STUDIES ON THE LIPOLYTIC ENZYME ACTION

I. KINETICS OF PANCREATIC LIPASE ACTIVITY ON AN ESTER IN HOMOGENEOUS AQUEOUS SOLUTION

by

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Pancreatic lipase catalyzes the hydrolysis of a series of esters, whether they are present in homogeneous, aqueous solution or emulsified in water. In this paper it is intended to report some studies on the action of pancreatic lipase on a simple ester in homogeneous, aqueous solution.

Several investigations have been published on the ester decomposition by pancreatic lipase. For various reasons, however, many of the well-known examinations are without importance to the closer understanding of the reaction mechanism because p_H varied considerably during the course of the hydrolysis, and the real substrate concentration was not known, the experiments being performed on oil emulsions, KANITZ[1], ENGEL[2], KASTLE AND LOEVENHART[3], DIETZ[4]. These investigators followed the enzymatic process titrimetrically. The same principle was employed by WILLSTÄTTER, WALDSCHMIDT-LEITZ AND MEMMEN[5], who measured lipatic activity on oil emulsions, buffered by NH_3–NH_4Cl. The hydrolysis was followed to about 24%. It is not possible from these experiments, no more than from those reported by UMSCHWEIF[6] to draw conclusions regarding the reaction mechanism. HOLWERDA, VERKADE AND DE WILLIGEN[7] have demonstrated that in heterogeneous systems the velocity and degree of hydrolysis catalyzed by pancreatic lipase are only slightly or not dependable on the amount of ester, but are primarily a result of the intensity and duration of the shaking. The authors mentioned were able to state that the salts of the fatty acids exert a smaller or larger inhibition of the hydrolysis of tricaprylin. FRAZER AND WALSH[8] have shown that in heterogeneous systems the lipatic activity is proportional to the surface of the fatty particles. BALLS, MATLACK AND TUCKER[9] found that under special conditions the enzymatic hydrolysis of monostearin may proceed according to the equation of the monomolecular reaction.

WILLSTÄTTER AND MEMMEN[10] followed titrimetrically the enzymatic hydrolysis of triacetin and methyl butyrate to relatively low degrees of hydrolysis. They state that the reaction course fits SCHÜTZ's equation approximately and point out that other investigators have found the same equation to hold for the ester hydrolysis brought about by serum lipase and liver lipase. WILLSTÄTTER AND MEMMEN believe that the validity of SCHÜTZ's equation may be explained by enzyme destruction during the reaction. WILLSTÄTTER AND MEMMEN[11] have also followed the lipatic hydrolysis by stalagmometry, using saturated tributyrin solution as substrate. Although some experiments may be found in which the process has been carried to a comparatively high degree of hydrolysis, the number of observations in each experiment is small and the initial substrate concentration uncertain, for which reason the experiments are not suitable for a closer analysis of the reaction kinetics. The object of stalagmometric measurements as well as of almost all other methods has in the first place been to determine the strength of the enzyme activity without going into details with regard to the nature of the process. This also holds for the manometrical measurements of lipase activity, WEBER AND AMMON[12], MURRAY[13].

SUGIURA, NOYES AND FALK[14] followed titrimetrically the decomposition of triacetin. As lipase they employed extracts of tissues. They make an attempt at fitting their results to three different equations (the monomolecular reaction equation, SCHÜTZ's equation and NORTHROP's equation $EKt = a \cdot \ln \frac{a}{a-x} - x$, a = amount of substrate, x = amount of acid liberated). The chemical

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change of hydrolysis was limited to the saponification of at most one acid equivalent in each molecule of the ester. They state that in certain more or less extended ranges of the experiments the equations mentioned were satisfied, but the work does not contain any solution of the reaction kinetic problem. SOBOTKA AND GLICK[15] have studied the action of pancreatic lipase on a series of esters, especially tri-, di-, and monobutyrin. They find that the reaction curves for all substrates flatten shortly after a steep rise and follow an almost horizontal course when a few per cent of the total possible hydrolysis have been effected. SOBOTKA AND GLICK believe that the phenomenon mentioned is not due to inhibition by split products. They maintain that the percentage of the hydrolysis of di- and monoesters is considerably below the expected, even if only one acid equivalent is hydrolyzed. In their opinion the explanation of the peculiar reaction curve was afforded by consideration of the rôle of inactive areas on the colloidal enzyme particles. WEBER AND KING[16] have examined the inhibitory influence of salts of fatty acids on the hydrolysis (pancreatic lipase and liver esterase) of ethyl butyrate. The salts of acetic, propionic, and butyric acids showed no inhibition in the concentrations used, but salts of caproic, caprylic, capric and lauric acids showed increasing inhibition as the length of the carbon chain increased. The paper mentioned, however, apparently only contains a documentation in the case of the liver enzyme. In previous papers (SCHØNHEYDER AND VOLQVARTZ[17]) concerned with enzymatic ester hydrolysis in homo- and heterogeneous systems, the authors have mainly been interested in initial velocity determinations.

It appears from the review that the reaction kinetics of the pancreatic lipase action are not known, for which reason it seems desirable that this problem should be made the subject of investigation.

When one examines the kinetics of the lipolytic reaction it is important to have a simple, absolutely pure, water-soluble ester, which does not undergo any perceptible spontaneous hydrolysis at neutral reaction. The low molecular, water-soluble triglycerides are not suitable because the decomposition comes to a standstill, possibly after one acid equivalent has been split off, and there is the possibility of a series of different split products being formed, as shown by DESNUELLE, NAUDET AND ROUZIER[18] for triolein and natural purified fats. The low molecular esters like methyl and ethyl butyrate are rather volatile, and therefore it is inevitable that a certain loss of substrate will take place during the reaction. These esters require relatively large amounts of pancreatic lipase to be hydrolyzed. As we intend to follow the hydrolysis by measuring the acid liberated this is a complication. The enzyme material contains protein with buffer effect, and this will involve the withdrawal of some of the acid which is liberated in the hydrolysis from the measurements (see later). Rac. 1-caprylyl glycerol has been found to be very suitable for enzymatic experiments because this compound can be obtained pure in crystalline state; it is non-volatile, water-soluble and does not undergo any appreciable spontaneous hydrolysis at p_H about 7.1. Furthermore, with the aid of small amounts of enzyme the hydrolysis of this ester goes to completion.

PROCEDURES

Rac. 1-caprylyl glycerol has been prepared according to principles given by AVERILL, ROCHE AND KING[19] for homologous compounds with more or less carbon atoms in the fatty acids. Acetone 1-caprylyl glycerol was prepared from rac. acetone glycerol and pure caprylyl chloride (prepared from methyl caprylate, fractionated by distillation in column). The following physical data were found for rac. acetone 1-caprylyl glycerol: $d_4^{20} = 0.9759$; b.p. 106 to 108° C at 1.5 mm Hg; $n_D^{24.95} = 1.4360$; mol. weight (determined by saponification) 259.5 (theoretical 258.36). By cautious hydrolysis this substance is transformed into rac. 1-caprylyl glycerol, which after repeated crystallizations from low boiling petroleum ether yielded a white substance which melted sharply at 39.5° C. Mol. weight 217.2 (theoretical 218.3). FILER, SIDHU, DAUBERT AND LONGENECKER[20] state the melting points of rac. 1-glycerides to be 53.0°, 63.0° and 70.5° for compounds containing 10, 12 and 14 carbon atoms in the fatty acid chain.

In order to find out whether it is of any importance to the reaction course, that the substrate used in our enzyme experiments is a mixture of two enantiomorphic forms, L(—)-1-caprylyl glycerol

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was prepared. D(+)-acetone glycerol was prepared according to BEAR AND FISCHER[21] from 1,2,5,6-diacetone-D-mannitol. Acetone L(—)-1-caprylyl glycerol* is an oily liquid at room temperature like the racemic form ($d_4^{20} = 0.9748$; b.p. 108 to 111° C at 2 to 2.5 mm Hg; $n_D^{24.95} = 1.4353$; mol. weight = 253.1 (theor. 258.36); $[\alpha]_D = +3.56°$ (in substance). By cautious hydrolysis of the acetone glyceride L(—)-1-caprylyl glycerol is obtained, a crystalline substance; m.p. 28° C; mol. weight 217.5 (theor. 218.3); $[\alpha]_D = -3.78°$ (in pyridin). The specific rotations found are somewhat smaller than those observed by BAER AND FISCHER[22]. This may be explained by a moderate racemization during the preparation of the substances.

