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INVESTIGATIONS ON THE GLYCOSIDASES OF MILK-SUGAR YEAST EMULSIN

BY

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NEUBERG and HOFMANN (1932) and HOFMANN (1932) have shown that milk-sugar yeast emulsin, in contrast to almond emulsin, provokes a quicker hydrolysis of β-galactosides than of β-glucosides.

Using an enzyme preparation from milk-sugar yeast (*saccharomyces fragilis Jørgensen*, one of the strains investigated by HOFMANN) we have to our astonishment found that β-galactosides were not hydrolysed at all, while on the contrary β-glucosides were hydrolysed, even if slowly, at a measurable velocity. Starting from this fact we have made a more thorough investigation of milk-sugar yeast emulsin in order to try to elucidate some problems concerning enzymic specificity. We are here presenting some results of this investigation.

The problem of the identity or non-identity of β-glucosidase and β-galactosidase has not yet been solved. For emulsin from almonds we know, principally from the close study made by HELFERICH and his co-workers (1938), that the two enzymes are, if not identical, at least so nearly related that all purifying or damaging manipulations which have been carried out have not been able to bring about a modification of the ratio between the β-glucosidatic and the β-galactosidatic effect. From this HELFERICH (1938) has drawn the conclusion that at any rate in almond emulsin the two enzymes are identical.

When emulsin from other sources than almond is used it is, however, no longer so. Through the investigations of NEUBERG and HOFMANN (see HOFMANN 1934, 1935) it was proved that the ratio between the β-glucosidatic and the β-galactosidatic effect of emulsin from milk-sugar yeast, from hip, from different *aspergillus*-species as well as of emulsin of animal origin (liver, kidney) is different from that found for almond emulsin. As an example may be mentioned that the β-glucosidatic effect, which

is the preponderant effect in almond emulsin, can barely be demonstrated in hip emulsin.

Later on HILL (1934) and HELFERICH and VORSATZ (1935) found that there is a still greater difference in alfalfa seed emulsin and in coffee emulsin, so that no β-glucosidatic effect at all is to be found. From this fact HELFERICH (1938, s. 96) draws the conclusion that there must exist two different β-galactosidases. One of them, which is present in almond emulsin, is identical with β-glucosidase, the other, which has been found e. g. in alfalfa seed emulsin, is different from β-glucosidase.

From the investigations hitherto mentioned no doubt has arisen as to the uniformity of the β-glucosidase from different sources.

ANTONIANI (1935), on the other hand, is of opinion that an enzyme preparation from the ungerminated seeds of *sorghum saccharatum* has only β-glucosidatic properties and no β-galactosidatic effect at all. This creates a doubt as to the uniformity of the β-glucosidase, which, according to HELFERICH, should be identical with one of the β-galactosidases, whereas ANTONIANI's β-glucosidase is obviously different from either of the two β-galactosidases.

HELFERICH is of opinion that the two β-galactosidases may be distinguished from each other by their behaviour toward o-cresol-β-d-galactoside and phenol-β-d-galactoside as expressed in the "Wertigkeit" of the two galactosides. (Wertigkeit $= k_{50\%}/e$ (log. 2)). For the β-galactosidase of the type found in almond emulsin the Wertigkeit of o-cresol-β-d-galactoside is considerably (some 8 times) greater than that of phenol-β-d-galactoside, just as it has been found for the two corresponding glucosides. For the β-galactosidase of the type found in alfalfa seed emulsin, on the contrary, the two Wertigkeits are nearly alike, that of o-cresol-β-d-galactoside being somewhat inferior to that of phenol-β-d-galactoside.

The significance of this is, however, reduced by the fact that MIWA and co-workers (1937) found similar differences for β-glucosidase from different sources. They use more dilute solutions than HELFERICH, hoping that possible differences in the affinities between the substrates and the enzyme will thereby better manifest themselves than in the more concentrated

solutions used by HELFERICH. From their investigations we learn that for emulsin from different *prunus*-species the ratio between the Wertigkeits of o-cresol-β-d-glucoside and phenol-β-d-glucoside is 25:1, for emulsin from *glucine hispada* it is 0.97:1, from *cucumbita moschata* 0.46:1 and for emulsin from different *aspergillus*-species the ratio drops to 0.025:1. These differences are greater than those stated for the two presumed β-galactosidases, and the findings of MIWA and co-workers render the assumption of only one β-glucosidase, common to all enzyme preparations with β-glucosidatic faculties, very unlikely.

The experiments described below prove that with emulsin from *saccharomyces fragilis Jørgensen* the finding of ANTONIANI that there exists a β-glucosidase which does not at the same time catalyse the hydrolysis of β-galactosides, as well as the findings of MIWA and co-workers, that for some β-glucosidase-preparations the Wertigkeit of phenol-β-d-glucoside is greater than that of o-cresol-β-d-glucoside, are corroborated.

One of the present authors (VEIBEL and ERIKSEN, 1937) has previously shown that velocity constants, k_{obs}, or Wertigkeits, as calculated directly from the hydrolysis experiments, are not suitable as a means of comparing the facility of hydrolysis of different substrates, since differences of k_{obs} may be caused either by differences of K_m, the dissociation constant of the enzyme-substrate compound, or by differences of k_3, the real unimolecular velocity constant for the fission of the enzyme-substrate compound into the products of hydrolysis, glucose, aglucone and enzyme. This will appear from the expression used for the calculation of k_3 (VEIBEL and LILLELUND 1938, 1940 1, 2):

$$k_3 = \frac{k_{obs}}{e\,(sal.f.)}\,(K_m + c + (K_m/K_{m_1} + K_m/K_{m_2} - 1)\,x)$$

or, in the initial stage of the hydrolysis,

$$k_3 = \frac{k'_{obs}}{e\,(sal.f.)}\,(K_m + c)$$

k_3 and K_m having the signification mentioned above, k_{obs} and k'_{obs} being the directly calculated unimolecular velocity con-

stants of the hydrolysis, k_{obs} calculated from point to point, k'_{obs} from time zero to time t, K_{m_1} and K_{m_2} being the dissociation constants of the compounds formed by the enzyme with the products of hydrolysis, *e* conventionally being g enzyme in 50 ml reaction mixture and sal.f. $= k_{obs\ 50\,\%}/e$ for a 0.139 n salicin solution in acetate buffer at p_H 4.4. Sal. f. may be calculated also from the velocity constant of hydrolysis of e. g. o-cresol-β-d-glucoside, see VEIBEL and LILLELUND (1938).

