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ENZYMIC HYDROLYSIS OF GLUCOSIDES I. HYDROLYSIS OF METHYL- AND ETHYL-β-d-GLUCOSIDES

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In a previous paper (VEIBEL, 1934) one of the authors showed that both d- and l-hexanol-(2)-β-d-glucoside are hydrolyzed under the influence of emulsin, but that the reaction constants for the hydrolysis of the two glucosides are not identical, the ratio k_d/k_l being 3.44. At the same time it was shown that for d,l-3-methylpentanol-(1)-β-d-glucoside the ratio k_d/k_l was 1.07. As a cause for the difference between the reaction constants was suggested a difference in the affinity of emulsin to the different glucosides or to the different alcohols.

In the present paper we have examined this question more carefully, starting with glucosides containing aglycones as simple as possible, in order to get an opportunity of observing the influence of the structure of the aglycone on the hydrolysis-constant and the affinity-constant.

With a view to get as complete a knowledge as possible of the enzymic hydrolysis of glucosides we have determined the hydrolysis constants and the affinity constants at 30° and at 20°, thus making it possible to calculate the heat of activation for the hydrolysis of the glucoside and the heat of formation for the addition compound enzyme-substrate.

We have furthermore examined the influence of the products of hydrolysis, glucose and the alcohol in question, on the rate of hydrolysis. This we have done by determining

the velocity constants in experiments where the solution besides glucoside contained one or the other of the products of hydrolysis in various concentrations. If an inhibiting action exists, it is in this way possible to determine an affinity constant for the union of emulsin with the products of hydrolysis.

The investigation is therefore divided into two parts.

A. Determination of the velocity constant of hydrolysis and the affinity constant at 30° and at 20°.

The velocity constants are determined for 0.04 M glucoside solutions. In order to compare velocity constants determined in different experiments it is necessary to know the concentration of emulsin, e.g. expressed as g emulsin in 50 ml. reaction mixture, and the enzymic power of the emulsin preparation used, e.g. expressed as sal. f. (Josephson, 1925). Sal. f. is determined by the action of emulsin on a standard solution of salicin at 30° and p_H 4.4. The quotients k/e·(sal. f.) are then directly comparable.

We have found for methyl-glucoside k/e·(sal. f.) at 30° $= 2.7 \cdot 10^{-2}$, at 20° $= 1.3_5 \cdot 10^{-2}$. k_{30}/k_{20} is 2 and the heat of activation is 12200 cal. For ethyl glucoside the corresponding figures are $k_{30} = 5.3 \cdot 10^{-2}$, $k_{20} = 2.2 \cdot 10^{-2}$. $k_{30}/k_{20} = 2.4$ and heat of activation 15400 cal.

The affinity constant is for methyl glucoside 1.6. (Josephson (1925) found the value 1.4) and for ethyl glucoside 4.0. In both cases k_{M30}/k_{M20} is 1.

It will be seen that the temperature coefficient for the velocity constants differs for the two glucosides examined, whereas the affinity constant is independent of the temperature. This means that it is only reasonable to examine whether proportionality exists between the affinity constant and the

velocity constant, when a standard temperature is fixed in advance. If the two constants are proportional at one temperature, this will not be the case at all other temperatures, as the temperature coefficients are different. From the values found for methyl and ethyl glucoside at 30° and at 20° it may be calculated that at 0° the two velocity constants ought to be of the same value. A control experiment carried out at 3—4° has shown that at this temperature the velocity constants for the two glucosides are practically identical.

Accordingly, the possibility mentioned in the previous paper (VEIBEL, 1934) that the difference of velocity of hydrolysis is exclusively dependent on differences in affinity constants cannot be maintained.

B. The influence of the products of hydrolysis.

The velocity constants were determined at 30° for solutions 0.04 M as to glucoside and at the same time 0, 0.01, 0.02, 0.04, 0.08 or 0.12 M as to one or the other of the products of hydrolysis. It was found that an inhibiting action of the two alcohols cannot be established with certainty. At all events it is impossible to calculate an affinity constant for the addition compound of emulsin and alcohol. JOSEPHSON (1925) has examined the hydrolysis of methyl glucoside in 50 % methyl alcohol and has found the affinity constant for the compound emulsin-methyl-alcohol = 0.32. As regards ethyl alcohol we have not been able to find any examination of it previous to ours.

The inhibiting action of glucose is vigorous enough to allow a calculation of the affinity constant for the compound emulsin-glucose. From the experiments with methyl glucoside we calculate for this constant the value 4.9, from

the experiments with ethyl glucoside the value 5.9. In experiments with different glucosides Josephson (1925) has found values differing from 5.1 to 6.2 for the affinity constant glucose-emulsin.

It is to be noticed that the glucose used in these experiments is equilibrium glucose. As the glucosides employed are β-glucosides, it is likely that the inhibition caused by the glucose set free during the hydrolysis is greater than the inhibition found here.

Experimental.

In order to compare constants found for different glucosides it is necessary to fix standard conditions for the concentration of glucoside, for the p_H of the solution and, to a certain extent, for the emulsin concentration. As standard substrate concentration we have chosen 0.04 M and as standard p_H 4.4 (acetate buffer), the optimal p_H for emulsin when the aglycone is neutral. The emulsin concentration used by us was 0.20—0.25 g of an emulsin preparation of sal. f. = 0.040—0.045 in 50 ml. of the solution. Under these standard conditions the hydrolysis of the glucosides proceeds at a rate that may be conveniently followed.

In another place (Veibel and Eriksen (1936, 2)) we have called attention to some sources of error which are to be taken into account in the polarimetric examination of the hydrolysis of glucosides, namely that (*a*) the specific rotation of glucosides and of glucose is dependent on the p_H of the examined solution. In hydrolysis experiments the rotation is determined in solutions of p_H 10—11, and the difference in rotation at this p_H and at p_H 4—7, at which p_H the specific rotation is generally determined, is some 3—4 %. (*b*) Solutions of glucose, which are kept at

p_H 10—11, are not quite stable. The rotation of such solutions decreases with time, for 0.1 M solutions the decrease is 0.0033° per hour. (*c*) The reaction constants determined in experiments where toluene has been added are not directly comparable with reaction constants determined in experiments without the addition of toluene, this addition causing an increase in reaction constants of some 25—33 %.

