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A Comparative Study of Inductive Influence on the Hydrolysis of Beta-Thioglucopyranosides by Beta-Thioglucosidase and Beta-Glucosidase

Robert W. Miller Jr.
Central Washington University

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A COMPARATIVE STUDY OF INDUCTIVE INFLUENCE
ON THE HYDROLYSIS OF BETA-THIOGLUCOPYRANOSIDES
BY BETA-THIOGLUCOSIDASE AND BETA-GLUCOSIDASE

by

ROBERT W. MILLER JR.

A THESIS
Submitted to the Graduate Faculty
in
partial fulfillment of the requirements
for the degree of
Master of Science in Chemistry
at
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Approved:
L. C. Duncan
Head, Major Department

Robert D. Gaines
Chairman, Examining Committee

Richard A. Neve
Dean, Graduate Division

W. W. Newschwander

Wilber V. Johnson

Ellensburg, Washington
January, 1967
Acknowledgements

I wish to thank at this time Dr. Robert D. Gaines for his guidance and encouragement during my years in graduate school.

In addition I would like to express my thanks to Jack Meeks, a fellow graduate student, for his gift of \( \beta \)-thioglucosidase which made possible the final completion of this thesis.

Finally I would like to thank my three roommates whose patience and encouragement made it possible for me to complete my graduate program.
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51. Phenyl-1-thio-β-D-glucopyranoside
52. 2,3,4,6-Tetraacetyl-α,β-D-glucopyranosyl bromide
Abstract

A series of phenyl-D-thioglucopyranosides were prepared and characterized in order to determine the absolute specificity of the $\beta$-thioglucosidase and $\beta$-glucosidase enzyme systems toward the thioglucosidic bond.

The specificity of $\beta$-glucosidase of almond emulsin toward oxygen linkage seems to be absolute, since no hydrolysis was observed by this enzyme system toward the phenyl-D-thioglucopyranosides.

The $\beta$-thioglucosidase system was found to hydrolyze only two of the thioglucopyranosides. It was apparent that only where the electron-attracting substituents in the phenyl group were strong enough to greatly weaken the strength of the sulfur linkage, as in the case of the nitro group, would there be any hydrolysis.

The lack of hydrolysis prevented application of the Hammett equation in testing the quantitative electronic effects of the substituents on the rate of bond hydrolysis.
Part I

The Preparation of Phenyl-D-thioglucopyranosides

A. Introduction

The influence of configurational changes in the aglucone moiety of β-D-glucopyranosides on the activity of sweet almond β-glucosidase has been known for some time (1). More recent work by Gaines and Goering (2) on the specificity of mustard β-thioglucosidase has indicated that changes in the carbohydrate moiety of β-D-thioglucopyranosides closely parallel the pattern of activity exhibited by the β-glucosidase system.

Beta-glucosidase, one of the oldest known enzymes, is the main constituent of sweet almond emulsin, a mixture of various carbohydrases. It is characterized by its ability to hydrolyze amygdalin and β-D-glucopyranosides. The general structure of these sugars is as follows;

![Chemical Structure]

The specificity of this enzyme has been found to be absolute toward configurational changes in the carbohydrate portion

1The name now agreed on by the Enzyme Commission (1962) for this enzyme is β-D-glucoside glucohydrolase.
of the glucoside; whereas, variation of the aglucone (R) group indicates that the inductive effect of these groups may alter the activity of the enzyme and also the affinity of the enzyme for the substrate (3). It was generalized by Pigman and Richtmyer (4) that the rate of hydrolysis increases with increasing number of carbon atoms, from 1 to 7 in the n-alkylglucoside series, and decreases in the glucosides of the higher normal alcohols. Branching of the alkyl groups sometimes decreases the rate of hydrolysis, probably due to steric hindrance (5). Nath and Rydon, studying the effects of systematic substitutions in the benzene ring on the cleavage of phenyl-D-glucopyranosides by emulsin, found that both the formation and breakdown of the enzyme-substrate complexes are facilitated by electron-attracting substituents (6).