Also when enzyme preparations of different origin are compared it seems likely that a comparison of the k_3-values of a deliberately chosen substrate is to be preferred to the comparison of the k_{obs}-values, as it may be presumed that K_m is determined not only by the prostetic group of the enzyme, but that it is also influenced by the nature of the colloidal carrier, and even if it is presumed that the prostetic group of the β-glucosidase is identical in all enzyme preparations with a β-glucosidatic effect, it will surely not be true that the colloidal carriers are also identical. The differences found by MIWA and co-workers may possibly be explained as differences in K_m caused by different colloidal carriers.

In the above-mentioned paper (VEIBEL and LILLELUND, 1940, 2) K_m, K_{m_1}, K_{m_2} and k_3 were determined for o-cresol-β-d-glucoside, using almond emulsin as enzyme. The determinations were made in different buffer solutions and at different p_H. In order to find an answer to the question as to the identity or non-identity of almond emulsin and milk-sugar yeast emulsin we have, with milk-sugar yeast emulsin as a catalyst, tried to determine K_m, K_{m_1}, K_{m_2} and k_3 for o-cresol-β-d-glucoside, this time confining ourselves to the use of phosphate-citrate buffer (MC. ILVAINE, 1921). The result of the investigation may be summarized as follows:

The p_H-optimum which, using almond emulsin, was found at p_H 4.4 (phosphate-citrate buffer) is, when milk-sugar yeast emulsin is used, at p_H 5.7—5.9.

The affinity constant of the o-cresol-β-d-glucoside K_m was determined at 0.050, using almond emulsin at p_H 6.0. When milk-sugar yeast emulsin is used the affinity between enzyme and substrate is appreciably greater, all of the enzyme being

combined with the substrate even in 0.01 m solutions of the substrate.

These differences may possibly be explained as being due to differences in the nature of the colloidal carrier. If the view put forward above is correct, the k_3-value is independent of the colloidal carrier, and if the two β-glucosidases are identical, we should find the same k_3-value in both cases. We have previously found (VEIBEL and LILLELUND, 1940, 2) $k_3 = 42.5 \cdot 10^{-2}$ (phosphate-citrate buffer, p_H 6.0, almond emulsin). Now we find, under identical experimental conditions, values for $\frac{k'_{obs}}{e}(K_m + c) = k_3$ (sal.f.) between $0.012 \cdot 10^{-2}$ and $0.0012 \cdot 10^{-2}$. In order to obtain a k_3-value of $42.5 \cdot 10^{-2}$ we must presume sal.f.-values between 0.0003 and 0.00003 for the milk-sugar yeast emulsin preparations examined. Calculation of sal.f. from the k_{obs}-values of 0.04 m solutions of o-cresol-β-d-glucoside have, however, given values between 0.0007 and 0.00007, and the k_3-value, when milk-sugar yeast is used, is consequently only about half of the value found when using almond emulsin, thus indicating a difference between the two β-glucosidases. Other peculiarities are, however, still more difficult to reconcile with the assumption of only one β-glucosidase, identical with one of the two β-galactosidases.

In almond emulsin the β-galactosidatic effect at the p_H-optimum of the β-glucosidase is usually about 1/6—1/13 of the β-glucosidatic effect. With the preparation of almond emulsin used by us we have found $k_{obs\ glucoside}/k_{obs\ galactoside}$ for 0.04 m solutions of o-cresol-β-d-glycosides (phosphate-citrate buffer) 7.1 at p_H 4.4, 6.3 at p_H 6.0. (VEIBEL and co-workers, not yet published). If the β-glycosidase in milk-sugar yeast emulsin were identical with the β-glucosidase of almond emulsin we should, therefore, expect a k_{obs}-value for the galactoside about 1/6—1/7 of the k_{obs}-value of the glucoside, presuming that the affinity constants of o-cresol-β-d-glucoside and of o-cresol-β-d-galactoside are not very different, for milk-sugar yeast emulsin as well as for almond emulsin. As we tried, however, to determine the velocity constant of the hydrolysis of o-cresol-β-d-galactoside with milk-sugar yeast emulsin as a catalyst, we

found that no measurable hydrolysis took place, even though the change in rotation was expected to be some 0.06°. This result was obtained with all milk-sugar yeast emulsin preparations examined, and it is, in our opinion, very difficult to explain, if the β-glucosidase present is identical with one of the β-galactosidases presumed by HELFERICH.

HOFMANN (1932), on the other hand, states that milk-sugar yeast emulsin hydrolyses β-galactosides more rapidly than β-glucosides. The only β-galactoside of which an investigation has been reported is, however, milk-sugar. We have, therefore, tried our preparations of milk-sugar yeast emulsin against milk-sugar. The result was the same as that obtained in trying it against o-cresol-β-d-galactoside, no hydrolysis whatever could be detected.

From these findings it results that, even if in accordance with HELFERICH we assume the β-glucosidase of almond emulsin to be identical with the β-galactosidase of the same enzyme preparation, we must admit that at least two β-glucosidases exist, since the β-glucosidase of the milk-sugar yeast emulsin has no β-galactosidatic properties.