The acetone 1-caprylyl glycerols are practically insoluble in water. It is difficult to determine the exact solubility of the 1-caprylyl glycerols in water. At room temperature one gets crystal-clear solution by dissolving up to 1.3 g per litre. With greater amounts of substance the solution starts giving opalescence.

The *enzyme material* used was obtained by extracting chopped, dried, defatted pig pancreas tissue with 1% NaCl solution. The extracts were filtered repeatedly through cotton-wool. p_H of the extract was brought to about 7.15 before adding it to the substrate mixture. It is important to use such amounts of enzyme that the hydrolysis does not proceed too fast during the first 10 to 15% of the reaction (see later). On the other hand, one has to use such amounts of enzyme, that a high degree of decomposition is reached in 4 or 5 hours.

The ester was dissolved in 400 to 700 ml 1% NaCl solution and a few ml of veronal buffer were added. The solution was placed in a three-necked round-bottomed flask. In one side-neck a protected glass electrode was introduced through a rubber stopper. In the other side-neck an agar-KCl bridge was placed through a rubber stopper. This bridge was connected with the reference electrode by means of a saturated solution of KCl. p_H was recorded on a Radiometer potentiometer. During and after the experiment it was ascertained that the asymmetric potential of the potentiometer did not change. All the experiments were carried out at 21° C. Before addition of enzyme, p_H in the substrate mixture was adjusted to about 7.20. By adding base p_H was kept as near as possible around 7.10. The base was added through a capillary tube which was introduced through a stopper in the centerneck. The capillary tube was connected with a Geissler burette, containing the base (divisions $^1/_{20}$ ml). The base was standardized against succinic acid and had such a strength that not more than 4 ml were added. The dilution caused was thus negligible. p_H was measured immediately after the addition of the enzyme and followed during the whole experiment, an adequate amount of NaOH being added after each fall in p_H below 7.10. For each addition of base is obtained a series of corresponding values of p_H and time. p_H is plotted as ordinate with time in minutes as abscissa, and the time corresponding to p_H 7.10 is determined (Fig. 1). Finally we obtain a time-course curve by plotting the amount of base added as ordinate and t corresponding to p_H 7.10 as abscissa. p_H 7.10 is chosen because at this p_H the veronal buffer has still a slight buffer capacity and the spontaneous hydrolysis of the ester used is negligible. The amount of acid liberated is recorded by the amount of NaOH added during the reaction. The time when the enzyme is added is the starting point of the reaction, $t = 0$. If then p_H of the system is above 7.10 a certain time will elapse before this p_H is reached the first time. During this time some of the substrate is hydrolyzed, and in order to obtain figures which are proportional to the acid split off it is necessary to add a correction to all the readings on the burette. This correction may be obtained either graphically or by means of one of the equations which satisfy the beginning of the process. The determination of this correction is of great importance to the mathematical treatment of the experiments. It is only possible to obtain a reliable value of this correction, if there is a sufficient number of observations in the beginning of the time-course curve.

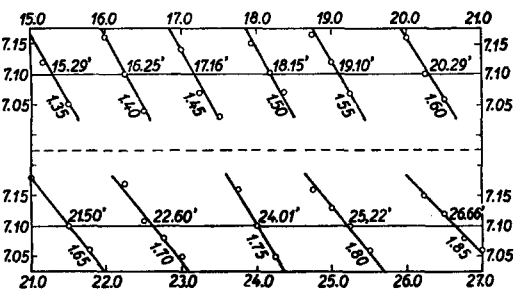

Fig. 1. Relation between p_H and t during a part of the hydrolysis of rac. 1-caprylyl glycerol (exp. 40). The figures at the end of each line denote the total ml NaOH added to neutralize the acid liberated. The figures at the two horizontal lines denote time corresponding to p_H 7.10.

RESULTS AND DISCUSSION

In the first experiments it was shown that no significant differences existed between

* The notations used by FISCHER AND BAER[23].

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the enzyme activities towards L(—)-1-caprylyl glycerol and rac. 1-caprylyl glycerol. Two of these experiments are given in Fig. 2. FISCHER AND BAER[23] have found that the acetone compounds of the enantiomorphic 1-caproyl glycerols are hydrolyzed by lipase of guinea pig serum with different velocities. We have been able to confirm this result for acetone compounds of L(—)-caprylyl glycerol and rac. 1-caprylyl glycerol, which were emulsified in water (guinea pig serum lipase). The experiments with the corresponding water-soluble 1-caprylyl glycerols seem to show practically the same time-course when the same amount of pancreatic lipase and substrate were used in the two experiments. This makes it very unlikely that there are any differences in the activities of pancreatic lipase towards the enantiomorphic forms in rac. 1-caprylyl glycerol.

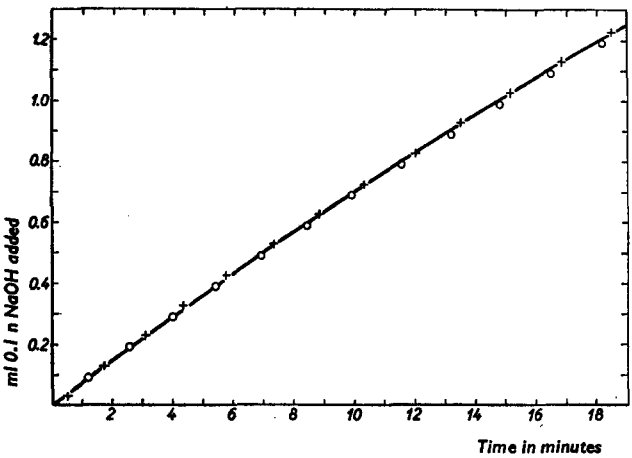

Fig. 2. Hydrolysis of L(—)-caprylyl glycerol (+) and rac. 1-caprylyl glycerol (o). 201.8 mg of the esters mentioned were dissolved in 400 ml 1% NaCl solution + 3 ml veronal buffer + 2 ml extract of pig pancreatic lipase. For neutralization 0.1 *n* NaOH was used.

In the experiments recorded in Tables I and II v_o (the initial velocity) has been determined graphically by means of the tangent to the first part of the hydrolysis curve (up to about 5% decomposition). It appears from these tables that in the ranges examined one is justified in assuming proportionality between v_o and enzyme concentration at constant substrate concentration, and proportionality between v_o and substrate concentration at constant enzyme concentration.

TABLE I

HYDROLYSIS OF RAC. 1-CAPRYLYL GLYCEROL (EXP. 28)

DEPENDENCE OF THE INITIAL VELOCITY ON THE CONCENTRATION OF PIG PANCREATIC LIPASE EXTRACT

Exp. No.	Rac. 1-caprylyl glycerol mg	1% NaCl solution ml	Veronal buffer ml	Enzyme extract ml	v_o/10 min. ml 0.1000 *n* NaOH	$\frac{v_o}{\text{enz.}}$	Deviation from average in %
a	411.2	386	12	2	0.502	0.251	+6
b	411.2	384	12	4	0.974	0.244	+3
c	411.2	380	12	8	1.735	0.217	—8
d	411.2	376	12	12	2.761	0.230	—3
e	411.2	372	12	16	3.906	0.244	+3

It should be mentioned that very dilute enzyme extracts standing at the experimental temperature and at pH 7.12 to 7.02 do not show any inactivation for 11 hours. During this period samples of the extract were tested towards rac. 1-caprylyl glycerol without any significant differences between initial velocities being demonstrated.

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TABLE II

HYDROLYSIS OF RAC. 1-CAPRYLYL GLYCEROL (EXP. 32)

DEPENDENCE OF THE INITIAL VELOCITY ON THE AMOUNT OF SUBSTRATE AT CONSTANT CONCENTRATION OF PIG PANCREATIC LIPASE EXTRACT

Exp. No.	Rac. 1-caprylyl glycerol mg	1% NaCl solution ml	Veronal buffer ml	Enzyme extract ml	v_0/10 min. ml 0.2010 n NaOH	$\frac{v_0}{\text{mg ester}}$	Deviation from average in %
f	52.7	400	3	3	0.206	$0.391 \cdot 10^{-2}$	−4
a	105.4	400	3	3	0.458	$0.435 \cdot 10^{-2}$	+6.5
b	158.2	400	3	3	0.643	$0.406 \cdot 10^{-2}$	−0.5
c	210.9	400	3	3	0.858	$0.407 \cdot 10^{-2}$	0
d	263.6	400	3	3	1.067	$0.405 \cdot 10^{-2}$	−1
e	421.8	400	3	3	1.690	$0.401 \cdot 10^{-2}$	−2

In order to obtain a clear picture of the mechanism it is necessary to follow the reaction to a high degree of hydrolysis because only when a considerable part of the reaction course is known is it possible to decide what mathematical expression gives the best fit. Several experiments have been followed nearly to complete hydrolysis. Owing to space it is only possible to give one of these experiments together with some of the calculations carried out.