Mustard $\beta$-thioglucosidase$^2$, which possesses many similarities with the almond emulsin $\beta$-glucosidase system, is characterized by its ability to cleave mustard oil glucosides. The general structure of these glucosides is illustrated as follows;

---

$^2$The name now agreed on by the Enzyme Commission (1962) for this enzyme is $\beta$-D-thioglucoside glucohydrolase. Throughout the literature the name $\beta$-thioglucosidase has been used analogously with sinigrinase and myrosinase.
Mustard oil glucosides were first isolated by Bussy (7), who observed that ground seeds of black mustard, when treated with water release a volatile mustard oil. He also observed that when the seeds were previously treated with protein denaturing agents the addition of water failed to produce the volatile oil. Little was known about the enzyme itself until it was studied by Erikson and von Euler (8). The work of these two men and subsequent work has led many workers to conclude that the enzyme is composed of two entities, a thioglucosidase and a sulfatase (9). Recent work on $\beta$-thioglucosidase (2) has indicated that the differences between it and $\beta$-glucosidase are slight, and that aside from their specific preference for the glycone moiety, the two enzymes also resemble one another in their activity on their natural substrates.

Due to the similarity between the two enzyme systems, considerable work has been done using thioglucosides as substrates for $\beta$-glucosidase and glucosides as substrates for $\beta$-thioglucosidase. The substrates chosen for this study were phenyl-$\beta$-D-thioglucopyranosides. There were two reasons for selecting these particular substrates. The first was that variable inductive effects can be systematically brought about by introduction of various substituents into the benzene ring, and the electronic effects of such substituents measured quantitatively by means of the Hammett substitution constant ($\sigma$-values). Secondly, little is known
about the relative specificity of either enzyme system with regards to a sufficiently systematic structural variation in the aglucone of thioglucopyranosides.

This section of the thesis describes the synthesis of a series of phenyl-β-D-thioglucopyranosides, which in turn were used in investigating the specificity of the two enzyme systems toward hydrolysis of these compounds.
B. Experimental

The method involved in the synthesis of the phenyl-D-thioglucopyranosides was to obtain thiophenolates that would produce a range in inductive influence on the sulfur linkage, and then to couple these compounds with an aceto-halo derivative of the sugar.

Many of the desired thiophenols were obtained from suppliers, while others that were not available had to be synthesized.

The procedure used to prepare the unavailable thiophenols was identical with one exception and will be described in general terms to include all reactants. The alternate procedure will be described separately. Since it was necessary to slightly modify the procedures used in forming the products, the procedure and reference to the original work are both given.

The preparation of thiophenols by diazo reaction:

Substituted aniline (0.2 mole) was added slowly to 40 gm. of crushed ice and 40 gm. of concentrated HCl. The solution was cooled to -5°C. in a salt, ice, and acetone bath. A cold solution (-5°C.) of sodium nitrite (0.21 mole) in 34 ml. of water was added slowly to the aniline solution so that the temperature did not rise above 5°C.. The diazo-solution was then added slowly, over a 3 hour period with stirring, to a warmed (40-45°C.) solution of potassium ethylxanthate (0.23
mole) in 48 ml. of water. After all the diazo-solution had been added the reaction was stirred for one-half hour longer. The oily red xanthate layer was separated from the aqueous layer and the aqueous layer extracted twice with 50 ml. portions of ether. The combined oil and ether extracts were washed twice with 50 ml. portions of water. The ether solution was then dried over calcium chloride. The solvent was removed with a rotary evaporator leaving a reddish-brown liquid which was the xanthate.

The crude xanthate was taken up in 120 ml. of 95% ethanol and refluxed with constant stirring. The refluxing was maintained by slow addition of 47 gm. (0.9 mole) of potassium hydroxide pellets. After addition was complete, the reaction mixture was stirred for an additional ten hours. The mixture was poured slowly into a beaker containing 85 gm. of concentrated HCl and 120 gm. of ice. The resulting solution was extracted three times with 50 ml. portions of solvent (usually benzene), and the combined extracts dried over magnesium sulfate. The solvent was removed by use of a rotary evaporator and the product then fractionated three times with the fraction boiling at the required temperature being collected (10).

The preparation of 2-nitrothiophenol:

To a solution of 31.5 gm. of 2-nitrochlorobenzene in 50 ml. of boiling alcohol, an alcoholic solution of sodium disulfide, prepared from 35 gm. of sodium sulfide monohydrate
(Na₂S·9H₂O) and 4.68 gm. of sulfur, was added over a period of about ten minutes. Eight grams of 1.0 molar alcoholic NaOH was added dropwise over a period of about 20 minutes as the reaction mixture was refluxed. The mixture was then cooled and poured into 200 gm. of ice and 300 ml. of water. The precipitate that formed was removed by filtration. The filtrate was acidified with HCl and the crude 2-nitrothiophenol collected by filtration and washed with 100 ml. of water. The crude 2-nitrothiophenol was dissolved in 30 ml. of alcohol. After the addition of 8.0 gm. of NaOH in 300 ml. of water, the solution was filtered and the 2-nitrothiophenol was reprecipitated with HCl, collected by filtration and dried in a vacuum desiccator (11).