1. Methyl-β-d-glucoside.

The enzymic hydrolysis of this glucoside has been examined previously, most carefully by JOSEPHSON (1925). It is possible, however, that his results may not be quite correct, as he seems to consider the methyl glucoside anhydrous. We have found that it contains ½ Mol of water as reported e. g. in BEILSTEIN. Neither Josephson nor other investigators seem to have taken any notice of the sources of error mentioned above.

The preparation of methyl glucoside used in the experiments described here showed M. P. 108—10° (corr.) and $[\alpha]_D^{20} = -32°.5$ (water, c = 2.835). The experimental method employed by us has previously been described (VEIBEL, 1934).

a. Determination of the velocity constant and its temperature coefficient. Tables I and II give the results of hydrolysis experiments at 30° and at 20°. All reaction constants are calculated with logarithms to base 10 and the minute as unit of time.

Table I.

Hydrolysis. 30°.

$c_{emulsin}$ 0.2233 g in 50 ml. $\alpha_{emulsin} = -0.380°$. Sal. f. = 0.043.

$c_{glucoside} = 0.0400$ M. $\alpha_{beg.} = -0.455°$, $\alpha_{end} = +0.620°$.

Time min.	Samples kept h.	α	corr.	α corr.	x	c—x	$k \cdot 10^4$
0	4.0	—0.835	—	—0.835	—	1.075	—
120	4.0	—0.745	—	—0.745	0.090	0.985	(3.16)
240	4.0	—0.680	—	—0.680	0.155	0.920	2.82
360	18.0	—0.635	0.005	—0.630	0.205	0.870	2.55
540	16.0	—0.555	0.005	—0.550	0.285	0.790	2.48
780	13.0	—0.450	0.005	—0.445	0.390	0.685	2.51
1440	4.0	—0.250	0.005	—0.245	0.590	0.485	2.40
1980	17.0	—0.145	0.015	—0.130	0.705	0.370	(2.34)
2880	4.0	—0.010	0.005	—0.005	0.830	0.245	(2.23)
						mean value...	2.6

$k/e \cdot (sal.\ f.) = 2.7 \cdot 10^{-2}$

Table II.

Hydrolysis. 20°.

$c_{emulsin}$ and $c_{glucoside}$ as in table I.

Time min.	Samples kept h.	α	corr.	α corr.	x	c—x	$k \cdot 10^4$
0	4.0	—0.835	—	—0.835	—	1.075	—
120	4.0	—0.770	—	—0.770	0.065	1.010	(2.26)
240	4.0	—0.750	—	—0.750	0.085	0.990	1.49
360	18.0	—0.720	0.005	—0.715	0.120	0.955	1.43
540	16.0	—0.670	0.005	—0.665	0.170	0.905	1.38
780	13.0	—0.630	0.005	—0.625	0.210	0.865	1.21
1440	4.0	—0.500	—	—0.500	0.335	0.740	1.13
1980	17.0	—0.420	0.010	—0.410	0.425	0.650	(1.10)
2880	4.0	—0.290	0.005	—0.285	0.550	0.525	(1.08)
						mean value...	1.3

$k/e \cdot (sal.\ f.) = 1.3_5 \cdot 10^{-2}$

From the values of $k/e \cdot (sal.\ f.)$ at 30° and at 20° k_{30}/k_{20} is seen to be 2.0. The heat of activation is calculated to be 12200 cal. per Mol from the expression

$$E = \frac{R \cdot T_1 \cdot T_2}{T_2 - T_1} \cdot \ln \frac{k_2}{k_1}$$

b. The affinity constant and its temperature coefficient. The affinity constant has been introduced by MICHAELIS and MENTEN (1913). We do not wish to discuss here whether this constant is a true thermodynamic constant or not, this question having been thoroughly discussed in a paper by BRIGGS (1931).

For the determination of the affinity constant K_M we have used the graphical method recommended in the book of HALDANE and STERN (1932, p. 119), which is discussed in a paper by LINEWEAVER and BURK (1934). For 6 different glucoside concentrations we have determined the initial rate of hydrolysis, the enzyme concentration being the same in all cases. For each glucoside concentration we have taken 4 readings at different times. From the expression $v = \frac{V \cdot c}{K_m + c}$, which may be written as $V/v = 1 + K_m/c$, (V the velocity when the enzyme is completely bound to the substrate) it may be seen that on plotting the values of 1/v against the values of 1/c a straight line is determined, and that the intercepts made on the axes by this line have the values 1/V and $-1/K_m = -K_M$.

We have determined the affinity constant at 30° and at 20°. Tables III and IV and fig. 1 give the results, which are that both at 30° and at 20° the affinity constant is 1.6. JOSEPHSON (1925) has found the value 1.4 at 30°; this is, within the limits of the experiment, the same value as ours.

When the affinity constant has, within the limits of the experiment, the same value at 30° as at 20°, the heat of formation for the enzyme-substrate-compound must be zero or very small. The value of the heat of formation for com-

Table III.

Affinity constant. 30°.

$c_{emulsin}$ 0.1976 g in 50 ml., $\alpha_{emulsin}$ —0.340° in all experiments.

Time min.	Samples kept h.	α	corr.	α corr.	x	1/v
I. 0.0204 M glucoside. 1/c = 49.0.						
0	4.0	—0.570	—	—0.570	—	—
120	—	—	—	—	—	—
240	5.0	—0.510	—	—0.510	0.060	16.67
360	20.0	—0.465	—	—0.465	0.105	9.52
480	20.0	—0.440	—	—0.440	0.130	7.69
II. 0.409 M glucoside. 1/c = 24.5.						
0	4.0	—0.805	—	—0.805	—	—
120	4.0	—0.730	—	—0.730	0.075	13.33
240	5.0	—0.665	—	—0.665	0.140	7.14
360	20.0	—0.620	0.005	—0.615	0.190	5.26
480	20.0	—0.565	0.005	—0.560	0.245	4.08
III. 0.0826 M glucoside. 1/c = 12.1.						
0	4.0	—1.280	—	—1.280	—	—
120	4.0	—1.145	—	—1.145	0.135	7.41
240	5.0	—1.030	—	—1.030	0.250	4.00
360	20.0	—0.915	0.010	—0.905	0.375	2.67
480	20.0	—0.820	0.010	—0.810	0.470	2.13
IV. 0.1672 M glucoside. 1/c = 6.0.						
0	4.0	—2.240	—	—2.240	—	—
120	4.0	—2.000	—	—2.000	0.240	4.17
240	5.0	—1.780	0.005	—1.775	0.465	2.15
360	20.0	—1.575	0.015	—1.560	0.680	1.47
480	20.0	—1.400	0.020	—1.380	0.860	1.16
V. 0.3334 M glucoside. 1/c = 3.0.						
0	4.0	—4.135	—	—4.135	—	—
120	4.0	—3.710	—	—3.710	0.425	2.35
240	5.0	—3.365	0.005	—3.360	0.775	1.29
360	20.0	—3.040	0.025	—3.015	1.120	0.89
480	20.0	—2.730	0.035	—2.695	1.440	0.69
VI. 0.6689 M glucoside. 1/c = 1.5.						
0	4.0	—7.950	—	—7.950	—	—
120	4.0	—7.380	0.005	—7.375	0.575	1.74
240	5.0	—6.840	0.005	—6.835	1.115	0.90
360	20.0	—6.360	0.040	—6.320	1.630	0.61
480	20.0	—5.850	0.050	—5.800	2.150	0.47

Table IV.

