XXVI. STUDIES ON THE ACTION OF EMULSIN.

III. SOME SOURCES OF ERROR IN THE POLARIMETRIC EXAMINATION OF ENZYMIC HYDROLYSIS OF β -GLUCOSIDES.

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In the examination of enzymic hydrolysis of β -glucosides the velocity constant for the hydrolysis and the affinity constant are often determined by following the hydrolysis of the glucoside polarimetrically. This may be done with great accuracy, but we wish to call attention to some sources of error which have not been sufficiently considered in these determinations, and to give material allowing a correction for these errors.

I. The rotation of solutions of glucosides and glucose is dependent on the p_H of the solutions. As the enzymic action of emulsin is stopped by addition of a solution of potassium carbonate to a sample of the glucoside-emulsin solution at p_H 4.4, and the p_H of the resulting mixture is 10.5–10.6 (colorimetrically determined), it is the rotation at the latter p_H which is to be taken into consideration. The difference in rotation at the two different values of p_H is some 3–4 %.

II. Glucose solutions are not stable at p_H 10.5–10.6. Michaelis and Rona [1912] have examined how the rotation of glucose solutions varies with the time at different p_H values and have found, for 10 % glucose solutions, that up to p_H 7.6 the solutions are quite stable, at p_H 10.15 the rotation decreases 0.012° per hour, at p_H 10.65, 0.022° per hour and at p_H 10.82, 0.037° per hour. We have found that for 0.1 *M* glucose solutions (1.8 %) at p_H 10.5–10.6 the decrease of rotation is 0.0033° per hour, *i.e.* sensibly the same result as Michaelis and Rona. In the examination of enzymic hydrolysis of glucosides samples containing glucose are kept for at least 3 hours at p_H 10.5–10.6 in order to allow the mutarotation to take place, and they may be kept a much longer time, up to some 20 hours, at this p_H before determination of the rotation. Under these circumstances the decrease in rotation of the solution may exceed the limit of error considerably, and a correction must be introduced. We have also examined whether glucose solutions containing emulsin change their rotation with time at p_H 4.4. It has been shown by Bourquelot [1914] and Bourquelot *et al.* [1920] that formation of gentiobiose and cellobiose may take place in solutions of glucose containing emulsin. Bourquelot, however, used glucose solutions much more concentrated than those used in our experiments, but we have thought it useful to examine if any trace of change in rotation may be observable. This was, however, not the case.

III. The standardisation of the emulsin preparation was carried out with salicin as standard glucoside. In a previous paper [Veibel, 1934] we have used the method of Helferich [1931], who quotes a paper of Weidenhagen [1929], published in a journal not very readily obtainable. We have now examined the

original paper of Weidenhagen and have found that the concentration of salicin used by him is not the same as that used by Helferich. Weidenhagen uses a salicin concentration of 3.972 % (in order to be in concordance with the "standard conditions" generally used in the hydrolysis of polysaccharides, *i.e.* a substrate concentration equivalent to a maltose concentration of 2.5 %). Helferich, on the other hand, uses a stock solution of salicin with the concentration 3.972 % and mixes 80 ml. of this solution with 20 ml. emulsin solution, so that the salicin concentration in the mixture is only 3.178 %. The reaction constant divided by the emulsin concentration, *i.e.* sal.f. , is in this way found too high, as the reaction constant increases when the salicin concentration decreases.

In a later paper [1932], dealing with the comparison of the rate of hydrolysis of different glycosides, Helferich seems to have used the salicin concentration indicated by Weidenhagen. He does not mention, however, that this concentration is different from that used in the standardisation of the emulsin preparation.

IV. In experiments dealing with enzymic hydrolysis of glucosides toluene is usually added to the solution in order to prevent the growth of micro-organisms during the experiment. We have examined whether addition of toluene causes any change of the rate of hydrolysis of glucosides and have found the reaction constants to be some 25–35 % higher in experiments with addition of toluene than in experiments without toluene. This means that it is not permissible to compare experiments in which toluene has been added with experiments without toluene, if the extent to which the rate of hydrolysis is altered by the addition of toluene is not stated.

EXPERIMENTAL.

I. Variation of rotation of solutions of glucosides and glucose with p_H .

In Table I are given the values of the rotation of different solutions at p_H 4.4 and at p_H 10.5–10.6. p_H 4.4 is the standard p_H in the hydrolysis experiments. In order to determine the progress of the hydrolysis samples of 5 ml. are

Table I. Values of α at different p_H values (2 dm. tubes).

