small quantity. It melts at 105°, dissolves readily in alcohol and also in an aqueous solution of sodium carbonate, at the ordinary temperature. It gives no coloration with an alcoholic solution of ferric chloride.

No definite compound could be isolated from the reaction products of *p*-aminobenzaldehyde and camphoroxalic acid, at 125–30°.

Some further experiments which have been made with acetylphenylhydrazine and camphoroxalic acid have failed to improve the yield of the condensation compound described by Bishop Tingle and Williams.[1]

Summary.

(1) We have studied the action of bromine, of the chlorides of phosphorus, of various oxidizing agents, of nitrous acid and of dimethyl sulphate on certain of the condensation compounds of camphoroxalic acid and amines, in order to obtain further data in respect to their constitution.

(2) The results which we have obtained are in accord with the formula, $C_8H_{14}\begin{cases} C:CR \\ | \quad | \\ CONR_1R_2 \end{cases}$, (R = H or CO_2H; R_1 and R_2 = H, alkyl or aryl), which has been previously assigned to these condensation compounds by the senior author and his colleagues.

(3) The interaction of thiosemicarbazine and camphoroxalic acid has been studied in order to compare the resulting compounds with those derived from semicarbazine. The replacement of CO (semicarbazine) by CS (thiosemicarbazine) greatly reduces the tendency of the primary condensation compounds to form cyclic derivatives.

(4) A considerable number of new condensation compounds have been prepared from camphoroxalic acid and the following amines: *1,3,4-xylidine, p-chloroaniline, dibenzylamine, m-aminobenzoic acid, benzidine* and *camphylamine*. Some of these new substances are well adapted for further study. No crystalline compound could be obtained from camphoroxalic acid and *p*-aminobenzaldehyde.

The investigation will be continued in various directions.

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THE DEVIATION OF FERMENT ACTION FROM THE MONOMOLECULAR LAW WITH ESPECIAL REFERENCE TO THE ESTERASES.

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During the course of an investigation into the effect of the fluorides on the action of lipase, it became necessary to study the kinetics of the

[1] *Am. Chem. J.*, 39, 120.

lipolytic action. The results obtained were so unexpected that it seemed worth while to publish them separately.

The reaction

$$\text{Ester} + \text{Water} \rightleftarrows \text{Acid} + \text{Alcohol}$$

is in its most general form a reaction of the second order, since we have two substances changing their concentrations in each semi-reaction. As usually studied, the water is present in large excess and may therefore be regarded as constant.

The semi-reaction

$$\text{Ester} + \text{Water}$$

should then be written

$$\text{Ester} + \text{Water (in excess)} \longrightarrow$$

and the whole reaction takes the form

$$\text{Ester} + \text{Water (in excess)} \rightleftarrows \text{Acid} + \text{Alcohol} + \text{Water (in excess)}.$$

Thus on the left side we have only one substance, the ester, changing its concentration, while on the right side we have two, the acid and the alcohol. Under these conditions, the reaction from left to right is better studied as a reaction of the first order. For such reactions we expect the following equation to hold:

$$\frac{1}{t} \log \frac{a}{a - x} = k.$$

Here k is a constant, a is the amount of ester present at the beginning of the reaction and x the amount of ester split up during the time t. In the following experiments a and x are measured in terms of 0.05 N NaOH.

The reaction from right to left has been studied qualitatively in several researches but so far as I know Bodenstein and Dietz[1] have been the only ones to investigate its kinetics. We will consider, therefore, in this discussion only the reaction from left to right or the hydrolytic reaction.

The researches fall into two divisions, reactions in homogeneous and reactions in heterogeneous solutions. We will consider briefly examples of each class and take up first the reaction in homogeneous solutions.

Kastle and Loevenhart[2] and later Kastle, Johnston and Elvove,[3] using a clear liver extract and ethyl butyrate found that when an attempt was made to calculate their results according to the first-order equation $k = 1/t \log [a/(a - x)]$, k fell off steadily from the beginning to the end of the reaction. They attributed this fact to the inhibiting influence of the acid on the enzyme. Towards the end of Kastle Johnston and Elvove's paper, however, occurs a series of experiments where the func-

[1] Bodenstein and Dietz, *Z. Elektrochem.*, 12, 605 (1906); Dietz, *Z. physiol. Chem.*, 52, 279 (1907).

[2] Kastle and Loevenhart, *Am. Chem. J.*, 24, 491 (1900).

[3] Kastle, Johnston and Elvove, *Ibid.*, 31, 521 (1904).

tion falls off only very slightly. In this series, moreover, the value of k, though nearly constant for any given concentration of ethyl butyrate, is, roughly speaking, inversely proportional to the initial concentration of the esterase, when this is varied. Thus though the monomolecular law is closely followed in one concentration of zymolyte, the reaction taken as a whole is at total variance with the law.

Euler[1] also obtained a constant value for k, using a clear esterase prepared from pig's adipose tissue and ethyl butyrate. As he gives only one experiment and did not vary the concentration of the butyrate, it is impossible to say that the monomolecular law is obeyed.