Possibly, however, two different types of milk-sugar yeast emulsin may be isolated, since the preparation of HOFMANN is in several respects different from our preparations. A closer study may perhaps show whether it will be possible to indicate procedures by which milk-sugar yeast emulsin with or without β-galactosidatic properties can be prepared at will.

NEUBERG and HOFMANN (1932) mention that their preparation of milk-sugar yeast emulsin is capable of hydrolysing raffinose, 1-α-galactosido < 1,5 > 6-α-glucosidose < 1,5 > 2-fructose. Our preparations, too, hydrolyse raffinose and the hydrolysis presumably leads to the formation of melibiose and fructose, this assumption according better with the changes in rotation observed than the assumption of the formation of galactose and sucrose or the complete splitting of the trisaccharide into 3 molecules of monosaccharide. A more thorough study of the action of milk-sugar yeast emulsin upon raffinose will be published elsewhere.

As mentioned above, HELFERICH is of opinion that a characteristic of the β-glucosidase of almond emulsin is that o-cresol-

β-d-glucoside is hydrolysed at a greater velocity than phenol-β-d-glucoside. In this respect too there is a difference between almond emulsin and milk-sugar yeast emulsin. We find that the k_{obs} of a 0.04 m solution of phenol-β-d-glucoside is about twice as great as the k_{obs} of a 0.04 m solution of o-cresol-β-d-glucoside. Much the same must be the case with the k_3-values of the two glucosides, if the K_m-values of o-cresol-β-d-glucoside and phenol-β-d-glucoside against milk-sugar yeast emulsin are not very different. (For a peculiarity concerning phenol-β-d-glucoside see the experimental part).

Experimental part.

Preparation of substrates. 1. Phenol-β-d-glucoside. Phenol-β-d-glucoside-tetracetate was prepared according to the method of HELFERICH and SCHMITZ-HILLEBRECHT (1933), which is quoted also in a paper by ZEMPLÉN (1938). Yield 42 % as stated by HELFERICH and SCHMITZ-HILLEBRECHT. F. 125.5°; $[\alpha]_D^{20} = -23.0°$ ($CHCl_3$, c = 0.999, $\alpha^{20} = -0.460°$, all measurements in 2 dm tubes) in fairly good accordance with the findings of HELFERICH and SCHMITZ-HILLEBRECHT, F. = 124—125°, $[\alpha]_D^{20°} = -22.0°$ ($CHCl_3$). In the paper of ZEMPLÉN $[\alpha]_D$ is erroneously given at −71°, the value found by HELFERICH and SCHMITZ-HILLEBRECHT for the free glucoside, not for the tetracetate.

The acetyl-groups were removed by means of sodium methylate in methyl alcohol (ZEMPLÉN and PASCU (1929)). The glucoside isolated showed F. 173—174° (capillary tube), $[\alpha]_D^{20°} = -64.1°$ (water, c = 0.963, $\alpha^{20} = -1.235°$). HELFERICH and SCHMITZ-HILLEBRECHT indicate F. = 144—145°, $[\alpha]_D^{20°} = -64.8°$. As we tried to determine the melting point on bloc maquenne we observed that at 145° the substance melted and after a moment solidified again and then melted at 173—174°. 145° is, therefore, the melting point of the glucoside with crystal water, 173—174° the melting point of the anhydrous glucoside. The water content of the glucoside was determined by drying it in vacuum over P_2O_5 at 78°. 1.2007 g lost 0.1483 g = 12.35 %. Kept at room temperature in an atmosphere saturated with water vapour 0.1465 g was regained in 4 days. No further water uptake could

be registered during the following 7 days. For $C_6H_{11}O_5OC_6H_5$, $2H_2O$ is calculated a water content of 12.33 %.

$[\alpha]_D^{20°}$ of the anhydrous glucoside is $-72.6°$ (water, c = 0.992, $\alpha^{20} = -1.440°$). HELFERICH and SCHMITZ-HILLEBRECHT indicate $[\alpha]_D^{20°} = -71.0°$ (water).

o-Cresol-β-d-glucoside, o-cresol-β-d-galactoside and phenol-β-d-glucoside were prepared analogically and showed the constants indicated in the literature.

Salicin, milk-sugar and raffinose were commercial preparations (MERCK).

Preparation and estimation of the enzymic force of emulsin from *saccharomyces fragilis Jørgensen.*

The emulsin preparations I—III described below are derived from a culture of *saccharomyces fragilis Jørgensen* which had kindly been placed at the disposal of one of the authors some years ago by Professor NEUBERG, Berlin-Dahlem, and which had since then been preserved at Alfred Jørgensens gæringsfysiologiske Laboratorium, Copenhagen. Preparations IV and V are derived from a culture of *saccharomyces fragilis Jørgensen, strain 14*, and preparations VI and VII from a Dutch strain of the same *fungus*, both belonging to the said laboratory and kindly placed at our disposal.

All cultures were grown on beer wort containing $^1/_2$ % milk-sugar. After some 3 weeks the cultures were filtered by suction. The cultivation and the filtration were carried out at the laboratory mentioned above. We are glad to take the opportunity of thanking the director of the laboratory, Mr. ALBERT HANSEN, and the head of the fermentation department, Mr. F. de FONTENAY, most cordially for all help in preserving and cultivating the yeast preparations.

The procedure adopted in isolating an emulsin preparation from the yeast cells was that described by NEUBERG and HOFMANN (1932) and by HOFMANN (1932) for the preparations I—IV. For the preparations V—VII we have examined the activity of the centrifuged and filtered plasmolysate and then from this solution prepared a dry preparation, using the method indicated by HELFERICH (1932) for the purification of almond emulsin, precipitating the enzyme by tannin.

The activities of the preparations examined are indicated in Table I (substrate o-cresol-β-d-glucoside).

Table I.