The properties of the thiophenols compared with those found in the literature are reported in Table I.

Preparation of 2,3,4,6-tetraacetyl-α,D-glucopyranosyl bromide:

Aceto-bromo-sugars have long been used to synthesize glucosides and thioglucosides (12). The reaction yields the beta series from the α-acetobromoglucose by inversion. The following procedure was used in the preparation of a tetra-acetyl-glucoside.

Anhydrous glucose, 50 gm., was placed in a 1000 ml. Erlenmeyer flask with 250 ml. of acetic anhydride that had been saturated with dry hydrogen bromide. The resulting straw-colored syrup was cooled and taken up in 600 ml. of
<table>
<thead>
<tr>
<th>Reference Number</th>
<th>Thiophenol</th>
<th>Yield</th>
<th>Melting Point</th>
<th>Boiling Point</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Observed</td>
<td>Reported</td>
</tr>
<tr>
<td>1</td>
<td>2-Aminothiophenol</td>
<td>---</td>
<td>26-28°C.</td>
<td>28°C.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(13)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4-Aminothiophenol</td>
<td>---</td>
<td>43-45°C.</td>
<td>46°C.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(14)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2-Chlorothiophenol</td>
<td>13 ml.</td>
<td>---</td>
<td>207-209°C.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47%</td>
<td></td>
<td>205-206°C.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(15)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3-Chlorothiophenol</td>
<td>7 ml.</td>
<td>---</td>
<td>206°C.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25%</td>
<td></td>
<td>205-207°C.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(16)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>4-Chlorothiophenol</td>
<td>---</td>
<td>53°C.</td>
<td>53-54°C.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(15)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>2-Mercaptobenzoic Acid</td>
<td>---</td>
<td>165°C.</td>
<td>164-165°C.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(17)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>3-Mercaptobenzoic Acid</td>
<td>6.8 gm.</td>
<td>146°C.</td>
<td>146-147°C.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22%</td>
<td>(18)</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>4-Mercaptobenzoic Acid</td>
<td>8 gm.</td>
<td>219°C.</td>
<td>216-219°C.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26%</td>
<td>(19)</td>
<td></td>
</tr>
</tbody>
</table>

1. Refers to the infrared spectrum number of compound
2. Uncorrected. Measured on a Fisher-Johns melting point apparatus
3. Obtained from City Chemical Corp., New York City, New York
<table>
<thead>
<tr>
<th>Reference Number</th>
<th>Thiophenol</th>
<th>Yield</th>
<th>Melting Point</th>
<th>Boiling Point</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Observed</td>
<td>Reported</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reported</td>
<td>Observed</td>
</tr>
<tr>
<td>25</td>
<td>2-Methoxythiophenol</td>
<td>5.1 ml.</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>3-Methoxythiophenol</td>
<td>7 ml.</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>4-Methoxythiophenol</td>
<td>6.4 ml.</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>2-Methylthiophenol(^3)</td>
<td>---</td>
<td>13°C.</td>
<td>15°C.</td>
</tr>
<tr>
<td>37</td>
<td>3-Methylthiophenol(^3)</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>40</td>
<td>4-Methylthiophenol(^3)</td>
<td>---</td>
<td>42-44°C.</td>
<td>42-43°C.</td>
</tr>
<tr>
<td>43</td>
<td>2-Nitrothiophenol</td>
<td>4.4 gm.</td>
<td>56°C.</td>
<td>56°C.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>4-Nitrothiophenol(^3)</td>
<td>---</td>
<td>76-77°C.</td>
<td>77°C.</td>
</tr>
<tr>
<td>49</td>
<td>Thiophenol(^4)</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

\(^1\)Refers to the infrared spectrum number of compound  
\(^2\)Uncorrected. Measured on a Fisher-Johns melting point apparatus  
\(^3\)Obtained from City Chemical Corp., New York City, New York  
\(^4\)Obtained from Pitt-Consol Chemical Co.
chloroform. The solution was washed twice with water, once with enough sodium bicarbonate solution to neutralize the dissolved acid, and again with water. The chloroform solution was dried over calcium chloride and the solvent removed with a rotory evaporator. The thick syrup which remained, was taken up in a little dry ether. Fifteen to twenty volumes of petroleum ether were added causing the bromoacetylglucose to precipitate as a thick syrup. The syrup upon cooling in a ice bath with vigorous stirring solidified in a few minutes to a crystalline mass. The crystals were filtered off with a Büchner funnel and recrystallized from 150 ml. of dry ether by rapidly evaporating the solvent. The yield was 96 gm., 84% of the theoretical, and the melting point of the product was 89°C.; reported 87-89°C. (30). The infrared spectrum of this compound is number 52 in the Infrared Data section.

