mixtures of products were obtained in all cases, it was necessary to fractionate repeatedly so as to obtain compounds sufficiently pure for analysis. All distillations were carried out at 6 mm., through suitable fractionating columns, and small fractions collected in 15-ml. vials. The yields of crude products were estimated from the first distillation. Ordinarily 10 to 20 fractions were collected. These were recombined for subsequent distillations on the basis of boiling range, refractive index, and chlorine content.

Summary

Dimethylethynylcarbinol and 2,5-dimethyl-3-hexyne-2,5-diol have been chlorinated, each in three solvents: carbon tetrachloride, methanol, and water. The products have been identified and characterized.

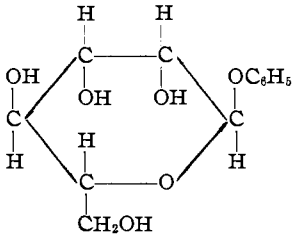
NOTRE DAME, INDIANA RECEIVED MARCH 2, 1940

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY, LEIPZIG, GERMANY]

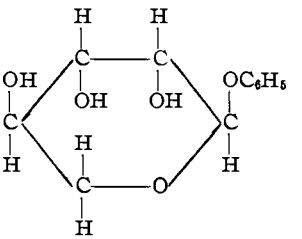
The Action of Almond Emulsin on α-Phenyl-*d*-lyxoside

BY WILLIAM WARD PIGMAN[1]

The occurrence in almond emulsin of enzymes which hydrolyze glycosides is well known. However, with very few exceptions, the previous work with these enzymes has been confined to glycosides of naturally occurring sugars. In an earlier paper,[2] the results of a study of the action of almond emulsin on a number of methyl glycosides of synthetic sugars were reported and in no instance was an hydrolysis observed which was larger than the experimental error. In general, the phenyl glycosides are much better materials for such studies since, when they are hydrolyzable, the rate of hydrolysis is much greater than that of the corresponding methyl glycosides. Also, a very sensitive method for the measurement of the degree of hydrolysis of the phenyl glycosides is available.[3] The negative results reported for α-methyl-*d*-lyxoside in the previous work[2] were somewhat surprising since α-phenyl-*d*-mannoside is known to be hydrolyzed by the enzymes in almond emulsin and as illustrated in the following formulas the pyranose rings of the phenyl-*d*-mannosides and lyxosides are identical except for the groups attached to the fifth carbon atom.



α-Phenyl-*d*-mannoside



α-Phenyl-*d*-lyxoside

In order to settle this question definitely α-phenyl-*d*-lyxoside was prepared according to the general method of Helferich and Schmitz-Hillebrecht.[4] The new substance which most probably has a pyranose structure and which has the expected rotation for the alpha isomer was found to be hydrolyzed by almond emulsin (the "Rohferment" of Helferich[5]). The rates of hydrolysis of β-phenyl-*d*-glucoside and α-phenyl-*d*-mannoside also were determined under the same conditions. The results are summarized in Table I.

TABLE I

Glycoside	$k \times 10^3$	Enzyme efficiency (Wertigkeit)
α-Phenyl-*d*-lyxoside	0.088[a]	9.4×10^{-4}
α-Phenyl-*d*-mannoside	3.5	0.15[b]
β-Phenyl-*d*-glucoside	7.3	.31[b]

[a] The enzyme concentration was four times as great for the lyxoside experiments as for the other experiments.

[b] The substrate concentrations were more dilute than those normally used but the values found are substantially the same as those reported by Helferich and Winkler.[6]

The value of 9.4×10^{-4} found for the α-phenyl-*d*-lyxoside is the smallest value reported for a hydrolyzable phenyl glycoside and compares with a value of 20×10^{-4} for β-phenyl-*d*-xyloside and a value of 3000×10^{-4} for β-phenyl-*d*-glucoside.

(1) This work was made possible by a grant from the Lalor Foundation of Wilmington, Del. The present address of the author is National Bureau of Standards, Washington, D. C.

(2) B. Helferich, W. W. Pigman and H. S. Isbell, *Z. physiol. Chem.*, **261**, 55 (1939).

(3) B. Helferich, H. Appel and R. Gootz, *ibid.*, **215**, 282 (1933).