Experiment No. 40. 0.6272 g rac. 1-caprylyl glycerol was dissolved in 715 ml 1% NaCl solution + 5 ml veronal buffer. 5 ml enzyme extract were added. Complete hydrolysis corresponds to 3.20 ml of the base employed (0.997 n NaOH was used).

The results are shown in Table III. It is seen that the hydrolysis has been carried through to 93.8%. Calculations were performed on the basis of the time-course material according to a considerable number of formulas some of which were purely empirical, while others had been deduced on a theoretical basis. Table III contains some of these computations. It is seen that SCHÜTZ's equation $k = \frac{x}{\sqrt{t}}$, (column IV), does not fit at all in the beginning and the end of the experiment. Further, we examined whether the reaction follows the bimolecular scheme. It appears from column V that the equation $k_{bi}\, t = \frac{1}{a-x} - \frac{1}{a}$ gives quite a good fit in the beginning, but later there is a steady increase in k_{bi}. Column VI shows that the monomolecular reaction equation does not give constant k_{mo} values, as they start decreasing quite early in the hydrolysis. The K values which can be computed from the equation $EKt = a \cdot \ln \frac{a}{a-x} - x$ are not constant (column VII). This equation NORTHROP[23] has found to hold for the entire course of the digestion of protein by pepsin (except for the first few minutes). The basis for NORTHROP's equation is the assumption that the reaction product (peptone) combines with pepsin to form an inactive compound, and K is equal to the product of the velocity constant (k_1) and the equilibrium constant of the enzyme-peptone complex (k_2).

As mentioned above the enzyme was found to be stable when standing at the experimental conditions, but in the abscense of substrate. This fact does not, however, exclude the possibility of enzyme inactivation in the presence of substrate, which would

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TABLE III

HYDROLYSIS OF RAC. 1-CAPRYLYL GLYCEROL (EXP. 40)

CALCULATION OF THE EXPERIMENT ACCORDING TO 7 DIFFERENT REACTION EQUATIONS

I	II	III	IV	V	VI	VII	VIII	IX	X
t min	x ml	a %	$\frac{x}{\sqrt{t}}$ 10^3	$\frac{\frac{1}{a-x}-\frac{1}{a}}{t}$ 10^4	$\frac{\Delta\ln}{t}$ 10^4	$\frac{a\,\Delta\ln - x}{t}$ 10^5	$\frac{\Delta\ln K_D}{1-e^{-K_D t}}$ 10^4	$\frac{\Delta\ln}{t-B_1 x}$ 10^4	$\frac{\Delta\ln}{t+Bx-C\left(\frac{1}{a-x}-\frac{1}{a}\right)}$ 10^4
1.50	0.20	6.3	163	139	431	467	437	222	295
2.82	0.36	11.3	214	140	423	773	432	222	296
3.62	0.45	14.1	237	141	419	961	431	222	297
4.59	0.55	17.2	210	141	411	693	410	222	297
5.63	0.65	20.3	274	142	404	1375	421	222	296
6.04	0.70	21.9	285	145	409	1502	427	224	301
7.14	0.80	25.0	299	146	403	1699	424	225	302
8.80	0.93	28.9	312	145	389	1925	414	223	299
10.49	1.05	32.8	325	145	379	2132	409	222	298
11.21	1.10	34.4	329	146	376	2217	407	222	298
12.04	1.15	36.0	332	146	370	2292	403	221	296
12.75	1.20	37.5	336	147	369	2392	404	221	298
13.59	1.25	39.1	339	147	365	2472	402	221	297
14.46	1.30	40.6	342	148	361	2553	400	220	297
15.29	1.35	42.2	345	149	358	2642	400	220	298
16.25	1.40	43.8	347	150	357	2808	401	221	299
17.16	1.45	45.3	350	151	352	2810	398	220	298
18.15	1.50	46.9	352	152	349	2894	397	220	298
19.10	1.55	48.4	355	154	347	2986	397	220	299
20.29	1.60	50.0	356	154	342	3047	394	219	298
21.50	1.65	51.6	356	155	337	3119	393	218	297
22.60	1.70	53.2	357	157	335	3209	393	219	299
24.01	1.75	54.7	357	157	330	3264	391	217	297
25.22	1.80	56.2	358	159	328	3354	391	218	299
26.66	1.85	57.8	358	161	324	3424	390	217	299
28.07	1.90	59.4	359	163	321	3502	391	217	299
29.82	1.95	60.9	357	163	315	3551	388	215	298
31.60	2.00	62.5	356	165	310	3606	387	214	297
33.36	2.05	64.1	355	167	307	3674	387	214	298
35.26	2.10	65.7	354	169	303	3736	387	213	298
37.32	2.15	67.2	352	171	299	3797	387	212	298
39.68	2.20	68.8	349	173	293	3839	386	210	297
42.00	2.25	70.3	347	176	289	3899	386	210	297
44.55	2.30	71.9	344	179	285	3950	387	208	297
47.46	2.35	73.5	341	182	279	3990	387	207	297
50.60	2.40	75.0	338	185	274	4026	387	205	296
54.00	2.45	76.6	334	189	269	4062	388	203	296
57.80	2.50	78.2	329	193	263	4078	388	201	295
61.90	2.55	79.7	324	198	258	4123	390	199	296
66.56	2.60	81.3	319	203	252	4143	392	197	297
71.80	2.65	82.8	313	210	245	4160	395	194	297
77.70	2.70	84.4	306	217	239	4172	398	192	298
84.25	2.75	86.0	299	227	233	4189	403	189	301
92.70	2.80	87.6	291	236	224	4159	408	185	302
102.65	2.85	89.2	282	248	216	4124	414	180	304
115.35	2.90	90.7	268	262	205	4054	423	174	306
129.70	2.95	92.2	259	284	197	4017	436	169	317
149.60	3.00	93.8	245	313	185	3927	454	145	335

$$\Delta\ln = \ln \frac{a}{a-x}$$

involve decrease in k_{mo} with time. If the hydrolysis follows the equation of the monomolecular reaction and there is simultaneously a monomolecular enzyme inactivation,

$$k_o = \frac{k \cdot t \cdot K_D}{1 - e^{-K_D t}} = \frac{\ln \frac{a}{a-x} \cdot K_D}{1 - e^{-K_D t}} \qquad (1)$$

k = apparent velocity constant of substrate decomposition, K_D = velocity constant of enzyme inactivation, $k_o = k$ at time $t = 0$.

The basis of the calculation of k_o and k_D is as follows. For a pair of times (t_1, t_2):

$$\frac{k_2 t_2}{k_1 t_1} = \frac{1 - e^{-K_D t_2}}{1 - e^{-K_D t_1}} = F\left(\frac{t_2}{t_1}\right)$$

k_t is plotted as ordinate with t as abscissa and k_t is interpolated corresponding to t = 10–20 – – 100–150–200 – – minutes. For a pair of times (t_1, t_2) $F = k_2 t_2 / k_1 t_1$ is computed. Then K_D is interpolated from a curve which has been constructed for the same time pairs (t_1, t_2) by plotting F as ordinate with K_D as abscissa. F is calculated from other time pairs and K_D values interpolated from similar F, K_D curves. Finally k_o is computed for all observations by entering the most plausible average of K_D into equation (1).

In a single experiment the calculated k_o values are fairly constant over the whole range of the experiment (column VIII). When K_D is computed for different initial amounts of substrate it is found that K_D increases with the substrate concentration, which does not agree with the assumptions on the basis of which equation (1) is deduced. This fact appears from experiments 56a, 56b and 56c reported in Table IV. These experiments are to be discussed later in this work.