Affinity constant. 20°.

$c_{emulsin}$ 0.2082 g in 50 ml., $\alpha_{emulsin}$ −0.355° in all experiments.

Time min.	Samples kept h.	α	corr.	α corr.	x	1/v
I. 0,0200 M glucoside. 1/c = 50.0.						
0	4.0	−0.585	—	−0.585	—	—
120	4.0	−0.555	—	−0.555	0.030	33.33
240	5.0	−0.535	—	−0.535	0.050	20.00
360	20.0	−0.515	—	−0.515	0.070	14.29
480	20.0	−0.500	—	−0.500	0.085	11.76
II. 0.0400 M glucoside. 1/c = 25.0.						
0	4.0	−0.810	—	−0.810	—	—
120	4.0	−0.765	—	−0.765	0.045	22.22
240	5.0	−0.730	—	−0.730	0.080	12.50
360	20.0	−0.690	0.005	−0.685	0.125	8.00
480	20.0	−0.660	0.005	−0.655	0.155	6.45
III. 0.0800 M glucoside. 1/c = 12.5.						
0	4.0	−1.265	—	−1.265	—	—
120	4.0	−1.180	—	−1.180	0.085	11.76
240	5.0	−1.115	—	−1.115	0.150	6.67
360	20.0	−1.055	0.005	−1.050	0.215	4.65
480	20.0	−1.005	0.005	−1.000	0.265	3.77
IV. 0.1600 M glucoside. 1/c = 6.25.						
0	4.0	−2.175	—	−2.175	—	—
120	5.0	−2.015	—	−2.015	0.160	6.25
240	5.0	−1.910	—	−1.910	0.265	3.77
360	20.0	−1.800	0.010	−1.790	0.385	2.60
480	20.0	−1.690	0.010	−1.680	0.495	2.02
V. 0.3142 M glucoside. 1/c = 3.18.						
0	4.0	−3.930	—	−3.930	—	—
120	4.0	−3.630	—	−3.630	0.300	3.33
240	5.0	−3.455	0.005	−3.450	0.480	2.08
360	20.0	−3.290	0.020	−3.270	0.660	1.52
480	20.0	−3.135	0.020	−3.115	0.815	1.23
VI. 0.6160 M glucoside. 1/c = 1.62.						
0	4.0	−7.360	—	−7.360	—	—
120	5.0	−6.820	0.005	−6.815	0.545	1.83
240	5.0	−6.530	0.005	−6.525	0.835	1.20
360	20.0	−6.265	0.030	−6.235	1.125	0.89
480	20.0	−6.020	0.030	−5.990	1.370	0.73

pounds of hydrolysing ferments with their substrates has been discussed, Euler and Laurin (1920) having found for the formation of a saccharase-saccharose-compound the

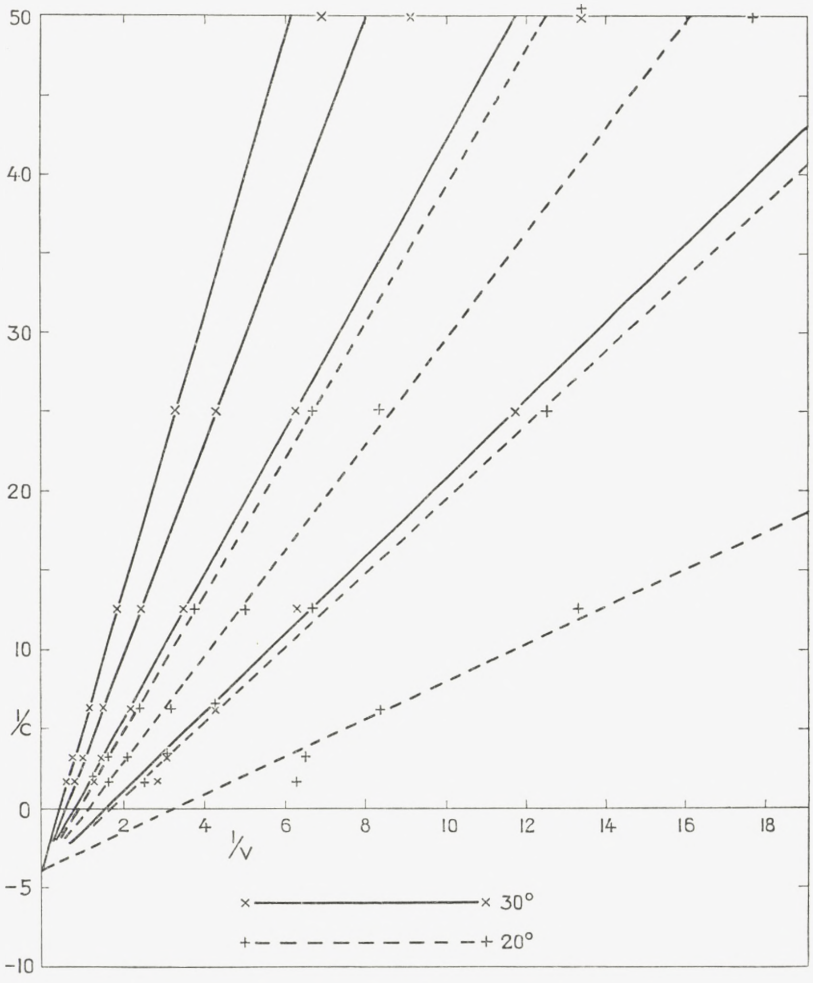

Fig. 1.

heat of formation to be 2000 cal., whereas Nelson and Bloomfield (1924) for the same compound found the value zero. Our results coincide with the results of Nelson and Bloomfield, both for methyl glucoside and for ethyl glucoside (see below).

c. The influence of the products of hydrolysis. The glucoside and the emulsin concentrations had the usual standard values, but so much glucose or methyl-alcohol was added that the concentration of the one or the other of these substances was 0.01, 0.02, 0.04, 0.08 or 0.12 M. The glucose was taken from a stock solution old enough to ensure that it contained equilibrium glucose.