Turning now to the reaction in heterogeneous solution, we will consider first the lipase from the castor oil bean (*Ricinus communis*). Armstrong and Ormerod[2] have shown that a slightly acid medium is necessary for the action of this enzyme, but that variations of 2500 per cent. in added acetic acid and of 500 per cent. in added citric acid make practically no difference in its hydrolysis of castor oil. Although this lipase has unfortunately been obtained only in insoluble form it should on account of its behavior towards acids be of great value for kinetic work. Animal lipases act best in a nearly neutral medium and apparently do not act on such a wide variety of esters as the ricinus lipase. The nature of the two lipases therefore may be fundamentally different. A. E. Taylor[3] in his work on this ferment found that in one instance it showed complete agreement with the monomolecular law. Working under rather difficult experimental conditions, he found that the values of k agreed very closely when triacetin was used as zymolyte. The extreme variation in each series was about 25 per cent., but for three different concentrations of ester, 0.5 per cent., 1 per cent. and 2 per cent., the mean values of k agreed very closely. When, however, he used ethyl acetate he found that k was about twice as great in the 2 per cent. as in the 1 per cent. solution. The result with triacetin is, of course, of the greatest importance. The result with ethyl acetate is practically unique, as in all other cases of enzyme action that are comparable with this k is greater in the weaker concentrations of the zymolyte. I cannot, however, pretend to have consulted more than a small part of the literature.

Very interesting and significant results have also been obtained by Bodenstein and Dietz,[4] using a turbid pancreatic extract and amyl butyrate They followed the reaction in both directions and obtained good agreement with the *mono-* and *bi-*molecular laws as long as they did not vary

[1] Euler, *Beitr. Chem. Physiol.* (*Hofmeister*), **7**, 1 (1905). Experiment referred to is on pages 13 and 14.

[2] Armstrong and Ormerod, *Proc. Royal. Soc.*, **78**, 376 (1906).

[3] Taylor, *J. Biol. Chem.*, **2**, 87 (1905).

[4] Bodenstein and Dietz, *Z. Elektrochem.*, **12**, 605 (1906); Dietz, *Z. physiol. Chem.*, **52**, 279 (1907).

the initial concentration of their zymolyte. When this was varied, however, they, too, found that k likewise varied in inverse proportion.

A great variety of other results have been obtained but the few researches given above are typical of the more careful work done during the past ten years. The investigators who consider that they have confirmed Schütz's rule (to be considered in the next paragraph) have for the most part worked with heterogeneous systems of at least three phases. This makes their work extremely difficult to interpret. It is noteworthy that practically everyone who has worked with the simpler solutions considers that Schütz's rule is, in the case of lipase at least, untenable, and that the amount of enzyme action is proportional to the mass of the enzyme. These results are scattered through the literature but some experimental evidence for the statement will be given later.

It may not be out of place in this connection to consider Schütz's rule. It may be given as follows: With a given reaction volume and a given initial concentration of zymolyte the amount of reaction products are proportional to the product of the square root of the enzyme mass and the square root of the time. Expressed in a formula this may be written $x = k\sqrt{Et}$ where x = the amount of reaction products, k = a constant for the stated volume and zymolyte concentration, E = the enzyme mass and t = the time.

Generally speaking, we cannot consider it improbable that the zymolyte and its reaction products will influence the activity of the enzyme and will do so differently in their different concentrations. Therefore it is better to compare the strength of the enzyme in two different solutions by the time taken to attain to a given stage of the reaction. In this way we shall eliminate as far as possible the different activity of the enzyme in different conditions of the medium. Working on this principle we can measure the activity of the enzyme by the reciprocal of the time taken to produce a given quantity of reaction products. (The reaction volume and initial zymolyte concentration are, of course, supposed to be constant.) For instance, if in one reaction mixture it takes twice as long to produce a given amount of acid as in another reaction mixture, we may say that the first reaction mixture has twice the enzymic activity of the second. We may formulate this by saying that Enzymic activity $= k/t$ or Enzymic activity $\times\ t = k$; where k is a constant for given initial conditions (with exception of enzymic mass) and given amount of reaction products. This is a general rule in the nature of a definition, and therefore independent of all experimental evidence.

If, however, in addition, we find that the enzymic activity as determined in this way is proportional to the enzymic mass, we will have a most valuable experimental rule. We may formulate it $Et = k$, where E = the enzyme mass (an experimentally given quantity). As we have

stated, this rule is of the greatest empirical value besides having abundant theoretical justification. Schütz's rule, on the other hand, requires a rather elaborate theoretical calculation to justify its existence and as the experimental work becomes more carefully planned is seen to have less and less empirical value.

Thus as a summary of the above researches we may say that the lipolytic reaction has frequently been shown to follow the monomolecular law so long as only one initial concentration of zymolyte was employed. In only one case (triacetin and ricinus lipase) was k shown to be the same with different concentrations of zymolyte. In another case k and the initial concentration of the zymolyte were directly proportional to each other. In all other cases k was larger in the weaker zymolyte concentrations, in some cases being inversely proportional to the concentration. The amount of enzymic action as measured by the reciprocal of the time to attain a given stage of the reaction was proportional to the mass of enzyme present.