Emulsin prp. nr.	I	II	III	IV	V	VI	VII	V	VI	VII
					Plasmolysate			Dry preparation		
$10^2 \cdot k/e$	0.34	0	0.04	0	0.02	—	0.2	0.03	0	0.12

These figures were obtained by carrying out hydrolysis experiments at 30° in phosphate-citrate buffer ($p_H = 6.0$). It was found later on that the emulsin powder loses in activity, rapidly at first, more slowly later on. These initial determinations carried out immediately after the preparation of the emulsin have, therefore, only a limited value.

0.1—0.3 g emulsin was dissolved in 25—45 ml of water. 2 ml were added, at 30°, to a 25 ml measuring flask containing a solution of o-cresol-β-d-glucoside in phosphate-citrate buffer (p_H 6.0), previously placed in a 30°-thermostat. Water was added to the mark, the glucoside concentration thus being regulated to 0.040 m.

At convenient intervals 5 ml samples were taken and added to 1 ml of a 20 % K_2CO_3-solution, all enzymic action being thus suppressed. The progress of the hydrolysis was followed polarimetrically, the measurements not being carried out till the mutarotation had been given time to come to an end (2—3 hours) and attention being paid to the sources of error pointed out by VEIBEL and ERIKSEN (1936). The actual content of emulsin in 50 ml of the solution in consideration (e in Table I) was determined by drying 10 ml of the emulsin solution at 105° and dividing the weight of dry material by 2.5.

Table II summarizes the results obtained.

It will be seen that within the first 24—33 hours the inactivation in solution is some 25—40 % but in the next 24 hours it is rather negligible. It will also be seen, on comparison of the values of k/e for the fresh emulsin-preparations and for the same preparations some days old, that these preparations, which lost a considerable part of their activity during the first few days, have conserved most of the remaining activity for a much longer period of time (some 30—50 days).

Table II.

	$10^2 \cdot k/e$				
	freshly prepared	some days old	30—50 days old	24—33 h. in solution at 30°	48 h. in solution at 30°
Prp. I	0.9	0.34	0.29	0.22	—
Prp. II	0	—	—	—	—
Prp. III	0.2	0.04	0.028	0.016	0.015
Prp. IV	0	—	—	—	—
Prp. V Plasmolysate	0.02	—	—	—	—
Prp. V Dry prp.	—	0.03	—	—	—
Prp. VI Dry prp.	0	—	—	—	—
Prp. VII Plasmolysate	0.2	—	—	—	—
Prp. VII Dry prp.	—	0.12	—	—	—

Determination of the p_H-optimum for milk-sugar yeast emulsin.

The velocity constants of hydrolysis of 0.04 m solutions of o-cresol-β-d-glucoside in phosphate-citrate buffer at p_H 5.0, 5.4, 5.8, 6.2, 6.6 and 7.0 (6.0 ml buffer solution in 25 ml reaction mixture) were determined. To each measuring flask were added 2 ml of an emulsin solution (preparation I). e was determined to be 0.0217, The hydrolysis was followed during some 6000 minutes, the degree of hydrolysis being then 45—65 %.

Table III and figure 1 show that the p_H-optimum is at p_H 5.7—5.9.

Table III.

p_H	5.0	5.4	5.8	6.2	6.6	7.0
$10^2 \cdot k/e$	0.29	0.31	0.32	0.29	0.26	0.20

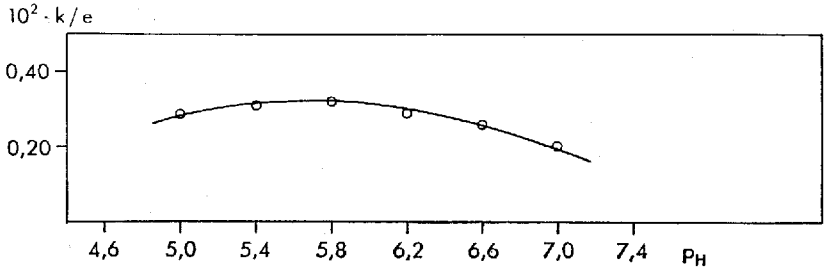

Figure 1.

This agrees fairly well with the statement of NEUBERG and HOFMANN (1932) that the optimal effect is obtained at p_H 6.3—6.0,

but is rather different from the p_H-optimum of almond emulsin, for which VEIBEL and LILLELUND (1940, 2) found p_H 4.4 in phosphate-citrate buffer.

Determination of K_m, the dissociation constant of the enzyme-substrate-complex. o-Cresol-β-d-glucoside, phosphate-citrate buffer, p_H 6.0.

The affinity between milk-sugar yeast emulsin and o-cresol-β-d-glucoside is so great that a determination by the method usually adopted by VEIBEL and co-workers (VEIBEL and LILLELUND 1940, I) could not be carried through, this method not being sufficiently sensitive. From the experiments registered in Table IV it will be seen, however, that the enzyme is practically completely bound to the substrate, even at a substrate concentration of 0.01 m. Table IV indicates, for a series of glucoside solutions of different concentrations containing emulsin in identical concentration, the change of rotation after 4 different intervals of time. The identity of these changes of rotation, within the limits of error, indicates that for all 6 concentrations the concentration of the enzyme-substrate compound, which is the velocity-determining substance, is identical.

Table IV.

Changes in rotation x of solutions of o-cresol-β-d-glucoside.

