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ACTION OF ALMOND EMULSIN ON THE PHENYL GLYCOSIDES OF SYNTHETIC SUGARS AND ON β-THIOPHENYL *d*-GLUCOSIDE

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ABSTRACT

The action of enzymes of almond emulsin on the phenyl glycosides of a number of sugars which are not naturally occurring has been investigated. The sugars used were synthetic heptoses (*d*-α-mannoheptose and *d*-α-glucoheptose) and *d*-talose. The results obtained indicate that almond emulsin does not hydrolyze all glycosides but instead only the glycosides of naturally occurring sugars or of sugars which may be considered to be derived from these sugars by a simple substitution outside of the pyranose ring. In the ordinary glucoside the two parts are connected through an oxygen atom. It has now been found that the substitution of this oxygen by sulfur atom reduces the hydrolytic action to such an extent that it cannot be readily measured. An explanation for this effect is given.

CONTENTS

I. "SUGAR SPECIFICITY" OF THE GLYCOSIDASES[1]

An explanation of the specificity to be expected of the glycoside hydrolyzing enzymes has been given previously.[2] The fundamental concept involved is that each of the alpha and beta isomers and *d* and *l* forms of the eight basic hexose types (glucosides, galactosides, mannosides, talosides, gulosides, idosides, altrosides, and allosides) requires a special enzyme. However, glycosides which have the same ring conformation and which may be considered as being derived from one of the basic hexose types by a substitution in the aglucon group or for the CH_2OH group will ordinarily be hydrolyzed by the same enzyme. Only those enzymes are to be expected in natural products which are capable of hydrolyzing glycosides of which the basic ring type is the same as that of a naturally occurring glycoside.

[1] This paper was read before the Division of Sugar Chemistry and Technology of the American Chemical Society at Detroit, Mich., September 1940.

[2] W. W. Pigman, J. Am. Chem. Soc. **62**, 1371 (1940).

Since the heptosides may be considered as being derived from the hexosides [3] by replacement of a CH_2OH group with a $CHOH\text{-}CH_2OH$ group, the phenyl glycosides of *d*-α-mannoheptose (*d*-galactose type) and *d*-α-glucoheptose (*l*-gulose type) were prepared [4] and their hydrolyzability by almond emulsin ("Rohferment" of Helferich) was determined. The inclusion of α-phenyl *d*-taloside in the study provided a new hexose type. The results are summarized in table 1.

TABLE 1.—*Action of almond emulsin on phenyl glycosides*

Glycoside	Enzyme Efficiency (E. E.)
β-Phenyl *d*-α-mannoheptoside	22×10^{-5}
α-Phenyl *d*-α-mannoheptoside	[a] 0.3×10^{-5}
β-Phenyl *d*-α-glucoheptoside	[a] 0.3×10^{-5}
α-Phenyl *d*-α-glucoheptoside	[a] 0.3×10^{-5}
α-Phenyl *d*-taloside	[a] 0.8×10^{-5}

[a] Maximum value. No hydrolysis observed.

The β-phenyl *d*-α-mannoheptoside contains the β-*d*-galactose ring and differs from β-phenyl *d*-galactoside in having a $CHOH\text{-}CH_2OH$ group attached to the fifth ring carbon instead of a CH_2OH group. For phenyl *l*-arabinoside, the CH_2OH group is replaced by hydrogen. Both β-phenyl *d*-galactoside and phenyl *l*-arabinoside are readily hydrolyzed by the enzymes in almond emulsin.[5] According to the specificity concept outlined above, the hydrolysis of the heptoside is readily understood and the enzyme responsible for the hydrolysis of the three glycosides is β-galactosidase. In the earlier publication and before these measurements were made, these results were semiquantitatively predicted (see footnote 2) in the statement that the heptosides would be more slowly hydrolyzed than the corresponding hexosides. This also agrees with the results of Helferich, Grünler, and Gnüchtel,[6] who found that the rate of splitting of the 6-substituted beta glucosides is a function of the size of the group substituted.