TABLE IV

k_o AND K_D CALCULATED ACCORDING TO EQUATION (1) FOR EXP. 56
SUBSTRATE: RAC. 1-CAPRYLYL GLYCEROL

Exp. No.	Substrate m.eqv.	$k_o \cdot 10^5$	$K_D \cdot 10^5$
56 c	0.5735	558	237
56 b	0.9253	553	290
56 a	1.869	569	390

The figures in column IX were obtained by means of the equation

$$Et = A_1 \ln \frac{a}{a-x} + B_1 x \qquad (2)$$

The values given are $1/A_1 = \ln \frac{a}{a-x} / (t - B_1 x)$. Equation (2) is analogous to the equation formulated by HENRI[24]

$$EtK_1 = K \ln \frac{a}{a-x} + x, \qquad (3)$$

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

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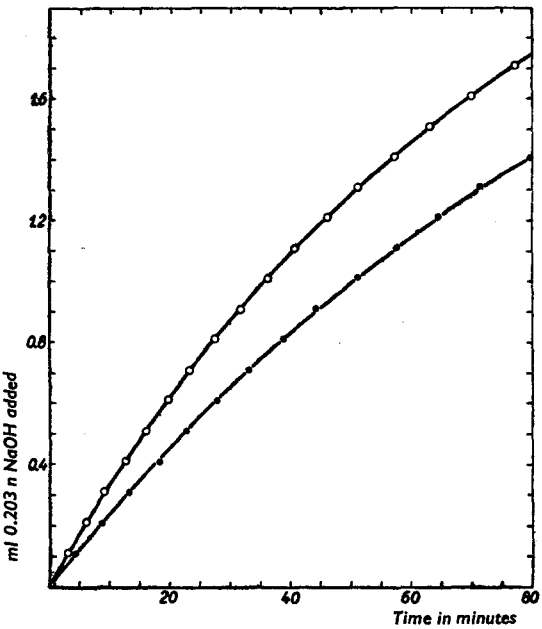

Fig. 3. Hydrolysis of rac. 1-caprylyl glycerol without and in the presence of sodium caprylate. o = 0.694 m. eqv. rac. 1-caprylyl glycerol + 405 ml 1% NaCl + 3 ml veronal buffer + 4 ml enzyme extract. · = 0.694 m. eqv. rac. 1-caprylyl glycerol + 403 ml 1% NaCl + 3 ml veronal buffer + 2 ml (1.387 m. eqv. caprylate) + 4 ml enzyme extract.

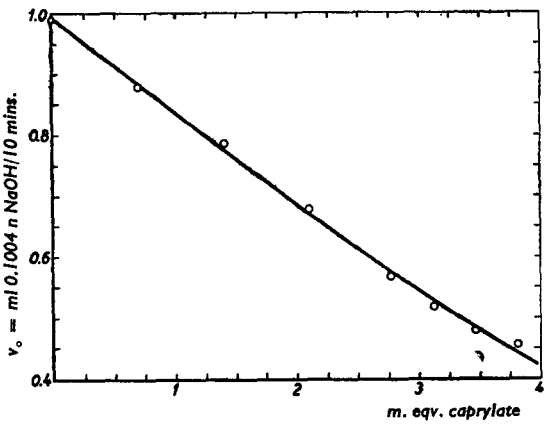

Fig. 4. Effect of increasing caprylate concentration on the initial velocity (v_0) of the hydrolysis of rac. 1-caprylyl glycerol. The reaction mixture in each experiment consisted of 0.5489 m. eqv. ester + 400 ml 1% NaCl + 3 ml veronal buffer + 2 ml enzyme + varying amounts of caprylate, made up with 1% NaCl to 412 ml.

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the substrate and enzyme. The velocity of hydrolysis is determined by the break-up of the enzyme-substrate compound, K = equilibrium constant for the first step in the reaction, and K_1 = velocity constant of the break-up of the intermediary complex. (3) is identical with (2) as $A_1 = K/K_1$ and $B_1 = 1/K_1$. When one tries to apply equation (2) to experiment 40 it appears from column IX that the equation agrees well with the first part of the reaction (up to about 40% hydrolysis), but B_1 *is found to be negative,* which is contrary to the assumption made by HENRI. Thus, the reaction mechanism suggested by HENRI is not valid for the hydrolysis of the ester studied. The question naturally presented itself whether it would be possible to deduce a differential equation which on integration leads to an expression with a negative value of the coefficient B_1.

The experiments plotted in Fig. 3 show a depressing effect on the enzyme activity exerted by sodium caprylate added to the enzyme-substrate solution before addition of enzyme. By increasing addition of caprylate (at constant amount of substrate and enzyme) the results plotted in Fig. 4 were obtained. In these experiments the initial velocity (v_0) is calculated from the inclination of the tangent to the curves (x, t) at the zero point.

Addition of glycerol was without inhibitory effect in the concentrations studied (up to 1.367 m.eqv. glycerol to 0.694 m.eqv. rac. 1-caprylyl glycerol).

In what follows it is shown how it is possible to obtain a negative coefficient to x in equation (2). The overall reaction $A \rightarrow B^- + G$ is thought to proceed by two steps (A = ester, B^- = caprylate ion, G = glycerol, X_1 and X_2 = different forms of the enzyme):

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

It appears from these equations that

the caprylate ion which inhibits the process enters into the reaction ($\pm$ 1), while G, which does not inhibit enters into the reaction ($+$ 2), which is supposed to be irreversible. CHRISTIANSEN[26] has treated such closed sequences by the method of stationarity to compute the reaction velocity $s = \frac{dx}{dt}$. For details and notations the reader is referred to his papers.

By applying the method of stationarity to the two partial reactions we get

$$s = x_1\omega_1 - x_2\omega_{-1} \tag{4a}$$

$$s = x_2\omega_2 \tag{4b}$$

Equations (4a) and (4b) are solved for x_1/s and x_2/s.

The solution may be written

$$Lx_1/s = \omega_2 + \omega_{-1}$$

$$Lx_2/s = \omega_1 \qquad L = \omega_1\omega_2$$

or, as $x_1 + x_2 = E$

$$LE/s = \omega_2 + \omega_{-1} + \omega_1 \tag{5}$$

$$\omega_1 = k_1(a - x);\ \omega_{-1} = k_{-1}(b + x);\ \omega_2 = k_2$$

Substituting ω_1, ω_{-1} and ω_2 in equation (5) we get

$$E\frac{dt}{dx} = \frac{1}{k_2} + \frac{1}{k_1}\cdot\frac{1}{a-x} + \frac{k_{-1}}{k_1k_2}\cdot\frac{b+x}{a-x} \tag{6}$$

which on integration ($b = 0$) gives

$$Et = \frac{1}{k_1}\left(1 + \frac{k_{-1}}{k_2}a\right)\ln\frac{a}{a-x} + \frac{1}{k_2}\left(1 - \frac{k_{-1}}{k_1}\right)x \tag{7}$$

E = total amount of enzyme, x_1 and x_2 amount of the two forms of enzyme. a = amount of substrate at $t = 0$, x = amount of substrate which has reacted at time t, b = excess of caprylate ion before starting the experiment. ω_i is a probability factor and k_i the velocity constant of reaction (i).

Equation (7) is analogous to (2). It is seen that B_1 is negative when $k_{-1}/k > 1$. The two-step reaction seems—as mentioned above—to agree well with the experimental facts for the first 40% of the decomposition, and equation (2) is very suitable for calculating initial velocities, when A_1 and B_1 have determined, $E(a/v_0) = A_1 + B_1a$.

Remarks concerning the practical determination of A_1 and B_1 in equation (2). For all corresponding values of t and x we compute $A_1 = t/\ln\frac{a}{a-x}$ for $B_1 = 0$, and $B_1 = t/x$ for $A_1 = 0$. In a diagram with abscissa A_1 and ordinate B_1 straight lines are drawn through the points B_1, 0 and 0, A_1. If equation (2) fits the reaction, then all the lines will meet in the same point of intersection. When the ordinate of the intersection point is negative, the point is determined by extrapolation. The intersection may be determined more accurately by interpolation, a new diagram being constructed on the basis of the preliminary diagram. B_1 values are computed for A_1 values on both sides of the intersection point. Such a graphical determination of A_1 and B_1 on the basis of data in experiment 40 is shown in Fig. 5. The straight lines are drawn through the ordinates computed for $A_1 = +30$ and $A_1 = +70$. It is seen that there is a marked tendency to form a point of intersection in the beginning of the process. As the hydrolysis proceeds there is more and more envelopment which means that the equation does not satisfy the whole reaction course.