Time min.	Concentration of glucoside solution 0.01	0.02	0.04	0.06	0.08	0.10
	Emulsin preparation I.					
150	0.060°	0.055°	0.055°	0.045°	0.045°	0.060°
300	0.105°	0.100°	0.095°	0.085°	0.085°	0.100°
480	0.155°	0.155°	0.125°	0.125°	0.140°	0.150°
1440	0.355°	0.380°	0.375°	0.365°	0.365°	0.390°
	Emulsin preparation V. Plasmolysate.					
1440	0.18°	0.19°	0.12°	0.14°	0.11°	
2880	0.31°	0.33°	0.31°	0.28°	0.28°	
4320	0.38°	—	0.45°	0.43°	0.42°	
5760	*0.45°	0.58°	0.58°	0.57°	0.58°	

* For 0.01 m solution the maximum change in rotation by complete hydrolysis is 0.49°.

From this it may be concluded that 0.001 is a maximum value for K_m, as the fraction of the emulsin present in the solution, which has combined with the substrate, is $c/K_m + c$, c being the concentration of the substrate. At the concentration 0.01 m a K_m-value of 0.001 indicates that 90 % of the enzyme have combined with the substrate, and a difference of combination from 90 % to 99 % is to be detected, even here where the exactitude of the readings is diminished owing to the darkness of the solutions of milk-sugar yeast emulsin.

For o-cresol-β-d-glucoside and almond emulsin VEIBEL and LILLELUND (1940, 2) found the K_m-value 0.050 in phosphate-citrate buffer at p_H 6.0. The affinity between the glucoside and milk-sugar yeast emulsin is, therefore, at least 50 times as great as that between the glucoside and almond emulsin, and a difference between the two enzymes is seen to exist in this respect as well as in regard to the p_H-optimum (see above).

Determination of K_{m_1}, the dissociation constant of the enzyme-glucose-compound, in phosphate-citrate buffer at p_H 6.0.

Here the usual technique was used, i. e. determination of the velocity constants of the hydrolysis of 0.04 m solutions of o-cresol-β-d-glucoside which at the same time are 0.00, 0.02, 0.04 or 0.08 m respectively with regard to glucose. The concentration of the enzyme (preparation I) was the same in all 4 solutions. K_{m_1} is then usually determined from the expression

$$K_{m_1} = \frac{K_m \cdot c_{glucose}}{(K_m + c)\ (k/k_H - 1)}$$

(VEIBEL 1937), k and k_H being the velocity constants for the hydrolysis of solutions without and with glucose respectively. Here no definite value of K_m is obtainable, but at all events K_m is negligible as compared with c and the above expression may be written

$$K_{m_1}' = \frac{K_m \cdot c_{glucose}}{c \cdot (k/k_H - 1)}.$$

Table V gives the result, which is $K_{m_1} = 7\ K_m$. For almond emulsin VEIBEL and LILLELUND (1940, 2) found $K_m = 0.050$,

$K_{m_1} = 0.35$, i. e. $K_{m_1} = 7\,K_m$. In this respect there is, consequently, no difference between the two enzymes.

Table V.

$c_{glucose}$	0.00	0.02	0.04	0.08
$10^4 \cdot k'$	0·51	0.48	0.45	0.38
$k/k_H - 1$	—	0.07	0.13	0.34
K_{m_1}	—	$6\,K_m$	$8\,K_m$	$6\,K_m$
	Average value of K_{m_1} ...			$7\,K_m$

Attempts at determination of K_{m_2}, the dissociation constant of the enzyme-o-cresol-compound, in phosphate-citrate buffer at p_H 6.0.

While the determination of K_{m_1} presented no difficulties whatever, the determination of K_{m_2} met with some anomalies which we did not encounter in the determination of K_{m_2} in the case of almond emulsin. The technique was exactly the same as that used in determining K_{m_1}, only with o-cresol instead of glucose, The results are indicated in Table VI (preparation I) and Table VII (preparation III).

Table VI.

Determination of K_{m_2} for o-cresol. Emulsin preparation I.

t	c—x	$10^4 \cdot k'$	c—x	$10^4 \cdot k'$	c—x	$10^4 \cdot k'$	c—x	$10^4 \cdot k'$
	0.00 m		0.02 m		0.04 m		0.08 m	o-cresol
0	2.245	—	2.245	—	2 245	—	2.245	—
533	2.105	0.53	2.110	0.51	2.155	0.34	2.185	0.22
1440	1.940	0.44	1.870	0.55	1.915	0.48	2.145	0.14
2880	1.665	0.45	1.555	0.55	1.685	0.43	2.175	—
5810	1.250	0.44	1.130	0.52	1.335	0.39	2.175	—
	average...	0.46		0.53		0.41		—

Table VII.

Determination of K_{m_2} for o-cresol. Emulsin preparation III.

t	c—x	$10^4 \cdot k'$	c—x	$10^4 \cdot k'$	c—x	$10^4 \cdot k'$	c—x	$10^4 \cdot k'$	c—x	$10^4 \cdot k'$	c—x	$10^4 \cdot k$
min.	0.000 m		0.005 m		0.010 m		0.015 m		0.020 m		0.040 m	o-cresol
0	1.945	—	1.945	—	1.945	—	1.945	—	1.945	—	1.945	—
440	1.820	0.200	1.810	0.217	1.820	0.200	1.810	0.217	1.790	0.250	1.830	0.183
880	1.710	0.194	1.705	0.199	1,705	0.199	1.710	0.194	1.700	0.203	1.720	0.185
320	1.650	0.165	1.610	0.190	1.615	0.187	1.670	0.153	1.615	0.187	1.660	0.159
760	1.570	0.161	1.530	0.181	1.510	0.191	1.550	0.171	1.575	0.159	1.640	0.129
average...		0.18		0.20		0.19		0.18		0.20		0.16

Both experiments show concordantly that o-cresol in small concentrations not only has no inhibiting effect but that, on the contrary, it seems to accelerate the hydrolysis of the glucoside. At higher concentrations, however, the inhibition is very considerable and it seems to be non-competitive, as the hydrolysis comes completely to a stand-still (see Table VI, 0.08 m o-cresol).