Preparation of phenyl-thio-acetylglucopyranosides:

The purified thiophenols were converted to their potassium salts and reacted with 2,3,4,6-tetraacetyl-α,β-D-glucopyranosyl bromide to form the phenyl-tetra-acetylthioglucopyranosides which were in turn deacylated just before they were to be used.

The procedure used to prepare the phenyl-thio-acetylglucopyranosides was invariant in all cases. It will be described, therefore, in general terms to include all of the reactants, even though the amount of starting material in some
cases was not the same.

2-Methoxythiophenol, 3.0 gm. (10% excess), was dissolved in 10 ml. of acetone and partially neutralized by the addition of 6.9 ml. of 3.1 N methanolic potassium hydroxide. Acetobromoglucose dissolved in 10 ml. of acetone, (0.9 equivalent based on the methoxythiophenol), was added to the reaction mixture. After stirring at room temperature overnight, the mixture was poured into 150 ml. of ice water, and allowed to solidify in the freezer. The product was filtered, dried, and recrystallized from absolute methanol. The resulting crystals were further purified by recrystallizing from another solvent, generally ethanol (31).

The yields and physical constants of the phenyl-thio-acetylglucopyranosides are summarized in Table II along with the physical constants of those compounds reported in the literature.

Saponification of the phenyl-thio-acetylglucopyranosides:

The procedure used to remove the acetyl groups from the acetylated glucosides was to dissolve 0.1-0.2 gm. of the product in 10 ml. of anhydrous methanol, which had previously been saturated with dry ammonia at 0°C. (32).

Generally about one hour is sufficient to complete the saponification. The mixture was then evaporated to dryness under vacuum and the residue recrystallized from absolute alcohol. The yield was generally 90-95% of the theoretical.
### Table II

<table>
<thead>
<tr>
<th>Reference Number</th>
<th>Acetylthioglucoside</th>
<th>Yield</th>
<th>Melting Point</th>
<th>Optical Rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2-Aminophenyl-1-thio-(\beta)-D-tetraacetylglucopyranoside</td>
<td>0.5 gm. 8%</td>
<td>108-109°C.</td>
<td>(\alpha_D^{23} = -10.8^\circ\text{in acetone}, c=0.4)</td>
</tr>
<tr>
<td>5</td>
<td>4-Aminophenyl-1-thio-(\beta)-D-tetraacetylglucopyranoside</td>
<td>0.5 gm. 8%</td>
<td>121-123°C.</td>
<td>(\alpha_D^{23} = -26.2^\circ\text{in acetone}, c=0.4)</td>
</tr>
<tr>
<td>8</td>
<td>2-Chlorophenyl-1-thio-(\beta)-D-tetraacetylglucopyranoside</td>
<td>1.9 gm. 22%</td>
<td>112-115°C.</td>
<td>(\alpha_D^{26} = 100.0^\circ\text{in CHCl}_3, c=0.4)</td>
</tr>
<tr>
<td>11</td>
<td>3-Chlorophenyl-1-thio-(\beta)-D-tetraacetylglucopyranoside</td>
<td>2.4 gm. 27%</td>
<td>112-114°C.</td>
<td>(\alpha_D^{26} = 112.5^\circ\text{in CHCl}_3, c=0.4)</td>
</tr>
<tr>
<td>14</td>
<td>4-Chlorophenyl-1-thio-(\beta)-D-tetraacetylglucopyranoside</td>
<td>2.2 gm. 25%</td>
<td>110-113°C.</td>
<td>(\alpha_D^{26} = 109.4^\circ\text{in acetone}, c=0.8)</td>
</tr>
<tr>
<td>17</td>
<td>2-Carboxyphenyl-1-thio-(\beta)-D-tetraacetylglucopyranoside</td>
<td>1.2 gm. 22%</td>
<td>160-163°C.</td>
<td>(\alpha_D^{23} = -22.5^\circ\text{in acetone}, c=0.4)</td>
</tr>
<tr>
<td>20</td>
<td>3-Carboxyphenyl-1-thio-(\beta)-D-tetraacetylglucopyranoside</td>
<td>1.0 gm. 18%</td>
<td>170-172°C.</td>
<td>(\alpha_D^{23} = -37.5^\circ\text{in acetone}, c=0.4)</td>
</tr>
<tr>
<td>23</td>
<td>4-Carboxyphenyl-1-thio-(\beta)-D-tetraacetylglucopyranoside</td>
<td>1.3 gm. 24%</td>
<td>204-205°C.</td>
<td>(\alpha_D^{23} = -36.2^\circ\text{in acetone}, c=0.4)</td>
</tr>
</tbody>
</table>