In my investigation I have used a carefully dried and twice redistilled ethyl butyrate and the lipase prepared from pig's liver. The enzyme solution was prepared as follows: Small pieces of freshly killed pig's liver were dissected free from vessels and connective tissue and rinsed off with distilled water. These pieces were then ground with sand and water and strained through cloth. The extract obtained from 50 cc. of liver was made up to 500 cc. and the resulting solution called "10 per cent." extract. After several days' standing under toluene at room temperature this was filtered through paper and a clear, highly refractive, deep straw-colored liquid obtained. It was again filtered after two and again after nine months. The filtrate was now much lighter than formerly, though still quite active, and was much more suitable for use with indicators. A small portion of this solution was mixed with 500 cc. of an ethyl butyrate solution previously warmed to 37° and the mixture kept at this temperature. The time of the beginning of the reaction (which was not more than 3 seconds in error) was taken as the time when one-half of the enzyme had flowed into the ethyl butyrate solution from a pipette. At suitable intervals 50 cc. of this solution was removed with a pipette, allowed to flow onto snow in a beaker and immediately titrated with 0.05 N NaOH free from carbonate, phenolphthalein being used as an indicator. The time of completion of the reaction was taken when one-half of the solution had gone onto the snow. The error in the measurement of t was probably not more than 6 seconds and the error in the measurement of x not more than 0.10 cc. though generally less than 0.5 cc. In all cases the acidity of the extract used has been deducted from the observed figures, so that the figures given are all corrected ones. For each 50 cc. of reaction mixture the initial acidity was 0.82 cc. 0.05 N

NaOH in the 0.476 per cent. solution, 0.34 cc. in the 0.196 per cent. solution and 0.17 cc. in the 0.099 per cent. solution.

The largest error is undoubtedly the temperature error. The temperature of the bath was within 0.1° of 37° during practically the entire reaction, but occasionally varied 0.2–0.4° for a few minutes. As the temperature coefficient of the reaction is about 10 per cent. for each degree, this introduces a slight factor of uncertainty, but it was not possible under my limitations of time to secure a better regulation, nor would the results to have been attained have warranted it. All experiments were done in duplicate, except the experiment in the 4th column in Table VII. As it differs slightly from the others, however, I did not feel justified in excluding it absolutely.

After considerable preliminary work in perfecting the technique, my first experiment showed that the reaction velocity fell off gradually from the beginning of the reaction.

TABLE I.

Enzyme = 0.476%[1] a = 42.77.

x in cc. 0.05 N NaOH.	t in min.	$1/t \log [a/(a-x)]$.
8.78	22.83	0.004371
8.71	22.83	0.004332
13.76	41.00	0.004112
13.13	40.33	0.003949
17.61	61.67	0.003736
17.70	62.33	0.003722
22.56	91.50	0.003558
22.56	93.33	0.003488
26.11	121.00	0.003383
25.88	121.33	0.003326
31.37	186.33	0.003082
31.53	187.08	0.003102
34.03	235.17	0.002932
34.01	234.50	0.002937
36.63	320.25	0.002632
36.88	321.33	0.002680
37.84	367.25	0.002555
37.68	366.58	0.002522
39.54	487.5	0.002301
39.43	488.0	0.002269

This falling off was supposed to be due to the accumulation of acid in the system, in accordance with the explanation proposed by Kastle, Johnston and Elvove. To test this hypothesis further, I used a much more dilute solution of enzyme (0.099 per cent.), so that the reaction could be better followed, and also used six different strengths of zymolyte.

[1] 25 cc. "10 per cent." liver extract + 500 cc. ethyl butyrate solution.

TABLE II.

Enzyme = 0.099%.

$a = 43.53$.			$a = 32.56$.		
x.	t.	$1/t \log [a/(a-x)]$.	x.	t.	$1/t \log [a/(a-x)]$.
1.07	9.63	0.001123	1.02	9.17	0.001507
1.14	10.03	0.001150	1.00	8.75	0.001548
1.95	18.88	0.001055	1.94	19.12	0.001395
2.00	19.95	0.001024	2.05	19.85	0.001423
2.81	30.00	0.000966	2.94	30.62	0.001342
2.94	31.33	0.000969	....	.....	.....
3.91	44.83	0.000912	3.80	42.53	0.001267
3.97	45.05	0.000922	3.98	45.27	0.001251
4.86	59.13	0.000870	5.23	62.95	0.001208
4.96	59.42	0.000884	5.20	62.43	0.001210
6.15	78.47	0.000843	6.16	77.23	0.001179
6.35	79.90	0.000857	6.26	78.47	0.001182
7.17	96.00	0.000814	7.08	91.10	0.001169
7.32	96.70	0.000827	7.07	91.25	0.001165
7.97	110.00	0.000798	7.96	106.03	0.001148
8.11	110.75	0.000808	8.12	108.40	0.001149
8.94	127.88	0.000781	9.04	124.62	0.001133
9.22	130.58	0.000792	9.05	124.60	0.001135
9.97	148.30	0.000762	9.93	141.62	0.001116
10.16	149.17	0.000774	9.97	142.63	0.001113