The α-phenyl *d*-galactoside is also hydrolyzed by almond emulsin ("Rohferment"), but the β-galactosidase activity (for splitting beta galactosides) is 6 to 10 times that of the α-galactosidase for substrates with the same aglucon groups.[7] The negative result obtained for the hydrolysis of α-phenyl *d*-α-mannoheptoside, which may be considered to be formally derived from α-phenyl *d*-galactoside by the replacement of the primary alcohol group by $CHOH\text{-}CH_2OH$, indicates that the influence of the increase in the size of the group attached to carbon 6 is more than 10 times as great for α-galactosidase as for the β-glucosidase. If the replacement of a hydrogen of the primary alcoholic group of α-phenyl *d*-galactoside by a CH_2OH group had the same effect on α-galactosidase action as it does on β-galactosidase action, the E. E. value ("Enzyme Efficiency" or "Wertigkeit") for α-phenyl *d*-α-mannoheptoside would be about 2×10^{-5}. Since the observed E. E. is not greater than 0.3×10^{-5}, the influence of the substitution is greater for the α-galactosidase than for the β-galactosidase.

[3] H. S. Isbell, J. Research NBS **18**, 505 (1937) RP990.
[4] W. W. Pigman and H. S. Isbell. Work not yet published.
[5] B. Helferich, Ergeb. Enzymforsch. **7**, 83 (1938).
[6] B. Helferich, S. Grünler, and A. Gnüchtel, Z. physiol. Chem. **248**, 85 (1937).
[7] B. Helferich, S. Winkler, R. Gootz, O. Peters, and E. Günther, Z. physiol. Chem. **208**, 91 (1932); W. W. Pigman, Z. physiol. Chem. **261**, 82 (1939).

The action of the almond emulsin on a number of methyl heptosides has been reported previously.[8] It was found that no appreciable action of almond emulsin could be detected on the alpha and beta methyl glycosides of the following sugars: *d*-gulose, *d*-α-glucoheptose, *d*-lyxose, and *d*-α-galaheptose (alpha isomer only). It was emphasized at that time that the phenyl glycosides are much better materials for such measurements than the methyl glycosides and that, particularly for the methyl lyxosides, the results were indecisive. A repetition of the work, using the phenyl derivatives, was indicated as being important. This was done for the α-phenyl *d*-lyxoside, and the results showed a positive action by the almond emulsin, the enzyme responsible being most probably α-mannosidase (see footnote 2). In the present paper the results obtained for the methyl *d*-α-glucoheptosides have been confirmed by the use of the phenyl glycosides. The values given in the table indicate that these glycosides can with reasonable certainty be classified as unhydrolyzable (E. E. $<10^{-5}$). As shown in table 2 (see Experimental Details) no detectable hydrolysis could be observed, in spite of the use of high enzyme concentrations and extended reaction periods.

The α-phenyl *d*-taloside differs from the α-phenyl *d*-mannoside only in the configuration of the second carbon atom of the ring. Although the latter substance is very easily hydrolyzed by almond emulsin (E. E. $=14{,}000\times10^{-5}$),[9] the taloside is not appreciably hydrolyzed and the maximum value for the enzyme efficiency (E. E.) is 0.8×10^{-5}, or less than 1/14,000th of that for the mannoside.

II. β-THIOPHENYL *d*-GLUCOSIDE

As a result of the cooperation of C. S. Hudson and C. B. Purves, it has been possible to study the action of the sweet-almond emulsin on β-thiophenyl *d*-glucoside. This substance differs from β-phenyl *d*-glucoside (a very easily hydrolyzable substance with an E. E. value of $33{,}000\times10^{-5}$), only in that the glucosidic linkage is through a sulfur rather than through an oxygen atom. A previous study [10] of the action of almond emulsin yielded negative results, but as the enzyme preparation now available is very much more active than those previously used, the earlier results could not be considered as conclusive. Thiophenol does not react quantitatively with iodine under the conditions used for the measurement of the enzymatic hydrolysis of the phenyl glycosides, and therefore the usual iodometric method could not be employed. Instead, the optical rotations before and after the reaction period were measured. The results given in the experimental part (table 3) show that if any hydrolysis takes place it is too small to be detected by the method used and a maximum value for the enzyme efficiency is 10^{-5}. However, in these experiments, in contradistinction to those of Fischer and Delbrück, a distinct odor of thiophenol developed in the reaction mixture. Apparently a little hydrolysis takes place, but even if the enzyme responsible is β-glucosidase, the rate is so slow as to place the thiophenyl glucoside at the border of the unhydrolyzable class. Additional investigation is required.

[8] B. Helferich, W. W. Pigman, and H. S. Isbell, Z. physiol. Chem. **261**, 55 (1939).
[9] B. Helferich and S. Winkler, Z. physiol. Chem. **209**, 269 (1932).
[10] E. Fischer and K. Delbrück, Ber. deut. chem. Ges. **42**, 1476 (1909).