In order to prevent the growth of microorganisms in the solutions containing glucose, 1 ml. of toluene was added to each measuring flask of 50 ml. As mentioned above, velocity constants found in experiments where toluene has been added are not to be compared with constants found in experiments without the addition of toluene.

In the experiments with addition of methyl alcohol no toluene was added.

Tables V—X give the results. The two first tables show the influence of glucose and methyl alcohol respectively. Tables VII—X give data from single experiments to show the accuracy which is to be expected in the determination of the reaction constants.

Table V. Influence of glucose.

$c_{emulsin}$ and $c_{glucoside}$ as in table VII. 1 ml. toluene added.

$c_{glucose}$	0.00	0.01	0.02	0.04	0.08	0.12 M
$k \cdot 10^4$	3.5	3.2	3.0	2.6	2.4	2.2

Table VI. Influence of methyl alcohol.

$c_{emulsin}$ and $c_{glucoside}$ as in table IX. No toluene added.

c_{CH_3OH}	0.00	0.01	0.02	0.04	0.08	0.12 M
$k \cdot 10^4$	2.7	2.7	2.6	2.7	2.6	2.6

Table VII.

Influence of glucose.

$c_{emulsin}$ 0.1943 g in 50 ml. $\alpha_{emulsin} = -0.330°$. 1 ml. Toluene added. $c_{glucoside}$ 0.0400 M. $c_{glucose}$ 0.0200 M. $\alpha_{beg.} = -0.475°$, $\alpha_{end} = +0.600°$.

Time min.	Samples kept h.	α	corr.	α corr.	x	c—x	k·10⁴
0	5.0	—0.480	0.005	—0.475	—	1.075	—
120	5.0	—0.385	0.005	—0.380	0.095	0.980	3.35
240	4.0	—0.305	0.005	—0.300	0.175	0.900	3.22
360	19.5	—0.255	0.015	—0.240	0.235	0.840	2.98
540	18.5	—0.175	0.015	—0.160	0.315	0.760	2.79
720	17.0	—0.095	0.015	—0.080	0.395	0.680	2.76
1440	7.0	+0.165	0.010	+0.175	0.650	0.425	2.80
						mean value...	3.0

Table VIII.

Influence of glucose.

$c_{emulsin}$ and $c_{glucoside}$ as in table VII. $c_{glucose}$ 0.1200 M. $\alpha_{beg.} = +1.030°$, $\alpha_{end} = +2.105°$. 1 ml. toluene added.

Time min.	Samples kept h.	α	corr.	α corr.	x	c—x	k·10⁴
0	5.5	+1.010	0.020	+1.030	—	1.075	—
120	5.0	+1.075	0.020	+1.095	0.065	1.010	2.26
240	4.5	+1.145	0.015	+1.160	0.130	0.945	2.33
360	20.0	+1.135	0.075	+1.210	0.180	0.895	2.21
540	19.5	+1.225	0.075	+1.300	0.270	0.805	2.33
720	17.5	+1.290	0.070	+1.360	0.330	0.745	2.21
1440	7.5	+1.545	0.030	+1.575	0.545	0.530	2.13
						mean value...	2.2

Table IX.

Influence of methyl alcohol.

$c_{emulsin}$ 0.2239 g in 50 ml. $\alpha_{emulsin} = -0.380°$. $c_{glucoside}$ 0.0400 M. $c_{methyl\ alcohol}$ 0.02 M. $\alpha_{beg.} = -0.835°$, $\alpha_{end} = +0.240°$.

Time min.	Samples kept h.	α	corr.	α corr.	x	c—x	k·10⁴
0	4.0	—0.835	—	—0.835	—	1.075	—
120	4.0	—0.755	—	—0.755	0.080	0.995	2.80
240	4.0	—0.685	—	—0.685	0.150	0.925	2.72
360	18.0	—0.625	0.005	—0.620	0.215	0.860	2.69
480	20.0	—0.575	0.005	—0.570	0.265	0.810	2.56
780	17.0	—0.460	0.010	—0.450	0.385	0.690	2.47
1440	5.0	—0.265	0.005	—0.260	0.575	0.500	(2.31)
						mean value...	2.6

Table X.

Influence of methyl alcohol.

$c_{emulsin}$ and $c_{glucoside}$ as in table IX. $c_{methyl\ alcohol}$ 0.12 M.

Time min.	Samples kept h.	α	corr.	α corr.	x	c—x	$k \cdot 10^4$
0	4.0	—0.835	—	—0.835	—	1.075	—
120	4.0	—0.755	—	—0.755	0.080	0.995	2.80
240	4.0	—0.690	—	—0.690	0.145	0.930	2.62
360	18.0	—0.630	0.005	—0.625	0.210	0.865	2.62
480	20.0	—0.580	0.005	—0.575	0.260	0.815	2.51
780	17.0	—0.465	0.010	—0.455	0.380	0.695	2.43
1440	5.0	—0.270	0.005	—0.265	0.570	0.505	(2.28)
						mean value...	2.6

These experiments show that methyl alcohol in the concentration used here has no retarding influence on the hydrolysis. JOSEPHSON (1925, p. 113) has found that methyl alcohol in the concentration 1.2—2.5 M acts as an inhibitor. We have restricted the examination to concentrations of methyl alcohol of the same order of magnitude as the glucoside concentration used in hydrolysis experiments, and for these concentrations the inhibiting influence is negligible.

Glucose, on the other hand, retards the hydrolysis considerably. Already at 0.02 M glucose the value of the reaction constant has diminished some 15 %. That means that the glucose set free during the hydrolysis will retard the reaction considerably. This may suffice to explain the steady fall in the reaction constant often observed in hydrolysis experiments.

JOSEPHSON (1925) has also examined the influence of an addition of glucose. He calculates the affinity constant for the addition compound of glucose and emulsin, making use of an equation given by MICHAELIS and MENTEN (1913)

$$K_{m_1} = \frac{G \cdot K_m}{(S + K_m)\ (v_o/v - 1)}$$

where K_{m_1} is the dissociation constant for the glucose-emulsin compound, K_m the dissociation constant for the glucoside-emulsin compound, S the concentration of glucoside, G of glucose, v_0 the velocity in the first stage of the reaction when glucose has not been added, v the corresponding velocity when glucose has been added. Velocity means here change in rotation in t minutes.