$a = 21.40$.			$a = 10.62$.		
x.	t.	$1/t \log [a/(a-x)]$.	x.	t.	$1/t \log [a/(a-x)]$.
1.26	11.45	0.002301	1.06	9.77	0.004675
1.21	10.82	0.002335	1.03	9.45	0.004689
2.21	21.75	0.002176	2.01	20.20	0.004511
2.26	22.07	0.002196	2.04	20.08	0.004614
3.19	33.37	0.002101	3.22	34.42	0.004558
3.25	33.73	0.002121	3.12	33.58	0.004497
4.04	43.62	0.002082	4.18	47.17	0.004577
4.01	43.53	0.002070	4.18	47.58	0.004538
4.94	55.75	0.002045	5.23	61.33	0.004802
4.98	56.07	0.002051	5.00	60.37	0.004578
6.02	70.47	0.002035	5.96	74.75	0.004785
6.02	70.65	0.002030	5.99	75.95	0.004747
7.03	85.63	0.002019	6.92	93.80	0.004882
7.16	87.57	0.002020	6.92	94.83	0.004829
8.13	103.62	0.002002	7.93	119.33	0.004997
8.09	102.87	0.002004	7.97	121.33	0.004969
9.10	121.00	0.001988	8.78	151.50	0.005025
9.20	121.75	0.002004	8.74	153.25	0.004906
10.11	140.58	0.001975	9.68	212.50	0.004955
10.19	140.12	0.002004	9.74	216.45	0.004998

TABLE II (*Continued*).

Enzyme = 0.099%.

$a = 5.66$.			$a = 2.74$.		
x.	t.	$1/t \log [a/(a-x)]$.	x.	t.	$1/t \log [a/(a-x)]$.
0.62	5.72	0.00881	0.64	5.65	0.0205
0.59	5.73	0.00834	0.60	5.38	0.0200
1.32	14.47	0.00797	1.10	13.15	0.0170
1.31	14.42	0.00793	1.02	11.97	0.0169
1.87	21.68	0.00804	1.70	21.07	0.0200
1.89	21.83	0.00809	1.66	21.98	0.0184
2.46	30.05	0.00824	2.06	31.23	0.0194
2.44	30.08	0.00814	2.14	32.88	0.0201
3.24A	43.05	0.00857	2.41	47.90	0.0192
3.47A	47.12	0.00875	2.44	52.88	0.0182
3.87	55.88	0.00895	2.57	77.32	0.0156
3.88	56.75	0.00885	2.53	78.05	0.0143
4.31	67.35	0.00924	....	.....	.....
4.30	67.67	0.00915	....	.....	.....
4.70	81.75	0.00943	....	.....	.....
4.76	83.43	0.00957	....	.....	.....
5.03	99.80	0.00954	....	.....	.....
5.04	101.17	0.00949	....	.....	.....
5.26B	134.8	0.00854	....	.....	.....
5.38B	156.5	0.00834	....	.....	.....

The two observations marked A were taken from the first reaction mixture and those marked B from the second.

TABLE II*a*.

Values of $1/t \log [a/(a-x)]$ for different values of x.

Enzyme = 0.099%.

x.	$a = 43.53$.	$a = 32.56$.	$a = 21.40$	$a = 10.62$.	$a = 5.66$.	$a = 2.74$.
1.00.....	0.001149	0.001529	0.002350	0.004688	0.00795*	0.00170*
2.00.....	0.001038	0.001409	0.002211	0.004565	0.00810	0.00198*
3.00.....	0.000960	0.001337	0.002125	0.004528*	0.00843	.....
4.00.....	0.000914	0.001253	0.002077	0.004543	0.00896	.....
5.00.....	0.000874	0.001217	0.002047	0.004683	0.00952*	.....
6.00.....	0.000856	0.001191	0.002033	0.004768	.....	.....
7.00.....	0.000828	0.001169	0.002021	0.004865	.....	.....
8.00.....	0.000804	0.001150	0.002004	0.004983*	.....	.....
9.00.....	0.000788	0.001135	0.001997	0.004966	.....	.....
10.00.....	0.000769	0.001114	0.001991	0.004977	.....	.....

These figures are obtained by interpolation and occasional extrapolation from Table II. The function changes slowly for any given series and the calculation therefore is generally not difficult. Whenever the observed figures show a maximum or minimum I have used that maximum or minimum value for the nearest corresponding value of x, as it

would probably lie nearer the true value than a figure obtained by interpolation. The six cases where this occurs are marked in the table by an asterisk (*).

I naturally expected that "k" would be the same in each concentration or very nearly so and that it would show a falling off from the beginning to the end of the reaction. Instead of this it was, roughly speaking, inversely proportional to the concentration of the ester.[1] Further, the function $1/t \log [a/(a-x)]$, though falling off markedly in the two stronger solutions, fell off only slightly in the "$a = 21.40$" solution, and even increased in the other three.

If we tabulate the time taken to split a definite amount of ethyl butyrate, the apparent law becomes clearly evident. We find that this time is practically independent of the concentration of the butyrate itself, whereas by the mass law it hould bear an inverse relation to it. The time taken to split up a given amount of ethyl butyrate first shows perceptible increase when the concentration of the ester falls below $N/200$ (*i. e.*, 5.00 cc. of 0.05 N NaOH per 50 cc.). These times are given in Table III. The figures are obtained by calculating backward from the values of $1/t \log [a/(a-x)]$ given in Table II*a*.