	Conc.	p_H 4.4	p_H 10.5–10.6
Salicin	5/6 3.972 %	–4.160°	–4.280°
β -Methylglucoside	5/6 0.04 <i>M</i>	–0.440	–0.455
β -Methylglucoside	5/6 0.10 <i>M</i>	–1.100	–1.140
β -Ethylglucoside	5/6 0.04 <i>M</i>	–0.535	–0.555
β -Ethylglucoside	5/6 0.10 <i>M</i>	–1.335	–1.385
Glucose	5/6 0.05 <i>M</i>	0.795	0.775
Glucose	5/6 0.10 <i>M</i>	1.585	1.530
Glucose	5/6 0.20 <i>M</i>	3.175	3.045

withdrawn and mixed with 1 ml. of 20 % potassium carbonate, the p_H being in this way brought to 10.5–10.6. In order to obtain values at p_H 4.4 comparable with the values at p_H 10.5–10.6 5 ml. are mixed with 1 ml. of water. The difference is in all cases 3–4 %.

II. Variation of rotation of glucose solutions with time.

Solutions of glucose, 0.05 *M*, 0.10 *M* and 0.20 *M* were prepared and were kept 24 hours to allow the mutarotation to take place. Samples of 5 ml. were then mixed with 1 ml. of 20 % potassium carbonate and kept different times before determination of the rotation. Table II gives the results.

Table II. *Variation with time of rotation of glucose solutions at p_H 10.5–10.6.*

Time after addition of K_2CO_3 (hours)	α	$\Delta\alpha$	$\Delta\alpha/\text{hour}$	$\Delta\alpha/\text{hour, 0.1 } M$
Glucose 0.05 M				
0	0.775°	—	—	—
3.25	0.770	0.005°	0.0015°	0.0030°
28.5	0.720	0.055	0.0019	0.0038
51.75	0.665	0.110	0.0021	0.0042
Glucose 0.10 M				
0	1.530	—	—	—
3.5	1.520	0.010	0.0029	0.0029
28.75	1.450	0.080	0.0028	0.0028
52.0	1.360	0.170	0.0033	0.0033
Glucose 0.20 M				
0	3.045	—	—	—
3.75	3.020	0.025	0.0067	0.0034
29.0	2.880	0.165	0.0057	0.0028
52.25	2.655	0.390	0.0075	0.0038
Mean value				0.0033°

Table III shows that the presence of emulsin causes no change in the rotation of glucose solutions up to 0.20 M and 54 hours.

Table III. *Rotation of glucose solutions containing emulsin.*

Time after addition of emulsin hours	0.05 M α	0.10 M α	0.20 M α
0	0.535°	1.290°	2.790°
30	0.535	1.290	2.795
54	0.535	1.290	2.795

The rotation of the emulsin control was -0.240° during the whole of the experiment.

III. *Standardisation of the emulsin preparation.*

As indicated above the rotation of the 3.972 % salicin solution, when 5 ml. are mixed with 1 ml. 20 % potassium carbonate, is -4.280° , and when they are mixed with 1 ml. water -4.160° , which is the value calculated from the $[\alpha]_D$ of salicin, -62.8° . The rotation of the solution after complete hydrolysis is at p_H 10.5–10.6 $+2.150^\circ$, at p_H 4.4 $+2.190^\circ$. The total change of rotation is thus at p_H 10.5–10.6 6.430° , at p_H 4.4 6.350° .

If no regard is paid to the influence of p_H on the rotation, so that the value at time 0 is calculated to be -4.160° , it may be seen that the reaction constant found is too low. This is to some degree counterbalanced since the value for the total change of rotation is also calculated too low. In Fig. 1 we give the results of two experiments with different emulsin concentrations, calculated in both ways.

Table IV gives the times required for 50 % hydrolysis, the corresponding reaction constants (unimolecular, calculated with logarithms to base 10 and the minute as unit of time), sal.f., which is $k_{50\%}/e$ (e = g. emulsin in 50 ml. reaction mixture) and β -glucosidase values = sal.f./log 2.

The emulsin preparation is the same as that used in the previous paper [Veibel, 1934]. Its sal.f. was there determined by the method of Helferich [1931] and was found to 0.045. We now find that sal.f., if corrected for the influence of p_H , = 0.043; if not corrected, = 0.041.