It has been shown above that K_m for methyl glucoside has the value 0.62. Table XI shows that K_{m_1} has the value 0.20_5, i. e. that the affinity constant $K_{M_1} = 1/K_{m_1}$ is 4.9. Josephson (l. c.) has found the value 5.2, which within the limits of the experiment is the same as ours.

Table XI.

Affinity constant for the compound emulsin-glucose.

$K_m = 0.62$, $S = 0.04$, $G = 0.01—0.12$.

G...	0.00	0.01		0.02		0.04		0.08		0.12	
t	v_0	v	v_0/v	v	v_0/v	v	v_0/v	v	v_0/v	v	v_0/v
120	0.100	0.095	1.05	0.095	1.05	0.090	1.11	0.075	1.33	0.065	1.54
240	0.185	0.175	1.06	0.175	1.06	0.160	1.16	0.125	1.48	0.130	1.42
360	0.265	0.260	1.02	0.235	1.13	0.215	1.23	0.185	1.43	0.180	1.47
540	0.365	0.350	1.04	0.315	1.16	0.305	1.20	0.270	1.36	0.270	1.36
mean values ...			1.04		1.10		1.18		1.40		1.45
K_{m_1}			0.235		0.189		0.209		0.189		(0.252)
mean value...						0.20_5					

From the values of the term v_0/v for the different glucose concentrations it may be seen that up to a glucose concentration 0.08 M the retarding influence is proportional to the glucose concentration. At 0.12 M glucose this does not hold good, may be because the emulsin has been saturated with glucose. From a comparison of tables VII and VIII the same fact may be deduced, namely that the fall in the reaction constant during the experiment is not as great at the glucose concentration 0.12 M as at the glucose

concentration 0.02 M, which means that the glucose set free during the hydrolysis has not so great an influence in the former case as in the latter.

2. Ethyl-β-d-glucoside.

In the experiments described below a preparation with M. P. 81—83° and $[\alpha]_D^{20°} = -38.6°$ (water, c = 3.533) was used. These values differ rather much from those given by COIRRE (1913, 1, 2) and by BOURQUELOT and BRIDEL (1913), namely M. P. 73° and $[\alpha]_D = -36.5°$. In another place a more careful description of the preparation and the properties of ethyl-β-d-glucoside will be given. (VEIBEL and ERIKSEN, (1936 1)).

a. Determination of the velocity constant at 30° and its temperature coefficient.

Tables XII and XIII give the results of hydrolysis experiments at 30° and at 20°.

Table XII.

Hydrolysis. 30°.

$c_{emulsin}$ 0.2818 g in 50 ml. $\alpha_{emulsin} = -0.535°$. Sal. f. = 0.044.

$c_{glucoside}$ 0.0400 M. $\alpha_{beg.} = -0.555°$, $\alpha_{end} = +0.620°$.

Time min.	Samples kept h.	α	corr.	α corr.	x	c—x	k·10⁴
0	6.5	—1.090	—	—1.090	—	1.175	—
60	6.5	—0.980	—	—0.980	0.110	1.065	7.12
120	6.0	—0.890	—	—0.890	0.200	0.975	6.75
240	4.5	—0.750	—	—0.750	0.340	0.835	6.18
360	18.0	—0.615	0.010	—0.605	0.485	0.690	6.42
510	16.5	—0.520	0.010	—0.510	0.580	0.595	5.80
840	12.0	—0.320	0.010	—0.310	0.780	0.395	(5.64)
1440	4.5	—0.120	0.005	—0.115	0.975	0.200	(5.34)
1740	3.5	—0.080	0.005	—0.075	1.015	0.160	(4.98)
						mean value ...	6.5

$k/e \cdot (\text{sal. f.}) = 5.3 \cdot 10^{-2}$.

Table XIII.

Hydrolysis. 20°.

$c_{emulsin}$ and $c_{glucoside}$ as in table XII.

Time min.	Samples kept h.	α	corr.	α corr.	x	c—x	$k \cdot 10^4$
0	7.0	—1.090	—	—1.090	—	1.175	—
60	6.5	—1.045	—	—1.045	0.045	1.130	2.83
120	6.0	—1.005	—	—1.005	0.085	1.090	2.72
240	4.5	—0.925	—	—0.925	0.165	1.010	2.74
360	18.5	—0.845	0.005	—0.840	0.250	0.925	2.89
510	17.0	—0.775	0.005	—0.770	0.320	0.855	2.71
840	12.0	—0.665	0.005	—0.660	0.430	0.745	2.36
1440	4.0	—0.490	0.005	—0.485	0.605	0.570	(2.18)
1740	3.5	—0.430	0.005	—0.425	0.665	0.510	(2.08)
						mean value...	2.7

$k/e \cdot (sal.\ f.) = 2.2 \cdot 10^{-2}$.

In this case k_{30}/k_{20} is 2.4 and the heat of activation 15400 cal.

As the enzymic hydrolysis of ethyl glucocide has not been much investigated, we have examined if the rate of hydrolysis is proportional to the concentration of emulsin. Table XIV shows that this is the case.

Table XIV.

Proportionality between rate of hydrolysis and enzyme concentration.

$c_{emulsin}$	$c_{glucoside}$	$k \cdot 10^4$	$k/e \cdot (sal.\ f.)$
0.0919 g	0.0400 M	2.1	$5.3 \cdot 10^{-2}$
0.1838 g	0.0400 M	4.3	$5.4 \cdot 10^{-2}$
0.2757 g	0.0400 M	6.5	$5.5 \cdot 10^{-2}$

b. The affinity constant and its temperature coefficient. As for methyl glucoside the affinity constant has been determined at 30° and at 20°. Tables XV and XVI and fig. 2 give the results, which are that at 30° as at 20° the affinity constant is 4.0. K_{M30}/K_{M20} is 1 and the

Table XV.

Affinity constant. 30°.

$c_{emulsin}$ 0.2304 g in 50 ml., $\alpha_{emulsin} = -0.390°$ in all experiments.