TABLE III.

Time in minutes to produce x cc. of acid.

Enzyme = 0.099%.

x.	$a = 43.53$.	$a = 32.56$.	$a = 21.40$.	$a = 10.62$.	$a = 5.66$.	$a = 2.74$.
0.60	.....	.....	.....	.....	5.66	5.29
1.00	8.78	8.85	8.84	9.16	10.62	11.61
1.50	.....	.....	.....	.....	16.82	18.42
2.00	19.68	19.54	19.27	20.07	23.37	28.72
2.43	.....	.....	.....	.....	29.78	50.61
3.00	32.30	31.40	30.87	31.82	38.90	.....
4.00	45.80	45.43	43.26	45.17	59.45	.....
5.00	60.83	59.49	56.46	59.02	98.0	.....
6.00	75.25	74.27	70.29	75.78	133.0	.....
7.00	91.96	89.92	85.13	96.08	.....	.....
8.00	109.69	106.48	101.45	121.98	.....	.....
9.00	127.65	123.79	118.67	164.44	.....	.....
10.00	147.41	143.03	137.37	249.49	.....	.....

The table is divided into three parts by two broken lines. To the left of the heavy line where the ester concentrations is above $N/200$, the figures on each horizontal line are nearly the same (*i. e.*, within 8 per cent. of each other). To the right of the dotted line they are con-

[1] This statement is true only for the values corresponding to $x = 1.00$ but anyone who cares to do so can see that it is also nearly true for other values of x, if the expression

$$\frac{1}{t_2 - t_1} \log \frac{a - x_1}{a - x_2}$$

is used. Table III can be used as a basis for this calculation.

siderably larger. Between the heavy and the dotted lines they are only slightly larger (10–15 per cent.) than the figures to the left of the heavy line.

It will be noticed that it took longer to produce 10 cc. of acid in the $a = 43.53$ solution than in the $a = 32.56$ or $a = 21.40$ solutions. Whether much importance should be attributed to this fact I cannot say. It seems to be a greater difference than can be accounted for by experimental errors.

The facts brought out in Table III can be shown even more plainly if instead of the time taken to produce x cc. of acid we tabulate the time taken to produce 1 cc. of acid at different stages of the reaction. Thus the time taken to produce the first cc. of acid in the three stronger solutions is 8.78, 8.85 and 8.84 minutes, respectively, while the time taken to produce the second cc. is 10.90, 10.69 and 10.43 minutes.

TABLE IV.

Time in minutes to produce 1 cc. of acid in different acid concentrations.

Concentration of acid.	Enzyme = 0.099 per cent.					
	$a = 43.53$.	$a = 32.56$.	$a = 21.40$.	$a = 10.62$.	$a = 5.66$.	$a = 2.74$
$x = 0.00$–1.00	8.78	8.85	8.84	9.16	10.62	11.61
$x = 1.00$–2.00	10.90	10.69	10.43	10.91	12.75	17.11
$x = 2.00$–3.00	12.62	11.86	11.60	11.75	15.53	.....
$x = 3.00$–4.00	13.50	14.03	12.39	13.35	20.55	.....
$x = 4.00$–5.00	14.83	14.06	13.20	13.85	38.55	.....
$x = 5.00$–6.00	14.61	14.78	13.83	16.76	.....	.....
$x = 6.00$–7.00	16.71*	15.65	14.84*	20.30	.....	.....
$x = 7.00$–8.00	17.73	16.56	16.32	25.90	.....	.....
$x = 8.00$–9.00	17.96	17.31	17.22	42.76	.....	.....
$x = 9.00$–10.00	19.76	19.24	18.70	85.05	.....	.....

* These two asterisks note the largest deviation to the left of the heavy line.

Here we notice the important fact that the time taken to produce 1 cc. of acid depends mainly on the reaction of the medium and very little on the concentration of the ethyl butyrate. That is, as long as the concentration of the ester is above $N/200$ it takes practically the same time to produce 1 cc. of acid in a mixture of given acidity. Although above this limit the concentration of the ester makes very little difference, yet when it falls below this limit the time taken to produce 1 cc. of acid increases rapidly. This is seen very well by comparing the 3rd and 4th series in the above table. In contrast to this comparative independence of the ester concentrations, the reaction of the medium is of the greatest importance. For instance, in the three stronger solutions it takes about twice as long to produce the ninth cc. of acid as the first cc. In the $a = 21.40$ solution the ester concentration has by this time fallen to about five-eights its original value. It therefore makes little difference in this one case whether we attribute this increase in time to

the diminished ester concentration or the increased acidity. In the two stronger solutions, however, the ester concentration has fallen only to $^3/_4$ and $^4/_5$ of its initial value and we should therefore expect it to take only $^4/_3$ and $^5/_4$ as long to produce the ninth cc. of acid as the first. Instead we find that in these two solutions, as well, the time is approximately doubled. If we assume that the reaction would have followed the monomolecular law, but that the activity of the enzyme is decreased by the acidity of the solution, we see from Table II*a* that it is diminished in different amounts in these three solutions, and is actually increased in the $a = 10.62$ solution.