1. Refers to the infrared spectrum number of compound
2. Percentage based on acetobromoglucose
3. Uncorrected. Measured on a Fisher-Johns melting point apparatus
4. Measured in a Kern Polarimeter using a 1 dm. cell
5. Not reported in the literature
Table II (Cont.)

<table>
<thead>
<tr>
<th>Reference Number</th>
<th>Acetylthioglucoside</th>
<th>Yield</th>
<th>Melting Point</th>
<th>Optical Rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Observed</td>
<td>Reported</td>
</tr>
<tr>
<td>26</td>
<td>2-Methoxyphenyl-1-thio-β-</td>
<td>2.5 gm.</td>
<td>122-124°C.</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>D-tetraacetylglucopyranoside $^5$</td>
<td>28%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>3-Methoxyphenyl-1-thio-β-</td>
<td>1.5 gm.</td>
<td>122-124.5°C.</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>D-tetraacetylglucopyranoside $^5$</td>
<td>33%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>4-Methoxyphenyl-1-thio-β-</td>
<td>2.7 gm.</td>
<td>103-105°C.</td>
<td>101-102°C.</td>
</tr>
<tr>
<td></td>
<td>D-tetraacetylglucopyranoside</td>
<td>31%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>2-Methylphenyl-1-thio-β-</td>
<td>2.0 gm.</td>
<td>104-106°C.</td>
<td>104-106°C.</td>
</tr>
<tr>
<td></td>
<td>D-tetraacetylglucopyranoside</td>
<td>48%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>3-Methylphenyl-1-thio-β-</td>
<td>1.7 gm.</td>
<td>93-94°C.</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>D-tetraacetylglucopyranoside $^5$</td>
<td>26%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>4-Methylphenyl-1-thio-β-</td>
<td>1.9 gm.</td>
<td>118-120.5°C.</td>
<td>118°C.</td>
</tr>
<tr>
<td></td>
<td>D-tetraacetylglucopyranoside</td>
<td>29%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>2-Nitrophenyl-1-thio-β-</td>
<td>0.8 gm.</td>
<td>143-145°C.</td>
<td>144-145°C.</td>
</tr>
<tr>
<td></td>
<td>D-tetraacetylglucopyranoside</td>
<td>15%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>4-Nitrophenyl-1-thio-β-</td>
<td>0.6 gm.</td>
<td>183-185°C.</td>
<td>181-183°C.</td>
</tr>
<tr>
<td></td>
<td>D-tetraacetylglucopyranoside</td>
<td>11%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>Phenyl-1-thio-β-</td>
<td>2.2 gm.</td>
<td>118-120°C.</td>
<td>118°C.</td>
</tr>
</tbody>
</table>

$^1$Refers to the infrared spectrum number of compound

$^2$Percentage based on acetobromoglucose

$^3$Uncorrected. Measured on a Fisher-Johns melting point apparatus

$^4$Measured in a Kern Polarimeter using a 1 dm. cell

$^5$Not reported in the literature
The physical constants obtained from the deacetylation of the thioglucosides are summarized in Table III along with the physical constants of those compounds reported in the literature.
**Table III**