(4) B. Helferich and E. Schmitz-Hillebrecht, *Ber.*, **66**, 378 (1933).

(5) B. Helferich, S. Winkler, R. Gootz, O. Peters and E. Günther, *Z. physiol. Chem.*, **208**, 91 (1932).

(6) B. Helferich and S. Winkler, *ibid.*, **209**, 269 (1932).

Almond emulsin contains a number of enzymes and it was considered important to determine which of these is responsible for the hydrolysis of the α-phenyl-*d*-lyxoside. The similarity of the pyranose rings of mannose and lyxose made it seem probable that the enzyme in question was α-mannosidase. This was easily tested since α-mannosidase is more stable to heating than the other important enzymes found in almond emulsin.[7] Therefore, a solution of emulsin was heated and its action on α-phenyl-*d*-lyxoside, α-phenyl-*d*-mannoside, and β-phenyl-*d*-glucoside determined. The results are summarized in Table II. As seen

TABLE II

Glycoside	Enzyme	$k \times 10^3$	$\frac{k(\text{heated})}{k(\text{unheated})}$
α-Phenyl-*d*-lyxoside	Heated	0.071	0.81
α-Phenyl-*d*-lyxoside	Unheated	0.088	
α-Phenyl-*d*-mannoside	Heated	3.1	.89
α-Phenyl-*d*-mannoside	Unheated	3.5	
β-Phenyl-*d*-glucoside	Heated	3.9	.53
β-Phenyl-*d*-glucoside	Unheated	7.3	

from the table, the enzyme responsible for the hydrolysis of the α-phenyl-*d*-lyxoside is quite resistant to heat and is, therefore, most probably α-mannosidase.

The action of almond emulsin on the phenyl-*d*-xylosides and *l*-arabinosides has been studied previously.[8] The β-phenyl-*d*-glucoside, which except for a group substituted on the fifth carbon atom has the same pyranose ring as β-phenyl-*d*-xyloside, is hydrolyzed 126 to 165 times as fast as the xyloside. The enzyme responsible for the hydrolysis of the *d*-xylosides is most probably β-glucosidase or a special β-*d*-xylosidase. The presence of a special *d*-xylosidase, *i. e.*, an enzyme cleaving *only* *d*-xylosides, in a natural enzyme mixture would not be unexpected since the sugar *d*-xylose is a constituent of natural products. However, the *d*-lyxosides are derived from a sugar which is not found in naturally occurring materials and therefore the existence of a special *d*-lyxosidase seems improbable. The relative rates of hydrolysis of α-phenyl-*d*-mannoside and lyxoside (160:1) are approximately the same as those for β-phenyl-*d*-glucoside and xyloside (125–165:1). This approximate agreement of the relative rates when considered with the reasons given for ascribing the hydrolysis of α-phenyl-*d*-lyxoside to α-mannosidase, indicates that the postulation of a special *d*-xylosidase is not necessary and that the *d*-xylosides are hydrolyzed by β-glucosidase. On the other hand, β-phenyl-*d*-galactoside, which in the configuration of its pyranose ring closely resembles phenyl-*l*-arabinoside,[9] is hydrolyzed only 2.2 times more rapidly than the arabinoside. This small difference may be due to the presence of a special arabinosidase in the emulsin or to a closer configurational relationship between the galactosides and arabinosides than that between the glucosides and xylosides or the mannosides and lyxosides.

In the heptose series of sugars, there are found synthetic sugars with ring configurations similar to the naturally occuring hexose sugars. From the results reported in this paper, it is possible to indicate which of the glycosides of these sugars should be hydrolyzed by the enzymes of almond emulsin. It seems probable that the heptosides which have ring configurations similar to hydrolyzable hexosides also should be hydrolyzed. Thus, the β-*l*-β-galaheptosides (*d*-glucose ring) the α-*l*-α-galaheptosides (*d*-mannose ring) and the β-*d*-α-mannoheptosides (*d*-galactose ring) should be cleaved by β-glucosidase, α-mannosidase and β-galactosidase, respectively. In all of these cases, however, it is to be expected that the heptosides will be hydrolyzed at a slower rate than the corresponding hexosides since it is known that an increase in the size of the atom or group attached to the fifth carbon atom decreases the rate of hydrolysis.[10] Experiments to verify these predictions are now in progress in the laboratories of the National Bureau of Standards.