It is desirable, however, to explain this great difference in the enzymatic hydrolysis of the oxygen and sulfur glucosidic linkages. According to the classical conception of the mechanism of action of the carbohydrases, the substrate is adsorbed by the enzyme or forms an intermediate compound with the enzyme. The adsorption complex or intermediate compound upon decomposition yields the products of hydrolysis and the enzyme. The change in the glucosidic bridge from an oxygen to a sulfur atom could then affect (1) the formation of the enzyme-substrate intermediate or (2) the splitting of the glycoside. Since all the groups in the thiophenyl and phenyl *d*-glucosides are the same except the atom connecting the sugar and benzene residues, it is unlikely that the formation of the enzyme substrate intermediate would be greatly affected unless the bridge atom itself is directly involved in the formation of the complex. The results obtained with the phenyl and thiophenyl *d*-glucosides might be interpreted in favor of a bonding of the bridge atom of the glucoside to the enzyme, a mechanism which has been suggested by Josephson.[11] However, another possible basis for this great difference in the enzymatic hydrolyzability may lie in the effect of the splitting of the glucoside while it is a part of the enzyme-substrate complex. As shown by Purves,[12] β-thiophenyl *d*-glucoside is extremely resistant to acid hydrolysis; and under conditions which hydrolyze the disaccharide linkage of β-thiophenyl lactoside with the liberation of galactose, the β-thiophenyl glucoside, also formed, is unaffected. Although for most glucosides there is no simple correlation between the ease of acid and of enzymatic hydrolysis,[13] it seems probable that for the thiophenyl *d*-glucoside this factor becomes important. The implications of this possibility to the mechanism of the splitting process will be discussed in a future publication.

The measurements reported in this paper agree with the specificity theory previously described, but study of additional hexose types is desirable. The *d*-mannose, *d*-glucose, and *d*-galactose types have been thoroughly investigated by Helferich and coworkers. The α-*d*-talose type has been studied in the present report, and the *d*-gulose types, although they have been investigated (see footnote 8), require additional study. For one synthetic sugar at least, a complete investigation of the α- and β-phenyl *d*- and *l*-glycosides should be made. Since, according to the specificity concept previously expressed, pentosidases may exist, the enzymatic hydrolysis of the phenyl *d*-ribosides should be investigated. This sugar occurs in natural products, but the hexoses with similar ring conformations (*d*-allose and *l*-talose) [14], as far as is now known, are not found in natural products.

III. EXPERIMENTAL DETAILS

1. PREPARATION OF SUBSTRATE SOLUTIONS

The preparation and properties of the substrates are described in another place.[15] The particular samples of glycosides used for the present investigations were those for which the rotations have been

[11] K. Josephson, Z. physiol. Chem. **147**, 146 (1925).
[12] C. B. Purves, J. Am. Chem. Soc. **51**, 3627 (1929); see also Fischer and Delbrück (footnote 10).
[13] B. Helferich, H. Scheiber, R. Streeck, and F. Vorsatz, Liebigs Ann. Chem. **518**, 211 (1935); Josephson (page 88 of reference in footnote 11); R. Kuhn and H. Sobotka, Z. physik. Chem. **109**, 65 (1924).
[14] H. S. Isbell and W. W. Pigman, J. Research NBS **18**, 141 (1937) RP969; H. S. Isbell, J. Research NBS **18**, 505 (1937) RP990.
[15] W. W. Pigman and H. S. Isbell. Work not yet published.

reported, except the β-phenyl *d*-α-mannoheptoside, which contained about 5 percent of the alpha isomer ($[\alpha]_D^{20}=30.1$). The β-thiophenyl *d*-glucoside was a sample of the material made by C. B. Purves and was furnished by the courtesy of C. S. Hudson. The material had a specific rotation of $[\alpha]_D^{20}=69.9$.

The "standard concentration" for the measurements made by Helferich and coworkers has been 40 mg of phenyl glucoside (anhydrous) in 2 ml of approximately 0.2 *N* acetate buffer solution (pH 5.0 at 18° C). The concentration in the reaction mixture is 0.052 *M*. In the present work this or a molecularly equivalent concentration was adhered to only for the β-phenyl *d*-α-mannoheptoside and the β-thiophenyl *d*-glucoside because of the low solubilities of the other compounds. For the α-phenyl *d*-α-mannoheptoside and α-phenyl *d*-α-glucoheptoside, 0.2235 g was dissolved in 25 ml of the buffer solution; and for the α-phenyl *d*-taloside and β-phenyl *d*-α-glucoheptoside, 0.2000 g was dissolved in 25 ml of buffer solution.