Time min.	Samples kept h.	α	corr.	α corr.	x	1/v
I. 0.0200 M glucoside. 1/c = 50.00.						
0	4.0	—0.670	—	—0.670	—	—
60	4.0	—0.630	—	—0.630	0.040	25.00
120	4.0	—0.595	—	—0.595	0.075	13.33
180	4.0	—0.560	—	—0.560	0.110	9.09
240	4.0	—0.525	—	—0.525	0.145	6.90
II. 0.0400 M glucoside. 1/c = 25.00.						
0	4.0	—0.945	—	—0.945	—	—
60	4.0	—0.860	—	—0.860	0.085	11.76
120	4.5	—0.785	—	—0.785	0.160	6.25
180	4.5	—0.710	—	—0.710	0.235	4.25
240	4.5	—0.635	—	—0.635	0.310	3.23
III. 0.0800 M glucoside. 1/c = 12.50.						
0	4.0	—1.500	—	—1.500	—	—
60	4.0	—1.340	—	—1.340	0.160	6.25
120	4.5	—1.210	—	—1.210	0.290	3.45
180	4.5	—1.085	—	—1.085	0.415	2.41
240	4.5	—0.965	0.005	—0.960	0.540	1.85
IV. 0.1600 M glucoside. 1/c = 6.25.						
0	4.5	—2.610	—	—2.610	—	—
60	4.5	—2.375	—	—2.375	0.235	4.25
120	4.5	—2.145	—	—2.145	0.465	2.15
180	4.5	—1.930	0.005	—1.925	0.685	1.46
240	4.5	—1.725	0.005	—1.720	0.890	1.12
V. 0.3200 M glucoside. 1/c = 3.13.						
0	4.5	—4.830	—	—4.830	—	—
60	4.5	—4.500	—	—4.500	0.330	3.03
120	4.5	—4.150	0.005	—4.145	0.680	1.46
180	4.5	—3.825	0.005	—3.820	1.010	0.99
240	4.5	—3.545	0.005	—3.540	1.290	0.78
VI. 0.6400 M glucoside. 1/c = 1.56.						
0	5.0	—9.270	—	—9.270	—	—
60	5.0	—8.915	—	—8.915	0.355	2.82
120	5.0	—8.450	0.005	—8.445	0.825	1.21
180	5.0	—7.995	0.005	—7.990	1.280	0.78
240	5.0	—7.620	0.010	—7.610	1.660	0.60

Table XVI.

Affinity constant. 20°.

$c_{emulsin}$ 0.2096 g in 50 ml., $\alpha_{emulsin} = -0.355°$ in all experiments.

Time min.	Samples kept h	α	corr.	α corr.	x	1/v
I. 0.0200 M glucoside. 1/c = 50.00.						
0	3.5	—0.635	—	—0.635	—	—
60	3.5	—0.615	—	—0.615	0.020	50.00
120	4.0	—0.595	—	—0.595	0.040	25.00
180	4.0	—0.575	—	—0.575	0.060	16.67
240	21.0	—0.560	—	—.0560	0.075	13.33
II. 0.0400 M glucoside. 1/c = 25.00.						
0	4.0	—0.910	—	—0.910	—	—
60	4.0	—0.860	—	—0.860	0.050	20.00
120	4.5	—0.830	—	—0.830	0.080	12.50
180	4.5	—0.790	—	—0.790	0.120	8.33
240	21.0	—0.765	0.005	—0.760	0.150	6.67
III. 0.0800 M glucoside. 1/c = 12.50.						
0	4.0	—1.465	—	—1.465	—	—
60	4.0	—1.390	—	—1 390	0.075	13.33
120	4.5	—1.315	—	—1.315	0.150	6.67
180	4.5	—1.265	—	—1.265	0.200	5.00
240	21.5	—1.205	0.005	—1.200	0.265	3.77
IV. 0.1600 M glucoside. 1/c = 6.25.						
0	4.0	—2.570	—	—2.570	—	—
60	4.0	—2.450	—	—2.450	0.120	8.33
120	5.0	—2.335	—	—2.335	0.235	4.26
180	5.0	—2.250	—	—2.250	0.320	3.13
240	22.0	—2.155	0.010	—2.145	0.425	2.35
V. 0.3200 M glucoside. 1/c = 3.13.						
0	4.0	—4.790	—	—4.790	—	—
60	4.0	—4.635	—	—4.635	0.155	6.45
120	5.0	—4.460	—	—4.460	0.330	3.03
180	5.0	—4.305	—	—4.305	0.485	2.06
240	22.0	—4.180	0.015	—4.165	0.625	1.60
VI. 0.6400 M glucoside. 1/c = 1.56.						
0	4.0	—9.220	—	—9.220	—	—
60	4.5	—9.060	—	—9.060	0.160	6.25
120	5.0	—8.815	—	—8.815	0.405	2.47
180	5.0	—8.600	—	—8.600	0.620	1.61
240	22.0	—8 420	0.020	—8.400	0.820	1.22

heat of formation of the glucoside-emulsin compound is zero.

c. The influence of the products of hydrolysis. The influence of glucose and of ethyl alcohol on the hydro-

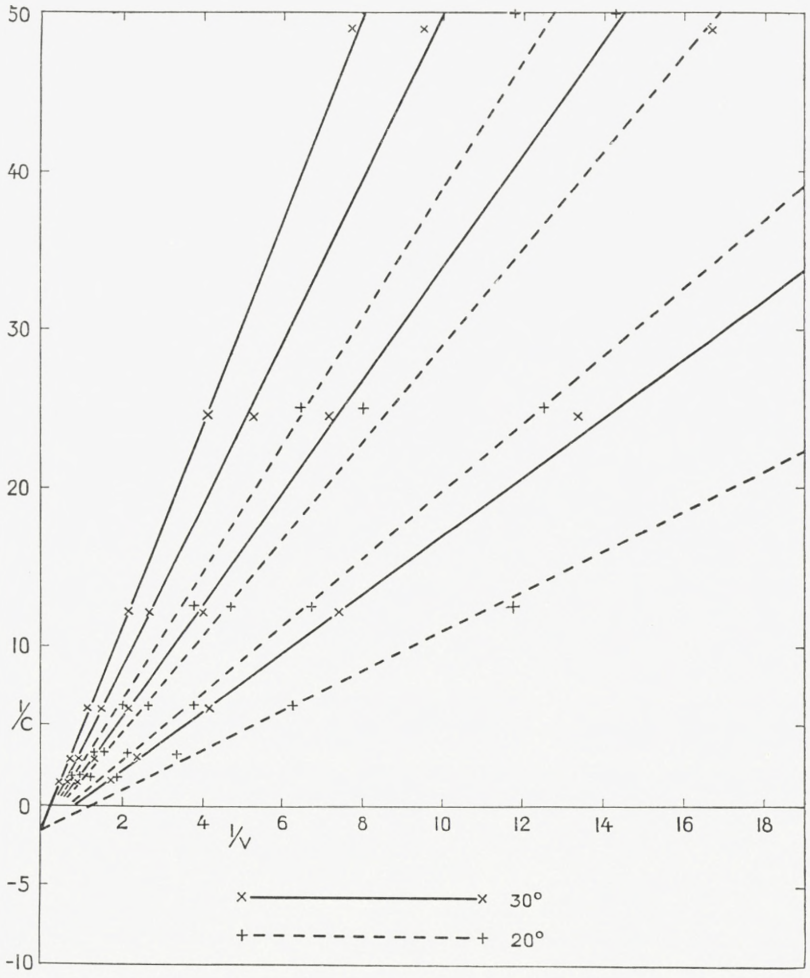

Fig. 2.