It was clearly seen that o-cresol in not too low concentrations has a special effect, for the addition of the enzyme-solution to the o-cresol-containing solution at once caused a turbidity, most distinct in the solution richest in o-cresol.

On account of these difficulties it has been impossible to determine any K_{m_2}-value.

Determination of k_3 for o-cresol-β-d-glucoside.

In previous papers (VEIBEL and collaborators 1937, 1938, 1940, 1) it was pointed out that the directly determined velocity constants of hydrolysis of glucosides are not very convenient as a basis of comparison. Instead it was recommended to use k_3, determined by the expression $k_3 = k_{obs}\,(K_m + c + (K_m/K_{m_1} + K_m/K_{m_2} - 1)\,x)/e$ (sal. f.), which is independent of the concentration of substrate used in the experiment, and in which the inhibiting effect of the products of hydrolysis are to a great extent accounted for.

As, however, it has not been possible to determine a K_{m_2}-value for o-cresol-β-d-glucoside and milk-sugar yeast emulsin, we must confine ourselves to the use of an approximative expression for the calculation of k_3: $k_3 = k'_{obs}\,(K_m + c)/e$ (sal. f.) or, if an inhibiting substance with dissociation constant of its enzyme-compound K_{m_h} is present in a concentration c_h, $k_3 = k'_{obs}\,(K_m + c + K_m/K_{m_h} \cdot c_h)/e$ (sal. f.). These approximative formulae are valid for the initial stage of the hydrolysis only, where the effect of the products of hydrolysis is not considerable.

In Table VIII we have calculated the k_3-values for all hydrolysis experiments at p_H 6.0, using the values $K_m = 0.001$ and $K_{m_1} = 7\,K_m$ in the calculations.

The mean value of all k_3-determinations is $19.2 \cdot 10^{-2}$. The values found with substrate concentrations 0.01 m and 0.02 m

are considerably higher than the mean value. This may mean that the K_m-value 0.001 is too high, but, as was pointed out above (p. 14), this value is a maximum value.

For the hydrolysis of o-cresol-β-d-glucoside catalysed by almond emulsin we previously found (VEIBEL and LILLELUND 1940, 2) $k_3 = 42.5 \cdot 10^{-2}$ (phosphate-citrate buffer, $p_H = 6.0$). The velocity of hydrolysis of the enzyme-substrate compound is, therefore, in the case of milk-sugar yeast emulsin only about half the value in the case of almond emulsin, another feature showing the difference between the two glucosidases.

Table VIII.

k_3-values of o-cresol-β-d-glucoside.

$c_{glucoside}$	$c_{glucose}$	$k' \cdot 10^4$	e	sal. f.	$k_3 \cdot 10^2$	Enzyme prp. nr.
0.0390	0.00	3.73	0.1104	0.0007	19.3	I
0.0098	0.00	3.76	0.0245	0.0007	23.7	I
0.0197	0.00	1.61	0.0245	0.0007	19.6	I
0.0394	0.00	0.70	0.0245	0.0007	17.0	I
0.0590	0.00	0.43	0.0245	0.0007	15.3	I
0.0789	0.00	0.33	0.0245	0.0007	15.5	I
0.0983	0.00	0.30	0.0245	0.0007	17.7	I
0.0460	0.00	0.51	0.0192	0.0007	17.8	I
0.0460	0.02	0.48	0.0192	0.0007	17.9	I
0.0460	0.04	0.45	0.0192	0.0007	17.7	I
0.0460	0.08	0.38	0.0192	0.0007	16.4	I
0.0400	0.00	0.59	0.0256	0.0004	20.5	III fresh
0.0400	0.00	0.18	0.0600	0.00007	17.6	III old
0.0400	0.00	0.26	0.0930	0.00007	16.4	III old
0.0100	0.00	2.49	0.1296	0.00006	35.2	V Plasmolysate
0.0200	0.00	1.05	0.1296	0.00006	28.4	V Plasmolysate
0.0400	0.00	0.40	0.1296	0.00006	21.1	V Plasmolysate
0.0600	0.00	0.27	0.1296	0.00006	21.2	V Plasmolysate
0.0800	0.00	0.21	0.1296	0.00006	21.9	V Plasmolysate
0.1000	0.00	0.18	0.1296	0.00006	23.4	V Plasmolysate
0.0400	0.00	0.09	0.0262	0.00007	20.1	V dry prp.
0.0400	0.00	3.1	0.1572	0.00004	20.0	VII Plasmolysate
0.0400	0.00	0.70	0.0584	0.00003	16.4	VII dry prp.
				average value...	19.2	

Investigation of the hydrolysability of milk-sugar.

HOFMANN (1932) has investigated the hydrolysis of milk-sugar, catalysed by milk-sugar yeast emulsin. He finds that at

37° and p_H 6.7 (phosphate buffer) a 0.045 m solution of milk-sugar is hydrolysed to 63 % in 105 min., to 82 % in 284 min. As his e-value is 0.05, $10^2 \cdot k_{obs}/e$ is 9.20 or 5.20. At 30° and p_H 6.0 a value for k_{obs}/e of about $8 \cdot 10^{-2}$ is to be expected for a 0.040 m solution, at least for hydrolysis to some 50 %.

We have placed a 0.1000 m milk-sugar solution (phosphate-citrate buffer, p_H 6.0) containing milk-sugar yeast emulsin (preparation I) at 30°. e = 0.0217. Even after 2980 minutes no change in rotation of the solution could be observed. This means that no greater change than 0.02° has taken place. As the change in rotation for complete hydrolysis of a 0.1000 m milk-sugar solution is 7.04° this means that k_{obs}/e as a maximum value is $0.002 \cdot 10^{-2}$, i. e. 1/4000 of the value found by HOFMANN for a 0.045 m solution.