2. ENZYME SOLUTION

A 2.5-g portion of sweet-almond emulsin (a sample of "Rohferment" furnished by the courtesy of B. Helferich [16]) was triturated with 100 ml of water and allowed to stand for 18 hours at 5° C. The supernatant solution was filtered through a thin layer of diatomaceous earth. The filtrate was the enzyme solution used. The residue which was obtained by evaporating 10 ml of this solution to constant weight in a vacuum desiccator over calcium chloride weighed 0.1782 g. The β-glucosidase value was 1.02.[17]

3. HYDROLYSIS MEASUREMENTS

(a) PHENYL GLYCOSIDES

The extent of hydrolysis of the substrates was measured by determination of the amount of sugar and phenol formed from the glycoside. This was done by measuring the amount of iodine which reacted in alkaline solution with an aliquot portion of the reaction mixture. It has been shown [18] that 1 mole of sugar and 1 mole of phenol react with 8 equivalents of iodine (6 for the phenol and 2 for the sugar). The experimental conditions used are similar to those described by Cajori,[19] except that potassium carbonate rather than sodium carbonate is used. The experimental details of the method of making the measurements will now be described.

Method.—The substrate and enzyme solutions were placed in a thermostat maintained at 30° C. When the solutions had reached the bath temperature, 6 ml of the substrate solution was placed in a test tube and 3 ml of the enzyme solution was added. For the β-phenyl *d*-α-mannoheptoside, 2 ml of substrate solution and 1 ml of enzyme solution were used. The solutions were mixed and the reaction mixture was kept at 30.1°±0.2° C for a measured time. The extent of hydrolysis was determined by pouring the contents of the test tube into a mixture of 7.5 ml of 15-percent potassium carbonate solution and 50 ml of 0.1 *N* iodine solution (containing 4 percent of potassium iodide). The solution remaining in the test tube was

[16] B. Helferich, S. Winkler, R. Gootz, O. Peters, and E. Gunther, Z. physiol. Chem. **208**, 91 (1932).
[17] R. Weidenhagen, Z. Ver. deut. Zuckerind. **79**, 597 (1929).
[18] B. Helferich, H. Appel, and R. Gootz, Z. physiol. Chem. **215**, 277 (1933).
[19] F. A. Cajori, J. Biol. Chem. **54**, 617 (1922).

washed into the iodine solution with 10 to 20 ml of water. After 30 minutes, 30 ml of 3 *N* sulfuric acid was added to the reaction mixture and the excess of iodine was titrated with 0.1 *N* sodium thiosulfate. The amounts of iodine used by the various glycosides are given in table 2. Blank experiments to determine the amount of iodine used up by the substrate and enzyme solutions alone were made on (1) a solution containing 6 ml of substrate solution and 3 ml of water, and (2) a solution containing 6 ml of buffer solution and 3 ml of enzyme solution (or 2 ml to 1 ml for the β-phenyl *d*-α-mannoheptoside). The column in table 2 headed "iodine corrected" indicates the amount of iodine used by the products of hydrolysis of the glycosides. All experiments were carried out in the presence of toluene, which was used as an antiseptic.

(b) β-THIOPHENYL *d*-GLUCOSIDE

The extent of hydrolysis was determined polarimetrically by measuring the rotation of a solution of the β-thiophenyl glucoside in the presence of almond emulsin. Although ordinarily 0.2 g of potassium carbonate is added to stop the hydrolysis and to hasten the mutarotation reaction, this was not necessary in these experiments and no alkali was used.

The reaction solution was prepared as described above by mixing 2 ml of substrate solution and 1 ml of enzyme solution and then adding 8 drops of toluene. The stoppered test tubes were kept for 11 or 18 days at 30.1°±0.3° C. The solutions, which were cloudy and which had a strong odor of thiophenol, were filtered, placed in 1-dm tubes, and read at 20° C with a Bates saccharimeter. The corrected values given were obtained by subtracting from the observed rotation the rotation of a similar solution containing no thiophenyl glucoside. The observed values are given in table 3.