Saponification Products of the Phenyl-thioglucosides

<table>
<thead>
<tr>
<th>Reference Number</th>
<th>Thioglucoside</th>
<th>Melting Point</th>
<th>Optical Rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2-Aminophenyl-1-thio-β-D-glucopyranoside</td>
<td>75°C.</td>
<td>$\alpha_D^{22} = -55°$ in pyr., c=0.2</td>
</tr>
<tr>
<td></td>
<td>(33)</td>
<td>71-75°C.</td>
<td>$\alpha_D^{20} = -60°$ in H$_2$O, (33)</td>
</tr>
<tr>
<td>6</td>
<td>4-Aminophenyl-1-thio-β-D-glucopyranoside</td>
<td>146-147°C.</td>
<td>$\alpha_D^{22} = -56°$ in pyr., c=0.2</td>
</tr>
<tr>
<td></td>
<td>(33)</td>
<td>146°C.</td>
<td>$\alpha_D^{22} = -63.5°$ in H$_2$O, (33)</td>
</tr>
<tr>
<td>9</td>
<td>2-Chlorophenyl-1-thio-β-D-glucopyranoside</td>
<td>syrup</td>
<td>$\alpha_D^{22} = 25.9°$ in MeOH, c=0.2</td>
</tr>
<tr>
<td>12</td>
<td>3-Chlorophenyl-1-thio-β-D-glucopyranoside</td>
<td>syrup</td>
<td>$\alpha_D^{22} = 27.0°$ in MeOH, c=0.2</td>
</tr>
<tr>
<td>15</td>
<td>4-Chlorophenyl-1-thio-β-D-glucopyranoside</td>
<td>syrup</td>
<td>$\alpha_D^{22} = 26.6°$ in MeOH, c=0.2</td>
</tr>
<tr>
<td></td>
<td>(34)</td>
<td>172-175°C.</td>
<td>$\alpha_D^{20} = -64.7°$ in pyr., c=1.5</td>
</tr>
<tr>
<td>18</td>
<td>2-Carboxyphenyl-1-thio-β-D-glucopyranoside</td>
<td>syrup</td>
<td>$\alpha_D^{22} = -50°$ in H$_2$O, c=0.2</td>
</tr>
<tr>
<td></td>
<td>(34)</td>
<td></td>
<td>(34)</td>
</tr>
<tr>
<td>21</td>
<td>3-Carboxyphenyl-1-thio-β-D-glucopyranoside</td>
<td>syrup</td>
<td>$\alpha_D^{22} = -20.5°$ in MeOH, c=0.2</td>
</tr>
<tr>
<td>24</td>
<td>4-Carboxyphenyl-1-thio-β-D-glucopyranoside</td>
<td>63-65°C.</td>
<td>$\alpha_D^{22} = -46.3°$ in MeOH, c=0.1</td>
</tr>
</tbody>
</table>

1. Refers to the infrared spectrum number of compound
2. Uncorrected. Measured on a Fisher-Johns melting point apparatus
3. Measured in a Kern Polarimeter using a 1 dm. cell
4. Not reported in the literature
### Table III (Cont.)

<table>
<thead>
<tr>
<th>Reference Number</th>
<th>Thiogluco side</th>
<th>Melting Point</th>
<th>Optical Rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>2-Methoxyphenyl-l-thio-β-D-glucopyranoside</td>
<td>57-60°C. heated rapidly</td>
<td>( \alpha^2_D = -61.3^\circ ) in MeOH, c=0.2</td>
</tr>
<tr>
<td>30</td>
<td>3-Methoxyphenyl-l-thio-β-D-glucopyranoside</td>
<td>45-50°C. heated rapidly</td>
<td>( \alpha^2_D = -50.0^\circ ) in MeOH, c=0.2</td>
</tr>
<tr>
<td>33</td>
<td>4-Methoxyphenyl-l-thio-β-D-glucopyranoside</td>
<td>101-102°C. 77°C. (34)</td>
<td>( \alpha^2_D = -48^\circ ) in MeOH, c=0.2</td>
</tr>
<tr>
<td>36</td>
<td>2-Methylphenyl-l-thio-β-D-glucopyranoside</td>
<td>152°C. 149-150°C. (35)</td>
<td>( \alpha^2_D = -51^\circ ) in H₂O, c=0.1</td>
</tr>
<tr>
<td>39</td>
<td>3-Methylphenyl-l-thio-β-D-glucopyranoside</td>
<td>103-104°C.</td>
<td>( \alpha^2_D = -66.7^\circ ) in H₂O, c=0.1</td>
</tr>
<tr>
<td>42</td>
<td>4-Methylphenyl-l-thio-β-D-glucopyranoside</td>
<td>151-152°C. 149°C. (35)</td>
<td>( \alpha^2_D = -51^\circ ) in H₂O, c=0.1</td>
</tr>
<tr>
<td>45</td>
<td>2-Nitrophenyl-l-thio-β-D-glucopyranoside</td>
<td>173-175°C. 154-155°C. (33)</td>
<td>( \alpha^2_D = -237^\circ ) in MeOH, c=0.2</td>
</tr>
<tr>
<td>48</td>
<td>4-Nitrophenyl-l-thio-β-D-glucopyranoside</td>
<td>164-166°C. 150-152°C. (33)</td>
<td>( \alpha^2_D = -104^\circ ) in MeOH, c=0.2</td>
</tr>
<tr>
<td>51</td>
<td>Phenyl-l-thio-β-D-glucopyranoside</td>
<td>133.5-135°C. 135°C. (36)</td>
<td>( \alpha^2_D = -55^\circ ) in MeOH, c=0.2</td>
</tr>
</tbody>
</table>