Table IV. *Corrected and uncorrected values for salicin.*

	50% hydrolysis		$k_{50\%}$		sal.f.		β -glucosidase values	
	Corr.	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.
$e=0.0691$	103	108 min.	29.2	$27.9 \cdot 10^{-4}$	0.042	0.040	0.140	0.134
$e=0.1382$	50	53 min.	60.2	$56.8 \cdot 10^{-4}$	0.044	0.041	0.145	0.136

Fig. 1.

IV. *The influence of addition of toluene.*

From a stock solution of methylglucoside in acetate buffer ($p_H=4.4$ and $0.1M$) and a stock solution of emulsin in water two measuring flasks of 50 ml. were filled at 30° . Each flask contained 0.2213 g. emulsin and was $0.04M$ with regard to methylglucoside. To one of the flasks 1 ml. of toluene was added. Samples were withdrawn as usual. Tables V and VI give the results.

In the experiment without toluene k/e (sal.f.) = $2.7 \cdot 10^{-2}$, whereas the corresponding value in the experiment with toluene is $3.6 \cdot 10^{-2}$. For ethyl- β -glucoside a corresponding difference was found: k/e (sal.f.) = $5.3 \cdot 10^{-2}$ without toluene, $7.6 \cdot 10^{-2}$ with toluene. We have not sought for an explanation of this effect of the addition of toluene. Our emulsin solutions are made by mixing 1 g. of emulsin with 100 ml. of water. After some time the solution is filtered. Some

Table V. *β -Methylglucoside. Hydrolysis without addition of toluene.*

Time min.	Samples kept at p_H 10.5 hours	α	$\alpha_{corr.}$	x	$c-x$	$k \cdot 10^4$
0	4.0	-0.810°	-0.810°	—	1.075°	—
120	3.5	-0.730	-0.730	0.080°	0.995	2.80
240	20.5	-0.680	-0.675	0.135	0.940	2.43
360	19.0	-0.615	-0.610	0.200	0.875	2.48
640	15.0	-0.460	-0.455	0.355	0.720	2.72
1440	5.5	-0.220	-0.215	0.595	0.480	2.43
Mean value						2.57

Table VI. *β -Methylglucoside. Hydrolysis with addition of toluene.*

Time min.	Samples kept at p_H 10.5 hours	α	$\alpha_{corr.}$	x	$c - x$	$k \cdot 10^4$
0	4.0	-0.810°	-0.810°	—	1.075°	—
120	3.75	-0.715	-0.715	0.095°	0.980	3.35
240	20.75	-0.635	-0.630	0.180	0.895	3.32
360	19.25	-0.545	-0.540	0.270	0.805	3.49
640	15.25	-0.380	-0.370	0.440	0.635	3.58
1440	5.75	-0.095	-0.090	0.720	0.355	3.34
2940	3.25	+0.095	+0.100	0.910	0.165	(2.77)
4320	4.25	+0.215	+0.220	1.030	0.045	—
5760	4.0	+0.235	+0.240	(1.050)	0.025	(—)
Mean value						3.42

The rotation of the emulsin control was in all cases but the last -0.355° . After 5760 min. the rotation was -0.340° . That is to say that the equilibrium point has been reached in about 4320 min.

0.7–0.8 g. is dissolved in this way. The filtrate is quite clear. When the emulsin solution is added to the buffer solution a slight flocculation usually takes place. It may be that some active substance is precipitated in this way and that it is then brought into solution by the addition of toluene. In a recent paper Willstätter and Rohdewald [1934] have called attention to the fact that toluene + water may be a different dispersion-medium from water alone. They state that the activity of lipase solutions decreases when toluene is added. They do not mention, however, if they have ever found an activation of ferments by toluene such as we have found here for emulsin.

SUMMARY.

Attention is called to some sources of error in the polarimetric examination of enzymic hydrolysis of glucosides, namely:

1. Solutions of glucose and glucosides have not the same rotation at p_H 10.5–10.6 as at p_H 4.4. The difference is some 3–4 %.
2. Glucose solutions kept at p_H 10.5–10.6 are not stable. Their rotation decreases with time; for 0.1 *M* solutions 0.0033° per hour.
3. The salicin concentration used by Helferich [1931] for standardising emulsin preparations is not the same as that indicated by Weidenhagen [1929].
4. When toluene is added to solutions of β -glucosides, used in the examination of the rate of enzymic hydrolysis, the reaction constants are found to be some 25–35 % higher than when no toluene is added.

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