lysis was examined in the way described for methyl glucoside. Tables XVII and XVIII show the influence of glucose and ethyl alcohol respectively. Tables XIX—XXII are examples showing the variations within single experiments.

Table XVII.

$c_{emulsin}$ and $c_{glucoside}$ as in table XIX 1 ml. toluene added.

$c_{glucose}$	0.00	0.01	0.02	0.04	0.08	0.12 M
$k \cdot 10^4$	8.5	8.0	7.7	7.0	5.9	5.7

Table XVIII.

Influence of ethyl alcohol.

$c_{emulsin}$ and $c_{glucoside}$ as in table XXI. No toluene added.

$c_{C_2H_5OH}$	0.00	0.01	0.02	0.04	0.08	0.12 M
$k \cdot 10^4$	4.6	4.7	4.7	4.8	4.6	4.6

Table XIX.

Influence of glucose. Toluene added.

$c_{emulsin}$ 0.2540 g in 50 ml. $\alpha_{emulsin} = -0.480°$. sal. f. = 0.044.
$c_{glucoside}$ 0.0400 M. $c_{glucose}$ 0.0200 M. $\alpha_{beg.}$ $-0.720°$,
α_{end} $+0.455°$.

Time min.	Samples kept h.	α	corr.	α corr.	x	c—x	$k \cdot 10^4$
0	6.0	—0.725	0.005	—0.720	—	1.175	—
60	6.5	—0.590	0.005	—0.585	0.135	1.040	8.84
120	6.5	—0.480	0.005	—0.475	0.245	0.930	8.46
240	21.0	—0.360	0.025	—0.335	0.385	0.790	7.18
360	21.0	—0.190	0.025	—0.165	0.555	0.620	7.71
510	20.0	—0.095	0.030	—0.065	0.655	0.520	6.94
720	18.0	+0.075	0.030	+0.105	0.825	0.350	7.31
1440	7.5	+0.270	0.015	+0.285	1.005	0.170	(5.83)
						mean value...	7.7

Table XX.

Influence of glucose. Toluene added.

$c_{emulsin}$ and $c_{glucoside}$ as in table XIX. $c_{glucose}$ 0.1200 M.
$\alpha_{beg.}$ $+0.795°$, α_{end} $+1.970°$.

Time min.	Samples kept h.	α	corr.	α corr.	x	c—x	$k \cdot 10^4$
0	7.0	+0.765	0.030	+0.795	—	1.175	—
60	7.0	+0.875	0.030	+0.905	0.110	1.065	(7.12)
120	7.0	+0.940	0.030	+0.970	0.175	1.000	5.84
240	21.5	+1.070	0.095	+1.165	0.370	0.805	6.84
360	21.5	+1.130	0.095	+1.225	0.430	0.745	5.50
510	20.0	+1.270	0.090	+1.360	0.565	0.610	5.58
720	19.0	+1.365	0.090	+1.455	0.660	0.515	4.98
1440	8.0	+1.695	0.040	+1.735	0.940	0.235	(4.85)
						mean value...	5.7

Table XXI.

Influence of ethyl alcohol.

$c_{emulsin}$ 0.2008 g in 50 ml. $\alpha_{emulsin} = -0.345°$, sal. f. 0.043.
$c_{glucoside}$ 0.0400 M. $c_{ethyl\ alcohol}$ 0.01 M. $\alpha_{beg.}$ −0.900°,
α_{end} +0.275°.

Time min.	Samples kept h.	α	corr.	α corr.	x	c−x	k·10⁴
0	4.0	−0.900	—	−0.900	—	1.175	—
60	5.0	−0.815	—	−0.815	0.085	1.090	5.44
120	6.0	−0.750	—	−0.750	0.150	1.025	4.94
240	21.0	−0.645	0.005	−0.640	0.260	0.915	4.54
360	20.5	−0.535	0.010	−0.525	0.375	0.800	4.64
540	19.5	−0.420	0.010	−0.410	0.490	0.685	4.34
720	17.5	−0.310	0.010	−0.300	0.600	0.575	4.31
					mean value...		4.7

Table XXII.

Influence of ethyl alcohol.

$c_{emulsin}$ and $c_{glucoside}$ as in table XXI. $c_{ethyl\ alcohol}$ 0.12 M.

Time min.	Samples kept h.	α	corr.	α corr.	x	c—x	k·10⁴
0	5.0	−0.900	—	−0.900	—	1.175	—
60	6.5	−0.820	—	−0.820	0.080	1.095	5.11
120	6.5	−0.755	—	−0.755	0.145	1.030	4.77
240	21.5	−0.640	0.005	−0.635	0.265	0.910	4.63
360	21.5	−0.545	0.010	−0.535	0.365	0.810	4.49
540	20.0	−0.435	0.010	−0.425	0.475	0.700	4.17
720	18.0	−0.320	0.010	−0.310	0.590	0.585	4.21
					mean value...		4.6

The result is, as in the previous case, that glucose acts as an inhibitor, although not quite as powerful in this case as in the case of methyl glucoside. A retarding influence of ethyl alcohol cannot be established for the concentrations used here.

From the experiments with addition of glucose the affinity constant for the emulsin-glucose compound was calculated in the same way as indicated for methyl glucoside.

Table XXIII gives the result, which is $K_{m_1} = 0.17$, $K_{M_1} = 5.9$. For K_m is used the value 0.25 determined above.

Table XXIII.

Affinity constant for the compound emulsin-glucose.

$K_m = 0.25$, S = 0.04, G = 0.01—0.12.

