$. The last column in Table IV shows A' values computed by means of the k values. A' agree quite well with the values determined graphically. The figures in Table III (columns 3, 6, 9, 12) represent $t_{calc.}/t_{obs.} = \left(A' \ln\frac{a}{a-x} + \bar{B}x\right)/t_{obs.}$. The average of $t_{calc.}/t_{obs.}$ only deviates a few per cent from unity and there is no trend in the relation computed, which indicates that the reaction mechanism suggested may be the right one.

Our studies on pancreatic lipase and liver esterase activity seem to show that there is a fundamental similarity in the kinetics of these enzymes. Both enzymes seem to act by a reaction mechanism which may be treated according to the principles which hold for chain reactions, and the reaction schemes put forward agree well with the findings that the hydrolysis is inhibited by caprylate ion but not by glycerol. The time-course curves for the hydrolysis conditioned by the two enzymes have a somewhat different shape, the curves for pancreatic lipase showing a much larger tendency to flattening

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than the curves for liver esterase. This difference may simply be explained by the different number of partial reactions in the hydrolysis catalyzed by the two enzymes (3 in the case of pancreatic lipase and 2 for liver esterase) and different orientation of the forms of enzyme. These phenomena also explain that there is not found any measurable "affinity" between pancreatic lipase and substrate, but apparently a large "affinity" between liver esterase and substrate. It does not seem possible to render the experimental results by means of MICHAELIS' K_m and V values.

SUMMARY

The analysis of the kinetics of hydrolysis of rac. 1-caprylyl glycerol by rabbit liver esterase has led to the conclusion that the experiments may be rendered by the equation

$$Et = \frac{1}{k_1}\left(1 + \frac{k_{-1}}{k_2}a\right)\ln\frac{a}{a-x} + \frac{1}{k_2}\left(1 - \frac{k_{-1}}{k_1}\right)x.$$

This equation corresponds to a closed sequence consisting of two partial reactions ($k_1 > k_{-1}$). On the basis of 4 experiments in which the initial substrate concentration is varied at constant enzyme concentration and putting $E = 1$ the following k values are calculated $k_1 = 0.04135$, $k_{-1} = 0.02432$, $k_2 = 0.00845$.

RÉSUMÉ

L'analyse de la cinétique de l'hydrolyse de 1-caprylyl glycérol racémique par l'estérase hépatique de lapin a conduit à la conclusion que les expériences peuvent être rendues par l'équation

$$Et = \frac{1}{k_1}\left(1 + \frac{k_{-1}}{k_2}a\right)\ln\frac{a}{a-x} + \frac{1}{k_2}\left(1 - \frac{k_{-1}}{k_1}\right)x.$$

Cette équation correspond à un cycle composé de 2 réactions partielles ($k_1 > k_{-1}$). Sur la base de 4 expériences dans lesquelles l'on fait varier la concentration initiale du substrat (à une concentration constante de l'enzyme) et en posant $E = 1$, nous calculons les valeurs de k suivantes $k_1 = 0.04135$, $k_{-1} = 0.02432$, $k_2 = 0.00845$.

ZUSAMMENFASSUNG

Die Analyse der Kinetik der Hydrolyse von rac. 1-Caprylyl-glycerol durch Kaninchenleberesterase hat zum Schluss geführt, dass die Experimente durch die Gleichung

$$Et = \frac{1}{k_1}\left(1 + \frac{k_{-1}}{k_2}a\right)\ln\frac{a}{a-x} + \frac{1}{k_2}\left(1 - \frac{k_{-1}}{k_1}\right)x.$$

wiedergegeben werden können.

Die Gleichung entspricht einer geschlossenen Reihenfolge, bestehend aus 2 Teilreaktionen. Auf Grund von 4 Versuchen, in welchen die Anfangssubstratkonzentration bei konstanter Enzymkonzentration geändert und $E = 1$ gesetzt wird, werden die folgenden k-Werte berechnet: $k_1 = 0.04135$, $k_{-1} = 0.02432$, $k_2 = 0.00845$.

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