We see, therefore, from this series of experiments, that on its face, at least, the reaction does not follow the monomolecular law. Even when only one reaction at a time is considered, the explanation required is complicated, while when the six series are considered together the difficulties become almost insuperable. The amount of material reacting per instant is not proportional to the amount of material present at that instant, even when due allowance is made for acidity. On the other hand, provided only that the acidity is the same in the two solutions to be compared, the amount of ester reacting per instant is practically independent of the ester concentration over considerable wide limits ($N/23$–$N/200$).

We may indeed well question whether the constancy of the function $1/t \log [a/(a - x)]$ for the two series $a = 10.62$ and $a = 21.40$ is not purely accidental.

The question then arises: how are we to explain this apparent deviation from the mass law? The answer is, I think, very simple. We may assume that the action takes place in two stages with the formation of an intermediate compound. The first stage is a reaction between the enzyme and the zymolyte (here the ethyl butyrate) with the formation of this hypothetical intermediate compound; the second stage is the splitting up of this intermediate substance into free enzyme, alcohol and acid. At wha stage of the reaction the water enters into this series of changes is not important for the following discussion, but we can represent the possibilities in two ways.

In the first equation the water does not enter into the formation of the intermediate compound, while it does in the second equation.

$$\text{Ester} + \text{Enzyme} + \text{Water} \rightleftarrows \left(\begin{smallmatrix}\text{Ester}\\ \text{Enzyme}\end{smallmatrix}\right) + \text{Water} \rightleftarrows \text{Alcohol} + \text{Acid} + \text{Enzyme}$$

or

$$\text{Ester} + \text{Enzyme} + \text{Water} \rightleftarrows \left(\begin{smallmatrix}\text{Ester}\\ \text{Enzyme}\\ \text{Water}\end{smallmatrix}\right) \rightleftarrows \text{Alcohol} + \text{Acid} + \text{Enzyme.}$$

By the mass law, the amount of acid production (which is what we measure) would be proportional to the concentration of this intermediate compound. If then our reaction constants bear such a proportion to each other that in ester concentrations from $N/23$ to $N/200$ most of the en-

zyme is held in such a compound, we can easily see that the amount of hydrolysis would be practically independent of our ester concentration.

With a little further consideration we can even make an approximate calculation of the percentage of enzyme present in the free and combined states. In the first place the amount of enzyme is so small that the total quantity present must react several times per second, and hence the system can never be very far from equilibrium so far as the enzyme itself is concerned. This being so, we have :

(Concentration free enzyme)^m $\times$ (Concentration ester)^n =

k_1 (Conc. intermediate compound)^p

where m, n and p represent the number of molecules of the substances entering into the reaction.

We have, however, absolutely no data for assigning values to m, n and p and hence can simplify the equation by making them equal to 1, that is by assuming that one molecule of enzyme reacts with one molecule of ester to form one molecule of the intermediate compound.

We have then:

Conc. free enzyme $\times$ Conc. ester = k_1 Conc. int. compd. (1)

and since the amount of acid produced per instant (or per small unit of time[1]) is proportional to the concentration of our intermediate compound we have:

k_2 Conc. int. compd. = acid p oduced per minute (2)

from which we can obtain by division a third equation:

Conc. free enz. $\times$ Conc. ester = k_1/k_2 acid produced per minute. (3)

Using these equations as a basis for calculation I obtained the figures in the following table (Table V). The mean amount of ester present during the production of the first cc. of acid is the acid equivalent of the ester present at the beginning of the reaction minus 0.5 cc. The rate of acid production is also a mean value, being merely the reciprocal of the time taken to produce the first cc. of acid.

TABLE V.

	Enzyme = 0.099%.					
	a = 43.53.	a = 32.56.	a = 21.40.	a = 10.62.	a = 5.66.	a = 2.74.
Mean concentration of ester during production of 1st cc. of acid.	43.0	32.1	20.9	10.1	5.16	2.24
Minutes taken to produce 1 cc. of acid.	8.78	8.85	8.84	9.16	10.62	11.61
cc. of acid produced per minute.	0.114	0.113	0.113	0.109	0.094	0.086
Free enzyme (per cent. of total enzyme).	1.7	2.3	3.7	7	12	25
Combined enzyme (per cent. of total enzyme)	98.3	97.7	96.3	93	88	75

[1] To avoid the difficulties of the differential notation, I have generally used a small but finite time and quantity unit (1 minute and 1 cc.). The error is, I think, very small.

The figures in the last two lines are obtained by successive approximations and it would not be of especial interest to give these at length. Instead I give a table which shows that they coincide very nearly with the observations.

TABLE VI.