For a 0.0400 m solution of o-cresol-β-d-galactoside containing milk-sugar yeast emulsin (preparation I, e = 0.0112) no change in rotation was observed during 2665 min. Assuming again 0.02° as the limit of change in rotation not observed, a maximum value for k_{obs} is $0.04 \cdot 10^{-4}$, for k_{obs}/e (sal. f.) $24 \cdot 10^{-2}$, i. e. 1/20 of the value found for o-cresol-β-d-glucoside.

We have repeated this experiment with other preparations of milk-sugar yeast emulsin, both plasmolysates and dry preparations. In no case have we observed any change in rotation of the milk-sugar solution. Practically, therefore, these enzyme preparations are inactive towards milk-sugar.

Investigation of the hydrolysis of raffinose.

NEUBERG and HOFMANN (1932) state that raffinose is hydrolysed by milk-sugar yeast emulsin. Their experiments were carried out at 22° in acetate buffer (p_H 4.7), the solution being 0.057 m as regards raffinose. The result was a k_{obs}/e-value of about $1.5 \cdot 10^{-2}$, assuming a hydrolysis to melibiose and fructose, i. e. the action of an α-glucosidase or a fructosidase.

We have examined the hydrolysis of a 0.0400 m solution of raffinose in phosphate-citrate buffer at p_H 6.0. Table IX gives the result.

It will be seen that the result is practically the same as that obtained by NEUBERG and HOFMANN. With other prepara-

tions of milk-sugar yeast emulsin we have obtained quite analogous results, but a more detailed description of the results will be published elsewhere.

Table IX.

Hydrolysis of raffinose (to melibiose and fructose).

t min		x	c−x	$k \cdot 10^4$
0	+4.105	—	1.955	—
235	+3.410	0.695	1.260	8.1
1433	+2.080	2.025	—	—

$e = 0.048$. $k/e = 1.7 \cdot 10^{-2}$.

Investigations of the hydrolysis of phenol-β-d-glucoside.

Table X records the determination of the velocity of hydrolysis of phenol-β-d-glucoside catalysed by milk-sugar yeast emulsin.

Table X.

Hydrolysis of phenol-β-d-glucoside. $c = 0.0400$. $e = 0.0217$. $\alpha_{beg} = -1.275°$. $\alpha_{end} = +0.620°$. $\alpha_{Emulsin} = 0°$ Phosphate-citrate buffer. p_H 6.0. Enzyme preparation I.

t min	α	c−x	$10^4 \cdot k'$
0	−1.275	1.895	—
240	−1.100	1.720	1.76
521	−0.950	1.570	1.57
1440	−0.535	1.155	1.46
2980	−0.110	0.730	1.39
6157	+0.355	0.265	1.39
		average...	1.51

$10^2 \cdot k'/e = 0.70$.

By comparison with e. g. Table III (p. 12) it is seen that a 0.04 m solution of phenol-β-d-glucoside is hydrolysed somewhat more than twice as quickly as an equimolar solution of o-cresol-β-d-glucoside, whereas HELFERICH and co-workers (1935) state that with almond emulsin as a catalyst o-cresol-β-d-glucoside is hydrolysed 13 times as quickly as phenol-β-d-glucoside. In order to find out whether this difference is due to a difference

in affinity between the two glucosidases and the substrates (different K_m-values) or possibly to a difference in the velocity of fission of the enzyme-substrate compounds of the two glucosides (different k_3-values) we have attempted to determine the K_m-values of phenol-β-d-glucoside, with almond emulsin as well as with milk-sugar yeast emulsin as catalyst.

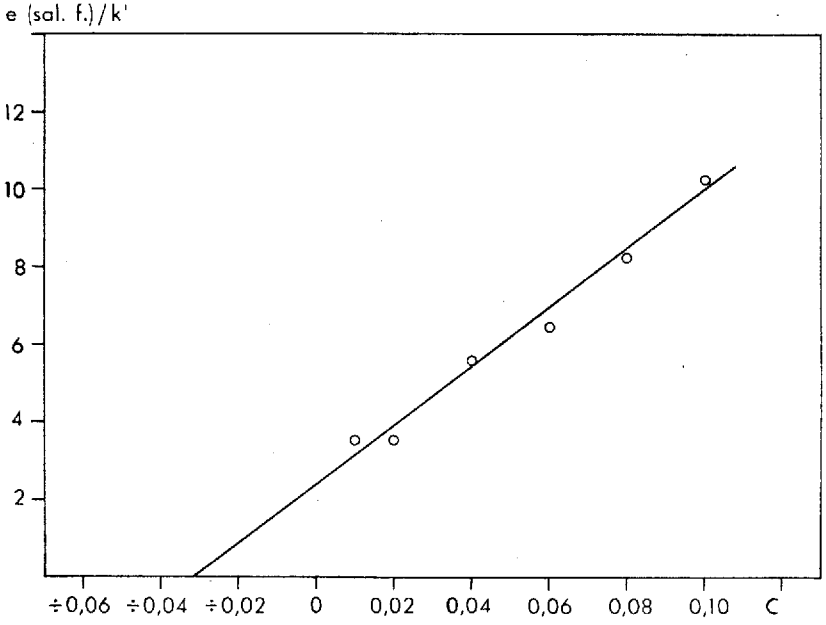

Fig. 2. Determination of K_m. Phenol-β-d-glucoside. p_H 6.0. Almond emulsin.

The determination of K_m, using almond emulsin, met with no difficulty whatever, the value found, 0.033 (for material see Table XI and fig. 2), shows that the affinity of phenol-β-d-glucoside to almond emulsin is somewhat greater than the affinity of o-cresol-β-d-glucoside to almond emulsin ($K_m = 0.050$).

A determination of K_m using milk-sugar yeast emulsin was, however, not possible as phenol-β-d-glucoside apparently has an inhibiting effect on the enzyme. The velocity of hydrolysis decreases rapidly with time, as will be seen from Table XII, which indicates the changes in rotation of 6 different glucoside solutions after 4 different intervals of time. For the more concentrated solutions the reaction comes to a complete standstill.