The prediction given above, as to the action to be expected of the enzymes of almond emulsin on the synthetic heptosides, may be extended to describe the specificity to be anticipated for the enzymes of this general type. In general it seems most probable in light of the existing information *that special enzymes are required to split each of sixteen fundamental hexose types,*[11] *(eight "d" and eight "l" isomeric aldohexopyranosides) as well as each of the alpha-beta isomers.* It is anticipated, however, that *naturally occurring enzymes are only such which will hydrolyze glycosides with pyranose rings identical (except for groups substituted on the*

(7) B. Helferich and U. Lampert, *Ber.*, **67**, 1667 (1934); B. Helferich, H. Heyne and R. Gootz, *Z. physiol. Chem.*, **214**, 139 (1933).

(8) B. Helferich and U. Lampert, *Ber.*, **68**, 1266 (1935); **67**, 1667 (1934); B. Helferich, E. Günther and W. Pigman, *ibid.*, **72**, 1953 (1939).

(9) This compound would be called "β" by Isbell and "α" by Hudson.

(10) B. Helferich, S. Grünler and A. Gnüchtel, *Z. physiol. Chem.*, **248**, 85 (1937).

(11) H. S. Isbell and W. Pigman, *J. Research Bur. Standards*, **18**, 141 (1937); H. S. Isbell, *ibid.*, **18**, 505 (1937).

fifth carbon atom) with those of naturally occurring glycosides. Enzymes hydrolyzing furanosides have been omitted from this classification since only one of this type, fructofuranosidase, is known. Disaccharides apparently fit into this scheme when considered as glycosides[12] although there is some objection to this.[13] According to this generalization, it may be expected that enzymes which split hexosides also will cause a cleavage of the pentosides, heptosides and higher homologs which have similar ring configurations. It seems probable that special pentosidases may exist which hydrolyze particular pentosides but the action of which on the hexosides is very weak. The generalizations here outlined are extensions of those proposed by Weidenhagen[12] and by Lettré.[14] Previous work, principally by Helferich and his coworkers,[15] also makes it possible to generalize as to the effect of structural changes of the substrate on the action of the enzyme. Thus it can be said that the substitution of the hydroxyls attached to carbons 2, 3, and 4 of "hydrolyzable" glycosides by other groups causes the substituted glycoside to be unhydrolyzable. Changes in the groups attached to carbons 1 and 5 cause only a change in the rate of hydrolysis. As shown above, configurational changes of the carbons forming the pyranose ring produce new hexose types each of which requires a special enzyme. It is possible that hexose types differing in configuration only at carbon 4 may be hydrolyzed by the same enzyme but as yet this problem is not settled since there is some doubt as to whether β-glucosidase and β-galactosidase are identical.

Experimental

A. Preparation of α-Phenyl-*d*-lyxoside.—A mixture of 7.4 g. of *d*-lyxose tetraacetate ($[\alpha]^{20}D$ +26.0, $CHCl_3$) and 9.5 g. of phenol was heated on a water-bath until melting occurred and after 2.5 g. of freshly fused zinc chloride had been added, the heating was continued for fifteen minutes. After the mixture had cooled, 50 cc. of benzene and 100 cc. of water were added and the sirupy phase was brought into solution. The layers were separated and the benzene solution was washed several times with water, then several times with *N* sodium hydroxide solution and again with water. The benzene was removed by vacuum distillation and the residue dried by dissolving it in absolute alcohol and then evaporating the solvent *in vacuo*. The residue was dissolved in 35 cc. of absolute methyl alcohol, 2 cc. of 0.1 *N* barium methylate was added and the solution was refluxed for ten minutes. When the solution had cooled, crystallization occurred, yield, 1.6 g. An additional 1.2 g. was obtained by evaporating the mother liquors. The material was recrystallized from a boiling mixture of equal amounts of alcohol and water. The new substance, after three crystallizations, melted at 178–181°. In aqueous solution, the phenyl lyxoside had a rotation of $[\alpha]^{20}D$ 123 (*c*, 0.6; 0.0307 g. substance; 5 cc. of solution; 2 dm.). The solubility in water at 20° is approximately 0.5 g. per 100 cc.