1. Refers to the infrared spectrum number of compound
2. Uncorrected. Measured on a Fisher-Johns melting point apparatus
3. Measured in a Kern Polarimeter using a 1 dm. cell
4. Not reported in the literature
5. 80% deacetylated derivative
C. Infrared Data

The infrared spectra of the prepared compounds have been grouped together so that each step in the formation of the thioglucoside can be observed. Definite similarities among the thioglucosides can be seen, especially among the isomers of the same substituted group; yet, differences brought about by variations of the aglycone moiety can easily be recognized.

In general very little has been reported on the infrared spectra of thioglucosidic compounds. The spectra given here were used primarily to follow the formation of a compound. In some cases, where it was possible to identify characteristic absorption peaks, the spectrum was also used as a method of identification.

From the steps in the formation of a thioglucoside, that is, from thiophenol to phenyl-acetothioglucoside to phenylthioglucoside, it was possible to determine whether a reaction had taken place, and in some cases, whether the reaction had reached completion. It can be observed that the absorption peaks at 1380 cm\(^{-1}\) and 1740 cm\(^{-1}\) are completely removed in the deacetylated products. The thiol peak at 2500 cm\(^{-1}\) is seen to disappear in the formation of the acetylthio-glucoside.

It was possible to identify many of the characteristic absorption peaks of the substituted thiophenols. The disubstituted benzene ring with its characteristic overtone
patterns between 1600-2000 cm\(^{-1}\), and strong absorption peaks between 1400-1550 cm\(^{-1}\) and 780-840 cm\(^{-1}\), can be seen on all the thiophenols. The thiol peak at 2500 cm\(^{-1}\) is also easily recognizable, as well as most of the functional groups used to produce the desired inductive effect.

In most cases the absorption peak at 880-900 cm\(^{-1}\), which is characteristic for the \(\beta\)-pyranoside (37) appears to be present. This indication was initially used as a criterion for the production of \(\beta\)-glucosides, since \(\alpha\)-pyranosides exhibit a peak at 840 cm\(^{-1}\) (37), but since some of the compounds have other functional groups that absorb close to these wavelengths, little consideration was given to this as a final method of identification.

The appearance of the weak hydroxyl band at 3300-3400 cm\(^{-1}\) on most of the acetylated glucosides was considered to be the result of water within the sample. This was found to be the case, since after subjecting one of the acetylated samples to harsh drying conditions it did not show an appreciable peak in this area. The strong absorption and broadened peaks in many of the glucopyranosides was a result of water in the sample. These compounds are very hygroscopic and are difficult to keep in the dry state for any length of time. In the short time necessary to run the infrared spectra (12 minutes), the initially clear KBr pellet became extremely opaque.

Polystyrene peaks were placed on each infrared curve
and used as reference peaks for phase correction. All previously mentioned wavelengths have been corrected to the polystyrene reference.

The liquid thiophenols were run as variable thickness samples. The acetylated thioglucosides, thioglucosides, and solid thiophenols were run as KBr pellets. All spectra were run on a Perkin-Elmer Model 137 Infracord.
1. 2-Amino thiophenol

2. 2-Aminophenyl-1-thio-β-D-tetraacetylglucopyranoside
3. 2-Aminophenyl-1-thio-\( \beta \)-D-glucopyranoside

4. 4-Aminothiophenol
5. 4-Aminophenyl-1-thio-β-D-tetraacetylglucopyranoside

6. 4-Aminophenyl-1-thio-β-D-glucopyranoside
7. 2-Chlorothiophenol

8. 2-Chlorophenyl-1-thio-β-D-tetraacetylglucopyranoside
9. 2-Chlorophenyl-1-thio-β-D-glucopyranoside

10. 3-Chlorothiophenol
11. 3-Chlorophenyl-1-thio-β-D-tetraacetylglucopyranoside

12. 3-Chlorophenyl-1-thio-β-D-glucopyranoside
13. 4-Chlorothiophenol

14. 4-Chlorophenyl-1-thio-β-D-tetraacetylglucopyranoside
15. 4-Chlorophenyl-1-thio-\( \beta \)-D-glucopyranoside

16. 2-Mercaptobenzoic Acid
17. 2-Carboxyphenyl-1-thio-β-D-tetraacetylglucopyranoside