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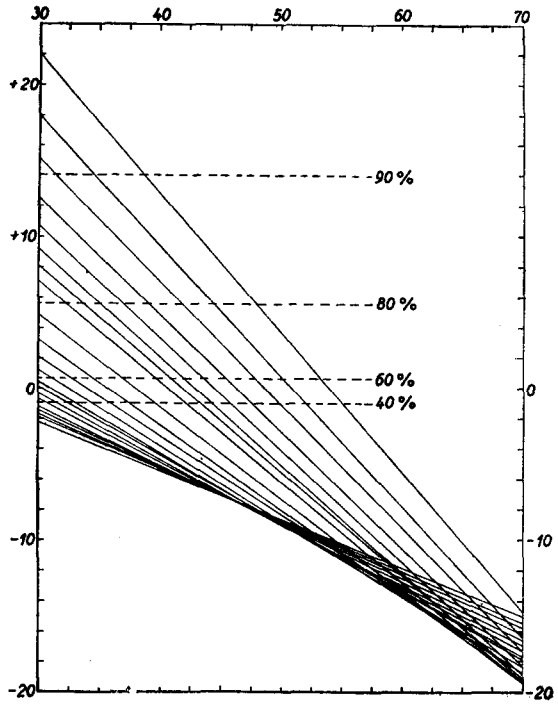

Fig. 5. Graphical determination of A_1 and B_1 in equation (2) by interpolation. Abscissa $= A_1$; Ordinate $B_1 = \frac{t - A_1(\ln a - \ln(a-x))}{x}$

The values of A_1 and B_1 used for computing the figures in column IX (Table III) were $A_1 = 45.0$ and $B_1 = -7.1$. $1/A_1 = 1/45 = 0.0222$, which agrees well with the constants up to 40% hydrolysis.

All those experiments which have been most carefully carried out show a fall in $1/A_1$ (equation 2), when the hydrolysis proceeds further than 40%. The phenomenon appears regardless of the amount of enzyme. This indicates the improbability that the fall is caused by enzyme inactivation. We therefore tried to extend the reaction mechanism by adding another partial reaction, and the following scheme was suggested:

$$A + OH^- + X_1 \rightleftharpoons X_2 + B^- \quad (\pm 1)$$
$$A + OH^- + X_2 \rightleftharpoons X_3 + B^- \quad (\pm 2)$$
$$X_3 \rightarrow X_1 + 2G \quad (+3)$$

According to CHRISTIANSEN[26] there are six different possibilities of orientation of the three forms of enzyme if we assume two different energy levels:

X_1 X_3 X_2	X_2 X_3 X_1	X_2 X_1 X_3	X_3 X_1 X_2	X_3 X_2 X_1	X_1 X_2 X_3
I	II	III	IV	V	VI

The last partial reaction $(+3)$ is supposed to be irreversible, and therefore the probability $\omega_{-3} = 0$, *i.e.* X_1 must be at a lower energy level than X_3. This involves that the possibilities I, III, IV and VI are ruled out. In order to decide, whether II and V or both are usable to express our experimental data it is necessary to deduce the respective differential equations and their integrated forms.

Applying the method of stationarity to the 3 partial reactions we obtain

$$s = x_1\omega_1 - x_2\omega_{-1} \quad (8a)$$
$$s = x_2\omega_2 - x_3\omega_{-2} \quad (8b)$$
$$s = x_3\omega_3 \quad (8c)$$

Solving for $\frac{x_1}{s}$, $\frac{x_2}{s}$ and $\frac{x_3}{s}$ we have

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$$\frac{Lx_1}{s} = \omega_2\omega_3 + \omega_{-1}\omega_3 + \omega_{-1}\omega_{-2}$$

$$\frac{Lx_2}{s} = \omega_3\omega_1 + \omega_{-2}\omega_1 \qquad L = \omega_1\omega_2\omega_3$$

$$\frac{Lx_3}{s} = \omega_1\omega_2$$

$$\frac{E}{s} = \frac{x_1 + x_2 + x_3}{s} = \frac{\omega_2\omega_3 + \omega_{-1}\omega_3 + \omega_{-1}\omega_{-2} + \omega_3\omega_1 + \omega_1\omega_2 + \omega_{-2}\omega_1}{\omega_1\omega_2\omega_3}$$

$$\omega_1 = k_1(a - x); \ \omega_{-1} = k_{-1}(b + x)$$
$$\omega_2 = k_2(a - x); \ \omega_{-2} = k_{-2}(b + x)$$
$$\omega_3 = k_3$$

Orientation II involves that ω_1 is negligible, consequently

$$\frac{E}{s} = \frac{\omega_2\omega_3 + \omega_{-1}\omega_3 + \omega_{-1}\omega_{-2}}{\omega_1\omega_2\omega_3} = \frac{1}{\omega_1} + \frac{\omega_{-1}}{\omega_1\omega_2} + \frac{\omega_{-1}\omega_{-2}}{\omega_1\omega_2\omega_3} \tag{9}$$

Substituting the values of ω in equation (9) we obtain

$$E\frac{dt}{dx} = \frac{1}{k_1(a-x)} + \frac{k_{-1}(b+x)}{k_1k_2(a-x)^2} + \frac{k_{-1}k_{-2}(b+x)^2}{k_1k_2k_3(a-x)^2}, \tag{10}$$

which on integration ($b = 0$) gives

$$Et = (P - (Y + 2Ra)) \ln\frac{a}{a-x} + Rx + a(Y + Ra)\left(\frac{1}{a-x} - \frac{1}{a}\right) \tag{11}$$

$$P = \frac{1}{k_1}; \quad Y = \frac{k_{-1}}{k_1k_2}; \quad R = \frac{k_{-1}k_{-2}}{k_1k_2k_3}$$

Equation (11) can also be written

$$Et = A_2 \ln\frac{a}{a-x} + B_2x + C_2\left(\frac{1}{a-x} - \frac{1}{a}\right) \tag{12}$$

Orientation V involves that ω_2 is negligible, consequently

$$\frac{E}{s} = \frac{\omega_{-1}}{\omega_1\omega_2} + \frac{\omega_{-1}\omega_{-2}}{\omega_1\omega_2\omega_3} + \frac{1}{\omega_2} + \frac{\omega_{-2}}{\omega_2\omega_3} \tag{13}$$

Substituting the values of ω in equation (13) we get

$$E\frac{dt}{dx} = \frac{1}{k_2(a-x)} + \frac{k_{-2}(b+x)}{k_2k_3(a-x)} + \frac{k_{-1}(b+x)}{k_1k_2(a-x)^2} + \frac{k_{-1}k_{-2}(b+x)^2}{k_1k_2k_3(a-x)^2} \tag{14}$$

which on integration ($b = 0$) gives

$$Et = (Z - Y + a(Q - 2R)) \ln\frac{a}{a-x} - (Q - R)x + a(Y + Ra)\left(\frac{1}{a-x} - \frac{1}{a}\right) \tag{15}$$

$$Z = \frac{1}{k_2}; \quad Q = \frac{k_{-2}}{k_2k_3}; \quad Y = \frac{k_{-1}}{k_1k_2}; \quad R = \frac{k_{-1}k_{-2}}{k_1k_2k_3} \tag{15a}$$

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Equation (15) can also be written

$$Et = A_3 \ln \frac{a}{a-x} - B_3 x + C_3 \left(\frac{1}{a-x} - \frac{1}{a} \right) \qquad (16)$$

In equations (12) and (16) C_2 and C_3 have to be positive. In equation (12) B_2 can only be positive. In equation (16) $-B_3$ is negative when $Q > R$. The problem is now to examine whether equation (12) or (16) agrees well with the experimental data.