	Enzyme = 0.099%.					
	$a = 43.53$.	$a = 32.56$.	$a = 21.40$.	$a = 10.62$.	$a = 5.66$.	$a = 2.74$.
$\frac{\text{Acid produced per minute}}{\%\text{ free enzyme} \times \text{mean amt. of ester}}$	0.0153	0.0153	0.0150	0.0154	0.0152	0.0154
$\frac{\text{Acid produced per minute}}{\text{Per cent. combined enzyme}}$	0.0116	0.0116	0.0118	0.0117	0.0107	0.0115

The agreement is thus seen to be very good except in the series $a = 5.66$. It was in this series that the calculation of the time taken to produce 1 cc. met with a little difficulty and here the discrepancy may be only an apparent one.

I wish to emphasize that the above calculation is to be regarded as purely hypothetical and only adopted for the purpose of giving a mechanism by which the reaction may be conformed to the mass law. The argument, of course, loses its validity if we do not assume the presence of an intermediate compound, but merely because the results obtained by a highly hypothetical calculation correspond fairly closely with the observations, we must not therefore conclude that an intermediate compound is necessarily present.

It is tacitly assumed in the above argument that the amount of enzyme action is proportional to the amount of enzyme present. This has been shown repeatedly, but it is so essential to my argument that I feel it necessary to give my own observations. We have seen that it is advisable to measure the enzymic activity by the reciprocal of the time taken to produce a given amount of acid, and formulated this as $E = k/t$ or $Et = k$, where E = the enzymic mass in a given volume or the enzymic concentration.

The following table summarizes my results with three different strengths of enzyme and various ester concentrations.

TABLE VII.

Time in minutes to produce x cc. in acid.

	Enzyme = 0.476%.	Enzyme = 0.196%.			Enzyme = 0.099%.			
x.	$a = 42.77$.	$a = 11.40$.	$a = 21.55$.	$a = 31.86$.†	$a = 10.02$.	$a = 21.40$.	$a = 32.56$.	$a = 43.53$.
2.....	.....	10.01	.....	8.92†	20.07	19.27	19.54	19.68
4.....	.....	22.41	.....	19.9†	45.17	43.26	45.43	45.80
6.....	.....	37.47*	.....	32.7†	75.78*	70.29	74.27	75.25
8.....	.....	57.11*	.....	46.8†	121.98*	101.45	106.48	109.69
10.....	27.14	114.*	66.63	62.2†	249.5*	137.37	143.03	147.41
16.....	53.27	.....	141.8	.....	.....	.....	.....	.....
20.....	75.40	.....	295.*	.....	.....	.....	.....	.....

† Temperature was inconstant and experiment was not done in duplicate.

* Concentration of ester is below $N/200$.

TABLE VII (*Continued*).

Time in minutes to produce x cc. of acid × per cent. of enzyme.

	Enzyme = 0.476%.	Enzyme = 0.196%.			Enzyme = 0.099%.			
x.	a = 42.77.	a = 11.40.	a = 21.55.	a = 31.86.	a = 10.62.	a = 21.40.	a = 32.56.	a = 43.53.
2.....	....	1.96	.....	1.75†	1.99	1.91	1.93	1.95
4.....	....	4.39	.....	3.90†	4.48	4.28	4.50	4.53
6.....	....	7.34*	.....	6.41†	7.50*	6.96	7.35	7.45
8.....	....	11.2*	.....	9.17†	12.1*	10.0	10.5	10.9
10.....	12.9	22.3*	13.1	12.2†	24.7*	13.6	14.2	14.6
16.....	25.4	.....	27.8	.....	.....	.....	.....	.....
20.....	35.9	.....	57.8*	.....	.....	.....	.....	.....

† Temperature was inconstant and experiment was not done in duplicate.

* Concentration of ester is below $N/200$.

Leaving out of consideration the figures marked with an asterisk (*) and considering the figures marked with a dagger (†) as only approximate, we see that Et is very nearly a constant for any given concentration of acid, although the enzyme is slightly more active in the 0.476 per cent. series. Practically every investigator who has done carefully planned work with lipase in one or two phase solutions has also concluded that the enzyme activity is proportional to the enzymic concentration.

The main thesis of this paper is that the amount of zymolyte hydrolyzed per instant is independent of the concentration of the zymolyte over a considerable range. This has been shown to be true for invertase by A. Brown,[1] Hudson[2] and Taylor,[3] for diastase by Taylor[3] and for lipase by Kastle and Loevenhart[4] and by Kastle, Johnston and Elvove.[5] Brown also gave a partial explanation of the phenomenon somewhat similar to the one I have given. The peculiarities in the hydrolyses of the ester and cane sugar are due to the enzyme and not to the substances hydrolyzed, since when acids are used as catalytic agents the progress of the action conforms absolutely to the mass law. This has been shown by numerous investigators beginning with Wilhelmy[6] in the case of cane sugar in 1857. Ostwald,[7] among others, has investigated the kinetics of both actions very completely and Cohen[8] has shown that the cause of the apparent deviation of the acid inversion of cane sugar from the monomolecular law is due to the volume of the solution occupied by the sugar. Morse and his co-workers have shown that the osmotic pressure of cane sugar is equal to the gas pressure of a gas occupying the vol-

[1] Brown, *J. Chem. Soc.*, 81, 373 (1902).

[2] Hudson, THIS JOURNAL, 30, 1564 (1908).

[3] Taylor, *J. Biol. Chem.*, 5, 405 (1909).