Anal. Calcd. for $C_{11}H_{14}O_5$: C, 58.40; H, 6.24. Found: C, 58.54; H, 6.31.

B. Action of Almond Emulsin on α-Phenyl-*d*-lyxoside

1. Preparation of Enzyme Solution.—Two and one-half grams of almond emulsin ("Rohferment" of Helferich[5]) was rubbed up in a mortar with 100 cc. of water, and after standing for two hours at 0° the solution was centrifuged until clear. Ten cc. of the solution when dried to constant weight in a vacuum desiccator over calcium chloride yielded a residue of 0.1886 g. The beta-glucoside-value (β-Gl.-V. or β-Gl.-W.[16]) of the solution was 0.85.

2. Method of Making Measurements.[3]—To 5 cc. of a solution of 0.0423 g. of α-phenyl-*d*-lyxoside in approx. 0.1 *N* acetate buffer[17] (*p*H 5.0 at 18°) was added 2.5 cc. of the enzyme solution. The reacting solution after several drops of toluene had been added was kept a measured time in a thermostat set at 30.0°. The reaction was then stopped by adding the solution to a mixture of 2.5% potassium carbonate solution and 50 cc. of 0.1 *N* iodine–potassium iodide solution. After fifteen minutes of standing the solution was acidified by the addition of 30 cc. of 2 *N* sulfuric acid solution and the excess iodine determined by titration with 0.1 *N* sodium thiosulfate solution.

Blank experiments also were made in a similar manner, in one experiment by replacing the substrate solution with water and in a second experiment by replacing the enzyme solution with water. These solutions were kept at 30° for 3780 minutes. The experimental results and calculations are given in Table III.

TABLE III

ACTION OF ALMOND EMULSIN ON α-PHENYL-*d*-LYXOSIDE

Time, min.	0.1 *N* I_2 used, cc. Total	Corrected[a]	% Hydrolysis	$k^b \times 10^4$	E. E.[c] $\times 10^4$
3780	9.74	8.04	53.7	88.5	9.4
5280	11.50	9.80	65.5	87.5	9.3
		(14.96)[d]	Average	88.0	9.4
5280[e]	10.20	8.65	57.8	71.0	

[a] The enzyme solution alone required 1.70 cc. of 0.1 *N* iodine solution. The heated solution used 1.55 cc. [b] Expressed in Briggs logarithms. [c] E. E. (enzyme efficiency or "wertigkeit") is calculated from:

$$\text{E. E.} = \text{Log}\frac{100}{100 - \%\text{ hydrolysis}}\frac{1}{tg\,(\log 2)} = \frac{k}{g(\log 2)}$$

where "*t*" is the time in minutes and "*g*" the weight of the enzyme in 50 cc. of the reaction solution. [d] Calculated value. [e] Heat treated emulsin used.

(12) R. Weidenhagen, *Ergebn. Enzymforsch.*, **1**, 168 (1932).

(13) J. Leibowitz and S. Hestrin, *Science*, **143**, 333 (1939); **141**, 552 (1938).

(14) H. Lettré, *Angew. Chem.*, **50**, 581 (1937).

(15) B. Helferich, *Ergebn. Enzymforsch.*, **7**, 83 (1938).

(16) R. Weidenhagen, *Z. Ver. deut. Zucker-ind.*, **79**, 591 (1929).

(17) The solutions were supersaturated but did not crystallize while the experiments were in progress. Since the solubility of the substrate is quite low, a lower concentration than usual was used.

C. Comparison of Rate of Splitting of the Phenyl Glycosides of Lyxose, Mannose and Glucose

1. Preparation of Enzyme Solutions.—Two solutions were used for this work. The one, called "unheated," was the same as that used in the above work. The second, called "heated," was prepared by heating the first solution in a soft glass bottle for thirty-six hours at 50° and then centrifuging. Some evaporation occurred during this process. The dry residue determined in the same manner as for the "unheated" solution was 0.1765 g. in 10 cc.