4. CALCULATION OF RESULTS

The results of Josephson [20] and others [21] have shown that in the concentration range ordinarily employed, the velocity constants for the splitting of a β-glucoside by β-glucosidase (*at a constant initial glucoside concentration*) are directly proportional to the enzyme concentration. In order to compare the rates of splitting of various glycosides, the "Enzyme Efficiency" (E. E., or "Wertigkeit" of Helferich) is calculated according to the following formula: [22]

$$\text{E. E.} = \frac{k}{g\ (\log 2)},$$

where k=the velocity constant in minutes and decimal logarithms for 0.052 *M* solution of substrate at 30° C and at pH=5.0, and g=grams of enzyme in 50 ml of reaction solution. This formula is a modification of the method suggested by Euler and Josephson [23] for the expression of enzyme activity. For the comparison of various substrates, molecularly equivalent concentrations are used when possible, although it has been pointed out that a better comparison for some purposes could be made by the use of equivalent concentrations of the enzyme-substrate compound.[24]

[20] K. Josephson, Z. physiol. Chem. **147**, 22 (1925).
[21] B. Helferich, unpublished results.
[22] B. Helferich and H. Appel, Z. physiol. Chem. **205**, 231 (1932).
[23] H. v. Euler and K. Josephson, Ber. deut. chem. Ges. **56**, 1749 (1923).
[24] K. Josephson, Z. physiol. Chem. **147**, 141 (1925).

When the solubilities of the substrates are too small for the usual concentration to be used, it is customary to measure salicin or a similare well-studied glucoside at the same concentration and then to assume that the relative rates observed at the lower concentration hold as a first approximation at the higher concentrations. This is, however, objectionable if the substrate concentrations of both glycosides are not great enough to practically saturate the enzyme.

TABLE 2.—*Action of almond emulsion on phenyl glycosides*

[t=30.1±0.3° C; 0.297 g of "Rohferment" in 50 ml of reaction mixture.]

Time	0.1 *N* iodine used	0.1 *N* iodine used (corrected)	Hydrolysis	k×10⁵	E. E.×10⁵
β-PHENYL *d*-α-MANNOHEPTOSIDE					
Minutes	*ml*	*ml*	*Percent*		
9,900	6.08	4.63	37.0	2.03	22.7
18,720	8.53	7.08	56.6	1.94	21.7
∞	----	[c] (12.50)	----	----	----
18,720 [a]	1.32	----	----	----	----
18,720 [b]	0.13	----	----	----	----
α-PHENYL *d*-α-MANNOHEPTOSIDE					
18,660	4.40	+0.26	----	----	----
30,240	3.92	−0.20	----	----	----
∞	----	[c] (14.99)	----	----	----
30,240 [a]	3.94	----	----	----	[d] 0.3
30,240 [b]	0.20	----	----	----	----
α-PHENYL *d*-TALOSIDE					
18,660	5.20	+0.94	----	----	----
30,240	4.97	+0.71	(4.8)	0.07	0.8
∞	----	[c] (14.99)	----	----	----
30,240 [a]	3.94	----	----	----	----
30,240 [b]	0.32	----	----	----	----
α-PHENYL *d*-α-GLUCOHEPTOSIDE					
18,660	5.13	+0.27	----	----	----
30,400	4.39	−0.47	----	----	[d] 0.3
∞	----	[c] (14.99)	----	----	----
30,400 [a]	3.94	----	----	----	----
30,400 [b]	0.92	----	----	----	----
β-PHENYL *d*-α-GLUCOHEPTOSIDE					
18,660	4.35	+0.05	----	----	----
30,400	4.04	−0.26	----	----	[d] 0.3
∞	----	[c] (13.42)	----	----	----
30,400 [a]	3.94	----	----	----	----
30,400 [b]	0.36	----	----	----	----

[a] Enzyme blank.
[b] Substrate blank.
[c] Calculated value for 100-percent hydrolysis.
[d] Calculated for 1.9 percent hydrolysis. This would correspond to a corrected iodine consumption of 0.25–0.30 ml.

TABLE 3.—*Action of almond emulsion on β-thiophenyl glucoside*

Time	Rotation (corrected)[a]	Maximum E. E.[b]
Minutes	°*S*	
0	−2.97	----------
15,800	−3.03	----------
25,900	−2.86	9×10^{-6}
∞	[c] (1.42)	----------

[a] In 1-dm tubes; corrected for rotation of enzyme (−0.30).
[b] Calculated for a decrease in rotation of 0.20°S.
[c] Calculated for 100-percent hydrolysis.

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