[4] Kastle and Loevenhart, *Am. Chem. J.*, 24, 491 (1900).

[5] Kastle, Johnston and Elvove, *Ibid.*, 31, 521 (1904).

[6] Wilhelmy, *Pogg. Ann.*, 81, 413 and 499 (1850).

[7] Ostwald, numerous articles in *J. prakt. Chem.* about the year 1885.

[8] Cohen, *Z. physik. Chem.*, 23, 442 (1897).

ume of water present in the sugar solution under consideration. Therefore, if in the expression $k = 1/t \log [a/(a - x)]$, we make a and x proportional to the osmotic pressure, instead of to the concentration of the sugar, we will have a very close agreement of k in the different concentrations. The osmotic pressure of the zymolyte cannot be invoked to explain the anomalies which I have noted in the above cases of enzyme action, as the variations of k are in the wrong direction. On the other hand the explanation which I have given or some modification of it, can be invoked to explain the anomalies of the action of invertase and diastase.

It may be of interest in this connection to give the results of other investigators bearing on the above statements.

(1) A. Brown.[1]

INVERTASE ACTING ON CANE SUGAR.

	Grams sugar per 100 cc.	Grams sugar inverted in 60 min.	$1/t \log [a/(a-x)]$.
A.	40.02	1.076	
	29.96	1.235	
	19.91	1.335	
	9.85	1.335	
	4.89	1.230	
B.	(2.0)	(0.308)	(0.00132)
	1.0	0.249	0.00219
	0.5	0.129	0.00239
	0.25	0.060	0.00228

Apparently two different enzyme solutions were used. The monomolecular law is followed only in dilute solutions. In the stronger solutions the absolute amount inverted in 60 min. is nearly independent of the sugar concentration. In the three most dilute solutions k is nearly the same but is much smaller in the 2 per cent. solution than in the 1 per cent. solution. Though not calculated for the stronger solutions, it would, of course, be about inversely proportional to the concentration of the sugar.

(2) Hudson.[2]

INVERTASE ACTING ON CANE SUGAR.

Cane sugar per 100 cc.	Grams inverted in 30 minutes per 100 cc. solution.	Grams inverted in 60 minutes per 100 cc. solution.
4.55	3.33	4.23[3]
9.09	4.12	6.74
27.3	3.06	6.00

(3) Kastle and Loevenhart.[4]

[1] Brown, *J. Chem. Soc.*, 81, 373 (1902), tables on 379 and 387.

[2] Hudson, THIS JOURNAL, **30**, 1575 (table).

[3] The concentration of cane sugar was only 1.22 per cent. at the beginning of the second 30 minutes of the reaction.

[4] Kastle and Loevenhart, *Am. Chem. J.*, **24**, 514; cf. Kastle, Johnston and Elvove, *Loc. cit.*, **31**, 546.

LIPASE ACTING ON ETHYL ACETATE.

Grams ethyl acetate per 5 cc.	Grams ethyl acetate hydrolyzed in 15 minutes per 5 cc. solution.
0.05656	0.003952
0.04242	0.003861
0.02828	0.003952
0.01414	0.003403

The agreement in the three stronger solutions is remarkable.

(4) Taylor[1] gives interesting figures for invertase and diastase. He gives only the reaction constants. These multiplied by the per cent. of the zymolyte show close agreement in the stronger solutions.

INVERTASE ON CANE SUGAR.

Per cent. sugar.	$k \times 10^4$.	$(k \times 10^4) \times$ per cent. sugar.
½	423	212
1	326	326
2	167	334

DIASTASE ON MALTOSE.

Per cent. maltose.	$k \times 10^5$.	$(k \times 10^5) \times$ per cent. maltose.
1	363	363
2	239	478
3	142	426

The enzyme action in these three cases is thus seen to differ markedly from the hydrolysis by acids.

Conclusions.

(1) In a solution of given volume and acidity the time taken to hydrolyze a given amount of ethyl butyrate is inversely proportional to the concentration of the enzyme. Under similar conditions of acidity each particle of enzyme hydrolyzes the same absolute amount of ester per instant no matter what the concentration of enzyme.

(2) With a given concentration of enzyme the time taken to hydrolyze a given amount of ethyl butyrate is dependent on the acid concentration but independent of the ester concentration, provided this is above $N/200$. In other words for each concentration of acid a given amount of enzyme hydrolyzes very nearly the same amount of ethyl butyrate over a wide range of ester concentration.

(3) This phenomenon can be conformed to the mass law by assuming that the enzyme and the ester form an intermediate compound, which in concentrations of the ester above $N/200$ contains most of the enzyme.

(4) Assuming that the amount of this intermediate compound at any instant is proportional to the product of the concentration of the free enzyme and the ester, we saw that the rate of acid production was proportional to this quantity. This hypothesis, therefore, is seen to be in perfect accordance with the mass law.

(5) No explanation is offered as to the inhibiting effect of the acid.

(6) The question is raised as to whether the constancy of the function $1/t \log [a/(a - x)]$ in certain cases is not purely accidental.

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[1] **Taylor,** *J. Biol. Chem.*, 5, 405 (1910).