2. Preparation of Substrate Solutions.—Equivalent solutions were prepared by dissolving the following amounts in 25 cc. of 0.1 *N* acetate buffer (*p*H 5.0): 0.240 g. α-phenyl-*d*-mannoside, 0.273 g. β-phenyl-*d*-glucoside-$2H_2O$ and 0.212 g. α-phenyl-*d*-lyxoside.

3. Method of Making Measurements.—To 1 cc. of the enzyme solutions, diluted 1:4 for the mannoside and glucoside experiments, there was added 2 cc. of the substrate solution and after the reaction was allowed to take place at 30° for the required time, it was stopped by the addition of 20 mg. of potassium carbonate. The optical rotation (D line of sodium) was then measured in 2-dm. tubes at 20° and the degree of hydrolysis calculated from the observed value and the known specific rotations of the unhydrolyzed glycosides and the corresponding sugars. The results are given in Table IV. The measurements on the lyxoside were carried out as described for the hydrolysis experiments reported in Table III and are given in that table.

TABLE IV

Enzyme solution	Time, min.	α_t	% Hydrolysis	$k \times 10^3$
β-Phenyl-*d*-glucoside, $\alpha_0 = -0.94$; $\alpha_\infty = +0.48$				
Heated	30.5	−0.60	23.9	3.90
Heated	80.0	− .22	50.7	3.84
Unheated	33.4	− .33	43.0	7.30
α-Phenyl-*d*-mannoside, $\alpha_0 = 1.39$; $\alpha_\infty = 0.12$				
Heated	33.0	1.10	22.8	3.41
Heated	79.8	0.87	40.9	2.87
Unheated	78.7	.79	47.2	3.53

Acknowledgment.—The writer wishes to express his appreciation of the coöperation, assistance and advice given by Professor B. F. Helferich during the progress of this investigation. The assistance given by the Lalor Foundation of Wilmington, Del., is also gratefully acknowledged.

Summary

The preparation of α-phenyl-*d*-lyxoside is reported. This substance is hydrolyzed by almond emulsin. The rate is the slowest reported for a hydrolyzable phenyl glycoside. The enzyme responsible for the hydrolysis is shown to be α-mannosidase. A prediction is made of the specificity to be expected of the enzymes found in almond emulsin and of other similar enzymes.

RECEIVED JANUARY 23, 1940

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

A Laccase from the Wild Mushroom, *Russula Foetens*

BY DONALD C. GREGG[1] AND WILBUR H. MILLER

Much of the early information regarding the enzyme, laccase, resulted from the researches of Bertrand and his students.[1a] Bertrand reported that laccase, the enzyme responsible for the blackening of lac tree juices, could be distinguished from tyrosinase by its ability to catalyze the oxidation of polyphenols such as hydroquinone, pyrogallol and guaiacol. In recent years, the enzymatic oxidation of these substrates, and in addition *p*-phenylenediamine, has become a criterion of laccase activity.[2-5] Most of this work has been concerned with an enzyme prepared from lac tree juices. In 1939, Graubard[5] reported limited studies on an enzyme that he obtained from the wild mushroom, *Russula foetens*. This enzyme was called laccase because of its ability to catalyze the aerobic oxidation of hydroquinone and *p*-phenylenediamine. In view of the present limited knowledge regarding the action of the plant oxidases, a more extensive study on the laccase from *Russula foetens* seemed desirable.

The enzyme preparations were obtained by the general method used in these laboratories for the preparation of oxidases from mushrooms. In a typical preparation, 100 g. of dried mushroom powder was suspended in 500 cc. of water in a refrigerator for seventy-two hours. To the resulting crude extract, solid ammonium sulfate was added to make the solution 0.5 saturated. The dark colored, gummy precipitate was triturated with a small quantity of disodium phosphate and dissolved in water. For each volume of solution, two volumes of acetone were added. At room temperature, the acetone not only precipitates the

(1) Geo. W. Ellis Fellow, 1939-1940.
(1a) G. Bertrand, *Compt. rend.*, **118**, 1215 (1894).
(2) K. Suminokura, *Biochem. Z.*, **224**, 292 (1930).
(3) G. Books, *La Nature*, No. 3011, 359-363 (1937).
(4) D. Keilin and T. Mann, *Nature*, **143**, 23 (1939).
(5) M. Graubard, *Enzymologia*, *V*, 332 (1939).