Remarks concerning the practical determination of A, B and C. In this case also, we have preferred a graphical determination to the time-consuming calculation by the method of the least sum of squares. For an adequate number of equations of the form (12) values of $\ln \frac{a}{a-x}$ and $\left(\frac{1}{a-x} - \frac{1}{a} \right)$ are calculated. C is eliminated by dividing each equation by the coefficient of C. We then obtain a series of equations:

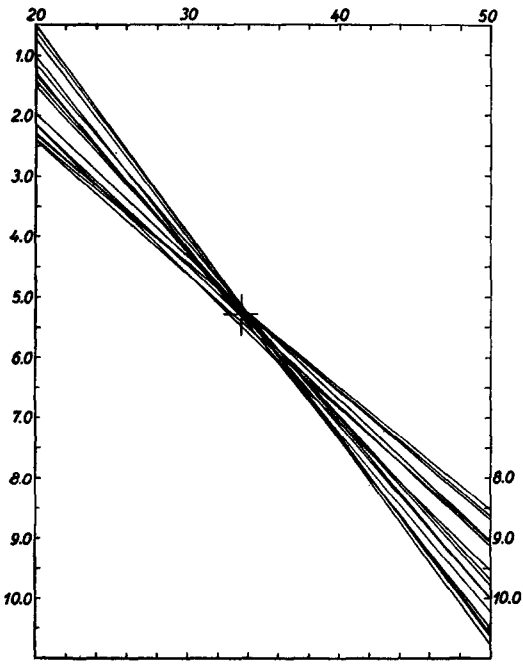

Fig. 6. Graphical determination of A_3 and B_3 in equation (16), by interpolation after elimination of C_3 from the equations. Abscissa $= A_3$. Ordinate $= B_3 = \frac{A_3 (\ln'' a - \ln'' (a-x)) - t''}{x}$

$$t' = A \ln' \frac{a}{a-x} + Bx' + C \qquad (17)$$

One of the equations in the middle is subtracted from all the others by which a new series of equations is obtained

$$t'' = A \ln'' \frac{a}{a-x} + Bx''$$

A and B are computed as previously mentioned, the zone of intersection first being decided by extrapolation and the point of intersection finally determined by interpolation.

Fig. 6 shows the graphical determination performed in experiment 40 according to equation (16). The final interpolation took place between $A = +20$ and $A = +50$. As seen in the figure there is a marked tendency to a narrow zone of intersection.

A probable solution is $A = 33.6$ and $B = +5.3$. By insertion of A and B in equation (17) we obtain an average value $C = 17.63$. The results of the calculations according to equation (16) are listed in Table V. As seen here $t_{calc.}$ and $t_{obs.}$ agree well except at the very end of the reaction, but there the expressions are so sensitive to small experimental errors that a great accuracy cannot be expected. Another check establishing that formula (16) fits experiment 40 well is the constancy of $\frac{1}{A} = \frac{\ln \frac{a}{a-x}}{t + Bx - C \left(\frac{1}{a-x} - \frac{1}{a} \right)}$ (column X, Table III).

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TABLE V

EXPERIMENT 40. COMPARISON BETWEEN t CALCULATED ACCORDING TO EQUATION (16) AND OBSERVED t

$A = 33.5$; $B = 5.3$; $C = 17.63$. $a = 3.20$ (ml 0.997 n NaOH corresponding to 0.6272 g rac. 1-caprylyl glycerol).

I	II	III			
$\ln \frac{a}{a-x} \cdot 33.6$	$x \cdot 5.3$	$\left(\frac{1}{a-x} - \frac{1}{a}\right) \cdot 17.63$	$t_{calc.}$ I + III − II	$t_{obs.}$	$\frac{t_{calc.} - t_{obs.}}{t_{obs.}} \cdot 100$
2.174	1.060	0.367	1.48	1.50	−1.3
4.008	1.908	0.698	2.80	2.82	−0.7
5.090	2.385	0.900	3.61	3.62	−0.3
6.344	2.915	1.144	4.57	4.59	−0.4
7.637	3.445	1.405	5.60	5.63	−0.5
8.303	3.710	1.543	6.14	6.04	+1.7
9.673	4.240	1.837	7.27	7.14	+1.8
11.491	4.903	2.248	8.84	8.80	+0.5
13.372	5.565	2.690	10.50	10.49	+0.1
14.159	5.830	2.886	11.22	11.21	+0.1
14.972	6.095	3.091	11.97	12.04	−0.6
15.802	6.360	3.306	12.75	12.75	0
16.652	6.625	3.531	13.56	13.59	−0.2
17.526	6.890	3.769	14.41	14.46	−0.3
18.416	7.155	4.029	15.29	15.29	0
19.491	7.420	4.295	16.37	16.25	+0.7
20.288	7.685	4.574	17.18	17.16	+0.1
21.265	7.950	4.870	18.19	18.15	+0.2
22.263	8.215	5.186	19.23	19.10	+0.7
23.292	8.480	5.509	20.32	20.29	+0.1
24.367	8.745	5.866	21.49	21.50	0
25.465	9.010	6.245	22.70	22.60	+0.4
26.604	9.275	6.650	23.98	24.01	−0.1
27.780	9.540	7.084	25.32	25.22	+0.4
29.010	9.805	7.549	26.75	26.66	+0.3
30.270	10.070	8.052	28.25	28.07	+0.6
31.594	10.335	8.595	29.85	29.82	+0.1
32.965	10.600	9.182	31.55	31.60	−0.2
34.396	10.865	9.822	33.36	33.36	0
35.881	11.130	10.518	35.27	35.26	0
37.454	11.395	11.281	37.34	37.32	+0.1
39.094	11.660	12.121	39.56	39.68	−0.3
40.817	11.925	13.048	41.94	42.00	−0.1
42.628	12.190	14.079	44.52	44.55	−0.1
44.557	12.455	15.232	47.33	47.46	−0.3
46.590	12.720	16.528	50.40	50.60	−0.4
48.757	12.985	17.997	53.77	54.00	−0.4
51.001	13.250	19.677	57.43	57.80	−0.6
53.572	13.515	21.614	61.67	61.90	−0.4
56.256	13.780	23.875	66.35	66.56	−0.3
59.190	14.045	26.545	71.69	71.80	−0.2
62.385	14.310	29.751	77.83	77.70	+0.2
65.930	14.575	33.668	85.02	84.25	+0.3
69.881	14.840	38.566	93.61	92.70	+1.0
74.370	15.105	44.861	104.13	102.65	+1.4
77.555	15.370	53.257	117.44	115.35	+1.8
85.683	15.635	65.011	135.06	129.70	+4.1
93.183	15.900	82.640	159.92	149.60	+6.9

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The calculation of experiment 40 indicates that orientation V and equation (16) give the explanation of the reaction, while orientation II and equation (12) must be excluded. The correctness of the reaction mechanism and orientation V, is however contingent on the different k values being constant under varying experimental conditions. The experiments in series 56 seem to show that this is the case.

Before presenting these experiments it ought to be mentioned that the possibility of a reaction mechanism by 4 steps was examined:

$$A + OH^- + X_1 \rightleftharpoons X_2 + B^- \quad (\pm 1)$$
$$A + OH^- + X_2 \rightleftharpoons X_3 + B^- \quad (\pm 2)$$
$$A + OH^- + X_3 \rightleftharpoons X_4 + B^- \quad (\pm 3)$$
$$X_4 \rightarrow X_1 + 3G \quad (+4)$$

Assuming two energy levels and $\omega_{-4} = 0$, *i.e.* X_1 at lower energy level than X_4, then the following orientations are possible:

X_2	X_3	X_4		X_4		X_3	X_4
	X_1		X_3	X_2	X_1	X_2	X_1

On integration the differential equations which may be deduced on the basis of these orientations all lead to equations of the following:

$$Et = A_4 \ln \frac{a}{a-x} + B_4 x + C_4 \left(\frac{1}{a-x} - \frac{1}{a} \right) + D_4 \left(\frac{1}{(a-x)^2} - \frac{1}{a^2} \right).$$

In all cases the coefficient D_4 ought to be positive according to the theory, D_4 only containing positive figures.

In experiment 40 it was tried to compute the constants (A_4, B_4, C_4 and D_4) in different ways, both graphically and by simple algebra. In the last case 2 sets of 4 equations spread over the reaction course were solved. It was not possible to make $t_{calc.}$ and $t_{obs.}$ agree relatively well unless D_4 was negative, which is against the theory of a reaction mechanism by 4 steps.

Attempts were made to deduce reaction equations on the assumption of one or more side-reactions in the process. It was for instance supposed that one or two caprylate ions unite with the enzyme by a reversible reaction to form an inactive compound. Neither the equations obtained on the basis of these assumptions, nor many other possibilities examined led to expressions which harmonize better with the experimental data than equation (16).

Attempt to compute the k values in equation (16) from experiment 56.

In 400 ml 1% NaCl solution + 3 ml veronal buffer were dissolved 1.869 m.eqv. (56a), 0.9253 m.eqv. (56b), and 0.5735 m.eqv. rac. 1-caprylyl glycerol (56c); 2 ml enzyme solution were added. The amount of enzyme was chosen very low so that the beginning of the experiments might be as accurate as possible. Between the experiments the enzyme preparation was kept in a cooling mixture. In order to carry out three experiments in the course of 12 hours it was not possible to let the hydrolysis proceed further than about 60%. The temperature of experiment was 21° C.