Table XI.

Determination of K_m. Phenol-β-d-glucoside. Almond emulsin. Phosphate-citrate buffer. p_H 6.0. 30°.

$c_{glucoside}$	$10^2 \cdot k'/e$ (sal. f.)	e (sal. f.)/k'	$10^2 \cdot k_s$
0.0100	28.2	3.55	1.21
0.0200	28.2	3.55	1.49
0.0400	18.1	5.54	1.31
0.0600	15.6	6.43	1.45
0.0800	12.2	8.22	1.38
0.1000	9.7	10.29	1.29
		average...	1.36

$K_m = 0.033$.

Table XII.

Changes in rotation x of solutions of phenol-β-d-glucoside. Milk-sugar yeast emulsin.

Time min	Concentration of glucoside solution 0.01	0.02	0.04	0.06	0.08	0.10
			Emulsin preparation I			
213	0.060°	0.085°	0.045°	0.050°	0.100°	0.015°
465	0.085°	0.120°	0.120°	0.100°	0.105°	0.075°
1440	0.320°	0.390°	0.295°	0.225°	0.205°	0.150°
2865	0.455°	0.540°	0.385°	0.300°	0.605° (?)	0.190°
			Emulsin preparation III			
1440	0.275°	0.280°	0.225°	0.190°	0.195°	0.205°
2880	0.375°	0.395°	0.300°	0.285°	0.280°	0.275°
4320	0.465°	0.505°	0.340°	0.285°	0.255°	0.250°
5760	0.455°	0.595°	0.385°	—	—	—

Discussion.

During this and previous investigations we have found the following values of k'_{obs}/e (sal. f.), valid for 0.0400 m glucoside solutions in phosphate-citrate buffer at p_H 6.0.

o-Cresolglucoside, almond emulsin $468 \cdot 10^{-2}$
o-Cresolglucoside, milk-sugar yeast emulsin $456 \cdot 10^{-2}$
Phenolglucoside, almond emulsin $18.1 \cdot 10^{-2}$
Phenolglucoside, milk-sugar yeast emulsin $1000 \cdot 10^{-2}$.

From these values we have calculated the k_s-values.

o-Cresolglucoside, almond emulsin $42.5 \cdot 10^{-2}$.
o-Cresolglucoside, milk-sugar yeast emulsin $19.2 \cdot 10^{-2}$.

Phenolglucoside, almond emulsin $1.36 \cdot 10^{-2}$
(Phenolglucoside, milk-sugar yeast emulsin $31 \cdot 10^{-2}$).

The value for k_3 for phenolglucoside, milk-sugar yeast emulsin is calculated, assuming a K_m-value not exceding 0.001, as was the case for o-cresolglucoside. It was shown above that a determination of the K_m-value is impossible.

The constants found for o-cresol-β-d-glucoside does not render impossible the identity of the glucosidases found in almonds and in milk-sugar. If, however, the constants found for phenol-β-d-glucoside also are compared, the differences are so great that it seems improbable that the two glucosidases can be identical. This is very clearly seen from Table XIII.

Table XIII.

Comparison of emulsin from almond and from milk-sugar yeast.

$\frac{k_{\text{obs cresol almond}}}{k_{\text{obs cresol milk-sugar yeast}}} = 1.0$	$\frac{k_{\text{obs phenol almond}}}{k_{\text{obs phenol milk-sugar yeast}}} = 0.02$
$\frac{k_{3\,\text{cresol a.}}}{k_{3\,\text{cresol m.}}} = 2.1$	$\frac{k_{3\,\text{phenol a.}}}{k_{3\,\text{phenol m.}}} = 0.04$
$\left[\frac{k_{\text{obs cresol}}}{k_{\text{obs phenol}}}\right]_{\text{almond}} = 26$	$\left[\frac{k_{\text{obs cresol}}}{k_{\text{obs phenol}}}\right]_{\text{milk-sugar yeast}} = 0.5$
$\left[\frac{k_{3\,\text{cresol}}}{k_{3\,\text{phenol}}}\right]_{\text{almond}} = 31$	$\left[\frac{k_{3\,\text{cresol}}}{k_{3\,\text{phenol}}}\right]_{\text{milk-sugar yeast}} = 0.6.$

Summary.

An investigation of a milk-sugar yeast emulsin preparation (from *saccharomyces fragilis Jørgensen*) has proved that by this enzyme

1) o-Cresol-β-d-glucoside is hydrolysed
2) o-Cresol-β-d-galactoside and milk-sugar are not hydrolysed
3) Raffinose is hydrolysed
4) Phenol-β-d-glucoside is hydrolysed twice as quickly as o-cresol-β-d-glucoside.

This is in partial disagreement with the findings of NEUBERG and HOFMANN who state that

1) Milk-sugar, salicin and raffinose are all hydrolysed
2) Milk-sugar is hydrolysed much more rapidly than β-d-glucosides.

Preliminary determinations of k_{obs}, K_m, K_{m_2} and k_3 for o-cresol-β-d-glucoside and k_{obs}, K_m and k_3 for phenol-β-d-glucoside have been given.

The results obtained are hardly reconcileable with the assumption of HELFERICH that there only exists one β-glucosidase, which is identical with one of the two galactosidases assumed by HELFERICH, as the β-glucosidase examined by us does not hydrolyse β-galactosides. Another difference from the β-glucosidase examined by HELFERICH is that it hydrolyses phenol-β-d-glucoside more rapidly than o-cresol-β-d-glucoside, whereas the β-glucosidase examined by HELFERICH hydrolyses o-cresol-β-d-glucoside some 13 times as quickly as phenol-β-d-glucoside.

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