G...	0.00	0.01		0.02		0.04		0.08		0.12	
t	v_0	v	v_0/v	v	v_0/v	v	v_0/v	v	v_0/v	v	v_0/v
60	0.135	0.125	1.08	0.135	1.00	0.120	1.13	0.085	1.59	0.110	1.23
120	0.260	0.265	0.98	0.245	1.06	0.205	1.27	0.175	1.49	0.175	1.49
240	0.435	0.415	1.08	0.385	1.16	0.400	1.09	0.380	1.15	0.370	1.18
360	0.610	0.550	1.12	0.555	1.10	0.525	1.16	0.455	1.35	0.430	1.41
mean value...			1.06		1.11		1.18		1.40		1.30
K_{m_1}			0.144		0.156		0.191		0.173		(0.344)
mean value...						0.17					

Josephson (1925) has determined the value of K_{M_1}, using different glucosides as test substances. The K_{M_1} values differ from 5.1 to 6.2. We have, with methyl glucoside and ethyl glucoside as test substances, found the values 4.9 and 5.9, i.e. within the limits of the experiment the same value as Josephson found.

It was mentioned above that the emulsin probably was saturated with glucose at a glucose concentration about 0.08 M. This is seen still more clearly here, the value of v_0/v being higher at 0.08 M glucose than at 0.12 M glucose.

The value for K_{M_1} found in these experiments is valid for equilibrium glucose. Josephson (1925) has found that for α-glucose the value for K_{M_1} is smaller, ca. 4.0. This means that the inhibiting power of the β-glucose set free during the hydrolysis is somewhat greater than calculated from the affinity constant found by the present examination.

Discussion.

At 30° the velocity constant for the enzymic hydrolysis of methyl glucoside is $2.7 \cdot 10^{-2}$, of ethyl glucoside $5.3 \cdot 10^{-2}$. The affinity constants for the compounds of emulsin and the two glucosides are 1.6 and 4.0. These values show that at 30° there is no proportionality between affinity constant and velocity constant.

As the temperature coefficient for the affinity constant of both glucosides is zero, whereas the temperature coefficients for the velocity constants are different from zero ($k_{30}/k_{20} = 2.0$ and 2.4 for methyl and ethyl glucoside respectively), a temperature where the velocity constant is proportional to the affinity constant may be calculated. This temperature is ca. 45°, i. e. too high to allow an experimental control on account of the thermal inactivation of emulsin. On the other hand a temperature may be calculated at which both glucosides are hydrolysed at the same rate. This temperature is ca. 0°. That the rate of hydrolysis at a low temperature is very nearly the same for the two glucosides we have found by means of an experiment where two measuring flasks, filled with solutions 0.0400 M as to the glucosides and with the same emulsin concentration, were placed in an ice-box at a temperature of about 3—4°. Samples were withdrawn at intervals and treated as usual. Tables XXIV and XXV show the result.

The values of k are identical within the limits of the experiment.

This control-experiment may prove that the relation between velocity constant and affinity constant is not a simple one.

Table XXIV.

Methyl glucoside. Rate of hydrolysis 4°.

$c_{emulsin}$ 0.1978 g in 50 ml. $\alpha_{emulsin} = -0.385°$. sal. f. = 0.044.

$c_{glucoside}$ 0.0400 M. $\alpha_{beg.}$ −0.840°, α_{end} +0.235°.

Time min.	Samples kept h.	α	corr.	α corr.	x	c—x	$k \cdot 10^4$
0	4.0	−0.840	—	−0.840	—	1.075	—
1440	3.5	−0.735	—	−0.735	0.105	0.975	0.31
3045	23.0	−0.645	0.005	−0.640	0.200	0.875	0.29
4320	3 5	−0.580	—	−0.580	0.260	0.815	0.28
5790	4.0	−0 520	—	−0.520	0.320	0.755	0.27
						mean value...	0.29

Table XXV.

Ethyl glucoside. Rate of hydrolysis 4°.

$c_{emulsin}$ and $c_{glucoside}$ as in table XXIV. $\alpha_{beg.}$ −0.940°, α_{end} +0.235°.

Time min.	Samples kept h.	α	corr.	α corr.	x	c—x	$k \cdot 10^4$
0	4.0	−0.940	—	−0.940	—	1.175	—
1440	3.5	−0.810	—	−0.810	0.130	1.045	0.35
3045	23.0	−0.725	0.005	−0.720	0.220	0.955	0.30
4320	3.5	−0.660	—	−0.660	0.280	0.895	0.27
5790	4.0	−0.580	—	−0.580	0.360	0.815	0.27
						mean value...	0.30

Summary.

The enzymic hydrolysis of methyl-β-d-glucoside and ethyl-β-d-glucoside has been examined. The reaction constants for 0.04 M solutions and the affinity constants have been determined at 30° and at 20°.

	$\frac{k_{30°}}{e \cdot (sal.\ f.)}$	$\frac{k_{20°}}{e \cdot (sal.\ f.)}$	Heat of activation	$K_{M30°}$	$K_{M20°}$
Methyl glucoside..	$2.7 \cdot 10^{-2}$	$1.3_5 \cdot 10^{-2}$	12200 cal	1.6	1.6
Ethyl glucoside ...	$5.3 \cdot 10^{-2}$	$2.2 \cdot 10^{-2}$	15400 cal	4.0	4.0

The inhibiting effect of the products of hydrolysis has been determined. For the two alcohols no such effect could be observed. Glucose, on the contrary, inhibits the hydrolysis, in the case of methyl glucoside to a higher degree than in the case of ethyl glucoside.

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(From the Chemical Laboraty, University of Copenhagen).

REFERENCES

Bourquelot and Bridel (1913). A. Ch. (8) 29, 145.
Briggs (1931). Biochem. J. 25, 1801.
Coirre (1913 1). These, Paris, p. 56.
— (1913 2). Journ. Pharm. Chim. (7) 8, 553.
Euler and Laurin (1920). Z. physiol. Chem. 110, 55 and 91.
Haldane-Stern (1932). Allgemeine Chemie der Enzyme p. 119.
Josephson (1925). Z. physiol. Chem. 147, 1 and 155.
Lineweaver and Burk (1934). J. Amer. Chem. Soc. 56, 658.
Michaelis and Menten (1913). Biochem. Z. 49, 333.
Nelson and Bloomfield (1924). J. Amer. Chem. Soc. 46, 1025.
Veibel (1934). Biochem. J. 28, 1733.
Veibel and Eriksen (1936 1) Bull. soc. chim. [5] 3, 277.
— (1936 2) Biochem. J. 30, 163.

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