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TABLE VI

EXP. 56. COMPARISON OF OBSERVED AND COMPUTED t VALUES IN EXPERIMENTS WITH 3 DIFFERENT SUBSTRATE CONCENTRATIONS. EQUATION (16)

Experiment 56a $A = 333$; $B = 105$; $C = 64.05$				Experiment 56b $A = 249$; $B = 105$; $C = 27.12$				Experiment 56c $A = 210$; $B = 105$; $C = 16.82$			
$t_{obs.}$ min	x m.eqv.	$t_{calc.}$	Deviation %	$t_{obs.}$ min	x m.eqv.	$t_{calc.}$	Deviation %	$t_{obs.}$ min	x m.eqv.	$t_{calc.}$	Deviation %
3.76	0.0381	3.55	−5.6	3.53	0.0178	3.54	+0.3	3.08	0.00962	3.04	−1.3
6.75	0.068	6.51	−3.6	6.52	0.0330	6.66	+2.1	4.78	0.01469	4.67	−2.3
9.48	0.096	9.33	−1.6	9.80	0.0481	9.85	+0.5	6.30	0.01975	6.33	+0.5
12.47	0.124	12.27	−1.6	13.15	0.0632	13.13	−0.2	9.70	0.02988	9.71	+0.1
18.36	0.181	18.59	+1.3	16.44	0.0783	16.51	+0.4	13.15	0.04001	13.18	+0.2
29.04	0.265	28.75	−1.0	19.88	0.0934	19.98	+0.5	16.80	0.05014	16.74	−0.4
40.56	0.350	40.18	−0.9	23.50	0.1085	23.55	+0.2	20.31	0.06027	20.48	+0.8
48.59	0.407	48.59	0	27.40	0.1236	27.25	−0.5	24.28	0.07040	24.22	−0.2
57.81	0.463	57.44	−0.6	31.18	0.1388	31.06	−0.4	27.89	0.08053	27.51	−0.1
67.21	0.519	67.01	−0.3	35.29	0.1539	34.98	−0.9	32.13	0.09066	32.13	0
82.84	0.604	82.92	+0.1	39.20	0.1690	39.04	−0.4	36.07	0.10079	36.26	+0.5
94.19	0.661	94.67	+0.5	43.39	0.1841	43.18	−0.5	40.33	0.11092	40.52	+0.5
106.66	0.717	107.18	+0.5	47.60	0.1992	47.48	−0.3	44.85	0.12105	44.91	+0.1
121.27	0.774	121.01	−0.2	52.30	0.2143	51.92	−0.7	49.35	0.13118	49.48	+0.3
133.22	0.819	132.74	−0.4	57.20	0.2295	56.56	−1.1	54.40	0.14131	54.16	−0.4
142.96	0.858	143.62	−0.4	62.10	0.2446	61.29	−1.3	59.00	0.15144	58.99	0
151.67	0.886	151.84	+0.1	66.60	0.2597	66.19	−0.6	64.10	0.16157	64.05	−0.1
160.47	0.915	160.74	+0.2	77.30	0.2899	76.53	−1.0	69.45	0.17170	69.22	−0.3
169.52	0.943	169.75	+0.1	82.70	0.3050	85.96	−0.9	74.65	0.18183	74.60	−0.1
179.77	0.971	179.14	−0.4	88.26	0.3201	87.62	−0.7	80.52	0.19196	80.82	−0.4
189.16	0.999	189.10	0	93.75	0.3353	93.50	−0.3	86.60	0.20209	85.98	−0.7
199.07	1.002	199.88	+0.4	99.73	0.3504	99.57	−0.2	92.41	0.21222	91.96	−0.5
210.94	1.056	210.82	−0.1	105.71	0.3655	105.88	+0.2	98.58	0.22235	98.21	−0.4
222.21	1.084	222.38	+0.1	112.34	0.3806	112.47	+0.1	105.20	0.23248	104.76	−0.4
234.42	1.112	234.55	+0.1	118.90	0.3957	119.69	+0.3	112.40	0.24261	111.55	−0.8
247.32	1.141	247.89	+0.2	126.66	0.4508	126.38	+0.1	119.60	0.25274	118.56	−0.8
261.49	1.169	261.52	0	134.10	0.4259	133.84	−0.2	126.50	0.26287	126.60	+0.1
275.50	1.197	275.99	+0.2	141.65	0.4411	141.64	0	134.00	0.27300	133.70	−0.2
290.79	1.225	291.38	+0.2	150.16	0.4562	149.75	−0.3	141.70	0.28313	141.77	0
	$a_{max.} = 65.5\%$			158.90	0.4713	158.23	−0.4	150.10	0.29326	150.30	+0.1
				165.98	0.4849	166.21	+0.1	158.20	0.30339	159.26	+0.6
				178.10	0.5045	178.35	+0.1	167.33	0.31352	168.58	+0.7
				186.10	0.5166	186.26	+0.1	178.00	0.32365	178.46	+0.3
				196.70	0.5318	196.67	0	188.00	0.33378	188.99	+0.5
				207.20	0.5469	207.59	+0.2		$a_{max.} = 58.2\%$		
				217.60	0.5620	219.12	+0.7				
				229.62	0.5771	231.33	+0.7				
					$a_{max.} = 62.4\%$						

The results of the experiments appear in Table VI. x is given in milliequivalents of acid liberated. It is seen from the table that $t_{calc.}$ and $t_{obs.}$ agree well.

Discussion of the results. For each experiment the constants A, B and C are computed by the procedure previously mentioned. From the graphical determinations it is found that the B values are about the same order of magnitude in the three experiments. As it appears from equations (15), (15a) and (16) that $B = Q—R$ contains only k values and is independent of the substrate concentration (a), the B value is chosen at which $t_{obs.}$ and $t_{calc.}$ agree best in the three experiments ($B = 105$).

The results of the calculations with regard to A and C are to be found in Table VII.

TABLE VII

CALCULATION OF THE CONSTANTS IN EQUATIONS (15), (15a), AND (16) AS APPLIED TO EXP. 56

Exp. No.	56c	56b	56a
a (m.eqv.)	0.5735	0.9253	1.869
A	210	249	333
B	105	105	105
C^*	16.82	27.12	64.05
$\frac{A}{B}$	2.000	2.371	3.171
$\frac{a}{v_o} = A - Ba + \frac{C}{a}$ **	179.1	181.2	171.0
$C = a\frac{1}{k_2}\cdot\frac{k_{-1}}{k_1}\left(1 + a\frac{k_{-2}}{k_3}\right)$ ***	13.59	25.62	71.78
$\frac{k_{-1}}{k_1} = 0.09731$	$\frac{k_{-2}}{k_3} = 0.6614$	$\frac{1}{k_2} = 175.8$	

* Computed by entering the values of A and B determined graphically in equation (16).
** Average = 177.1.
*** C computed from k values.

The problem is now whether a and the values found for A, B and C satisfy equation (15), in which

$$Z - Y + a(Q - 2R) = A = \frac{1}{k_2}\left(1 - \frac{k_{-1}}{k_1}\right) + a\frac{k_{-2}}{k_2 k_3}\left(1 - 2\frac{k_{-1}}{k_1}\right) \quad (18)$$

$$Q - R = B = \frac{k_{-2}}{k_2 k_3}\left(1 - \frac{k_{-1}}{k_1}\right) \quad (19)$$

$$a(Y + Ra) = C = a\cdot\frac{1}{k_2}\cdot\frac{k_{-1}}{k_1}\left(1 + a\frac{k_{-2}}{k_3}\right) \quad (20)$$

References p. 165.

On the basis of the values of A, B and C found in experiment 56 it is possible to compute $1/k_2$, k_{-1}/k_1 and k_{-2}/k_3. The simplest way is to use the following equation:

$$\frac{A}{B} = \frac{1}{\frac{k_{-2}}{k_3}} + a\left(1 - \frac{\frac{k_{-1}}{k_1}}{1 - \frac{k_{-1}}{k_1}}\right)$$

When A/B is calculated for each experiment and plotted as ordinate with a as abscissa the points ought to lie on a straight line with the inclination($1 - ((k_{-1}/k_1)/(1 - k_{-1}/k_1))$ and which intercepts $1/(k_{-2}/k_3)$ on the ordinate axis. It appears from Fig. 7 that the A/B values lie on a straight line. The inclination of this line is computed by the method of the least sum of squares.

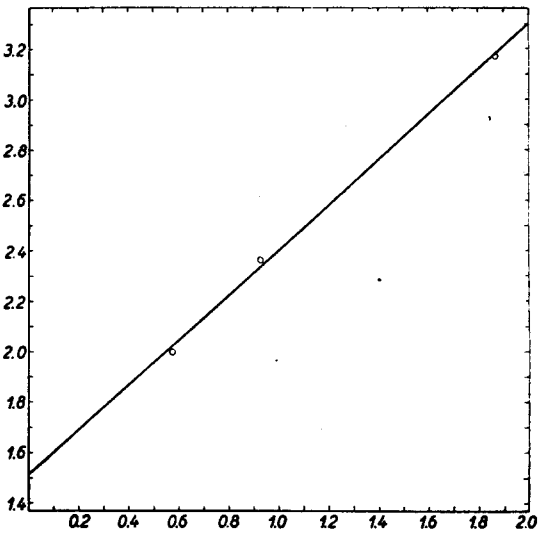

Fig. 7. Determination of k_{-1}/k_1, k_{-2}/k_3 and $1/k_3$ on the basis of the A_3 and B_3 values computed in exp. 56. Abscissa = m. eqv. of substrate in each experiment. Ordinate A_3/B_3

$1 - ((k_{-1}/k_1)/(1 - k_{-1}/k_1)) = 0.8922$,
and therefore $k_{-1}/k_1 = 0.09731$
$1/(k_{-2}/k_3) = 1.512$, then $k_{-2}/k_3 = 0.6614$

By insertion of values found for k_{-1}/k_1 and k_{-2}/k_3 in equation (19) we obtain $1/k_2 = 175.8$. $1/k_2$ may also be obtained directly in each experiment. It is seen from the differential equation (14) that $a/v_0 = 1/k_2$. From equation (16) we get on differentiation $a/v_0 = A - Ba + C/a$. In Table VII are reported a/v_0 values computed in this way. The average is 177.1, which agrees well with $1/k_2$ calculated from the other k values.

Check of the C values. Inserting $1/k_2 = \frac{1}{2}(177.1 + 175.8) = 176.5$, $k_{-1}/k_1 = 0.09731$, and $k_{-2}/k_3 = 0.6614$ in equation (20) one obtains $C = a \cdot 17.175\ (1 + a \cdot 0.6614)$. As seen in Table VII the C values computed by means of the k constants agree moderately well with the values calculated from equation (16) by inserting the values of A and B computed graphically.

The results of the calculations presented seem to confirm the theory advanced for the kinetics of the reaction studied. It has previously been found (p. 135) that the initial velocity is proportional to the substrate concentration, which agrees well with the theory for the reaction by three steps. If v_0 is calculated from the differential equation (6) valid for a reaction by two steps this could not be expected.

At the moment it is not possible to state whether the reaction scheme deduced holds for the action of pancreatic lipase on other esters than rac. 1-caprylyl glycerol in homogeneous solution.

References p. 165.

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SUMMARY

1. A review of previous works dealing with the decomposition of esters by pancreatic lipase shows that it has not been possible to put forward an equation which holds for the entire course of the reaction, and which allows one to draw conclusions with regard to the reaction mechanism.

2. Rac. 1-caprylyl glycerol seems to be a most suitable substrate for studying the kinetics of ester decomposition in homogeneous aqueous solution. Rac. 1-caprylyl glycerol can be obtained as a pure crystalline substance which is non-volatile and somewhat soluble in water. The enzymatic decomposition by OH^- of the ester goes to completion and the spontaneous hydrolysis is negligible at p_H values about 7.1.

3. Pancreatic lipase splits the enantiomorphic forms in rac. 1-caprylyl glycerol with the same velocity. The initial velocity is proportional to the enzyme concentration at constant substrate concentration and proportional to the substrate concentration at constant enzyme concentration.

4. Our experiments bring out that the hydrolysis of rac. 1-caprylyl glycerol by pancreatic lipase may be explained by a reaction mechanism corresponding to a closed sequence consisting of 3 partial reactions. By means of the principle of stationarity an integral equation of the form

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

was put forward. A, B and C are expressed by the velocity constants k_1, k_{-1}, k_2, k_{-2}, and k_3 in the partial reactions. A method for the graphical determination of A, B and C is stated. By varying the substrate concentration at constant enzyme concentration $1/k_2$, k_{-1}/k_1 and k_{-2}/k_3 are computed. In the experiments presented calculated and observed t values agree well.

RÉSUMÉ

1. Un exposé des travaux précédents sur la décomposition des esters par la lipase pancréatique montre qu'on n'a pas réussi à établir une équation valable pendant toute la durée de la réaction, et qui permet de tirer une conclusion en ce qui concerne un mécanisme de réaction.

2. Le 1-caprylyl glycerol racémique semble être un substrat très propre à des études cinétiques de la décomposition d'esters en solution aqueuse homogène. Le 1-caprylyl glycerol racémique peut être obtenu comme une substance pure cristalline non-volatile et assez soluble dans l'eau. La décomposition enzymatique par OH^- de l'ester se poursuit complètement et l'hydrolyse spontanée est négligeable à un p_H d'environ 7.1.

3. La lipase pancréatique décompose les formes énantiomorphes du 1-caprylyl glycerol racémique à la même vitesse. La vitesse initiale est proportionnelle à la concentration de l'enzyme quand la concentration du substrat demeure constante et proportionnelle à la concentration du substrat lorsque la concentration en enzyme est constante.

4. Nos expériences montrent que l'hydrolyse par la lipase pancréatique du 1-caprylyl glycerol racémique peut être expliquée par un mécanisme de réaction correspondant à une cycle composé de 3 réactions partielles. En utilisant le principe de stationarité nous proposons une équation intégrale de la forme

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

où A, B et C sont exprimés par les coefficients de vitesse k_1, k_{-1}, k_2, k_{-2} et k_3 des réactions partielles. Nous donnons un compte rendu d'un mode de calcul graphique d'A, B et C. En faisant varier la concentration du substrat (à concentration constante de l'enzyme), nous calculons $1/k_2$, k_{-1}/k_1 et k_{-2}/k_3. Dans les expériences présentées nous avons demontré une bonne concordance entre les valeurs de t observées et calculées.

ZUSAMMENFASSUNG

1. Eine Übersicht über frühere Arbeiten betreffs der Spaltung von Estern durch pankreatische Lipase zeigt, dass man nicht im stande gewesen ist eine Gleichung aufzustellen, die für den ganzen Reaktionsverlauf gilt, und die erlaubt, Schlüsse über einen Reaktionsmechanismus zu ziehen.

References p. 165.

2. Rac. 1-Caprylyl-glycerol scheint ein sehr geeignetes Substrat für Untersuchungen über die Kinetik der Esterspaltung in homogener, wässriger Lösung zu sein. Rac. 1-Caprylyl-glycerol kann man als eine reine krystallinische Substanz, die nichtflüchtig und mässig löslich in Wasser ist, gewinnen. Die enzymatische Spaltung des Esters durch OH^- ist vollständig, und die spontane Hydrolyse bei p_H ca 7.1 kann vernachlässigt werden.

3. Pankreaslipase spaltet die enantiomorphen Formen des rac. 1-Caprylyl-glycerols mit gleicher Geschwindigkeit. Die Anfangsgeschwindigkeit ist der Enzymkonzentration proportional, wenn die Substratkonzentration konstant gehalten wird, und proportional der Substratkonzentrazion, wenn die Enzymkonzentration konstant ist.

4. Unsere Versuche zeigen, dass die pankreaslipatische Hydrolyse von rac. 1-Caprylyl-glycerol durch einen Reaktionsmechanismus, der einer geschlossenen Reihenfolge, bestehend aus drei Teilreaktionen, entspricht. Mittels des Stationaritätsprinzips wird eine Integralgleichung von der Form

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

aufgestellt, wo A, B und C durch die Geschwindigkeitskoeffizienten k_1, k_{-1}, k_2, k_{-2} und k_3 der Teilreaktionen ausgedrückt sind. Eine Methode zur graphischen Bestimmung von A, B und C wird angegeben. Durch Änderung der Substratkonzentration (konstantes Enzym) werden $1/k_2$, k_{-1}/k_1 und k_{-2}/k_3 berechnet. Aus den beschriebenen Versuchen geht hervor, dass eine gute Übereinstimmung zwischen gefundenen und berechneten t-Werten nachgewiesen werden kann.

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