18. 2-Carboxyphenyl-1-thio-β-D-glucopyranoside
19. 3-Mercaptobenzoic Acid

20. 3-Carboxyphenyl-1-thio-β-D-tetraacetylglucopyranoside
21. 3-Carboxyphenyl-1-thio-β-D-glucopyranoside

22. 4-Mercaptobenzoic Acid
23. 4-Carboxyphenyl-1-thio-β-D-tetraacetylglucopyranoside

24. 4-Carboxyphenyl-1-thio-β-D-glucopyranoside
25. 2-Methoxythiophenol

26. 2-Methoxyphenyl-1-thio-β-D-tetraacetylglucopyranoside
27. 2-Methoxyphenyl-1-thio-\(\beta\)-D-glucopyranoside

28. 3-Methoxythiophenol
29. 3-Methoxyphenyl-1-thio-\(\beta\)-D-tetraacetylglucopyranoside

30. 3-Methoxyphenyl-1-thio-\(\beta\)-D-glucopyranoside
31. 4-Methoxythiophenol

32. 4-Methoxyphenyl-1-thio-β-D-tetraacetylglucopyranoside
33. 4-Methoxyphenyl-1-thio-\(\beta\)-D-glucopyranoside

34. 2-Methylthiophenol
35. 2-Methylphenyl-1-thio-\(\beta\)-D-tetraacetylglucopyranoside

36. 2-Methylphenyl-1-thio-\(\beta\)-D-glucopyranoside
37. 3-Methylthiophenol

38. 3-Methylphenyl-1-thio-β-D-tetraacetylglucopyranoside
39. 3-Methylphenyl-1-thio-β-D-glucopyranoside

40. 4-Methylthiophenol
41. 4-Methylphenyl-1-thio-β-D-tetraacetylglucopyranoside

42. 4-Methylphenyl-1-thio-β-D-glucopyranoside
43. 2-Nitrothiophenol

44. 2-Nitrophenyl-1-thio-β-D-tetraacetylglucopyranoside
45. 2-Nitrophenyl-1-thio-β-D-glucopyranoside

46. 4-Nitrothiophenol
47. 4-Nitrophenyl-1-thio-β-D-tetraacetylglucopyranoside

48. 4-Nitrophenyl-1-thio-β-D-glucopyranoside
49. Thiophenol

50. Phenyl-1-thio-$\beta$-D-tetraacetylglucopyranoside
51. Phenyl-1-thio-β-D-glucopyranoside

52. 2,3,4,6-Tetraacetyl-α,D-glucopyranosyl bromide
Part II

Specificity of Beta-thioglucosidase and Beta-glucosidase Toward Phenyl-thioglucosides

A. Introduction

Previously it had been reported that synthetic thioglucosides were not hydrolyzed by \( \beta \)-thioglucosidase (38) and that thioglucosides did not serve as substrates for almond emulsin (39). Gaines and Goering (2) reported the hydrolysis of gentibiose, amygdalin, phloridzin, and salicin by the highly purified enzyme \( \beta \)-thioglucosidase. They also observed that almond emulsin did not hydrolyze the mustard oil glucosides even though the glucosides did possess considerable affinity for the enzyme. Working with an induced \( \beta \)-glucosidase in yeast, Durksen and Halvorson (40) found the enzyme to be inactive toward a series of ring substituted phenyl-thioglucosides, but the ortho-para directing substituents positioned meta to the thioglucosidic linkage had a stronger affinity for the enzyme than those at other positions. Goodman et al (41) reported the hydrolysis of several \( \beta \)-thioglucosides by \( \beta \)-thioglucosidase and also found the almond emulsin system to possess hydrolytic activity toward substituted purinyl thioglucosides. Reese, Clapp and Mandels (42) reported the hydrolysis of 4-nitrophenyl-\( \beta \)-D-glucoside by the \( \beta \)-thioglucosidase system.

In spite of the large volume of work done, very few definite conclusions have been reached, partly because
structural variations in the aglucones have not been systematic. It is the purpose of this section of the thesis to investigate the specificity of $\beta$-thioglucosidase and $\beta$-glucosidase systems toward the thioglucosidic linkage.
B. Experimental

Assay procedures:

The products that are obtained by enzymatic hydrolysis of phenyl-thioglucosides are glucose and thiophenol. Unless otherwise specified, 4.0 mg. of substrate in 0.5 ml. of buffer were incubated with 0.2 ml. of enzyme solution for analysis of activity.

Acetate buffer, at a pH of 6.8, was used for the β-thioglucosidase and substrate solutions. The reaction vessels were incubated at 37°C. for a measured period of time, and the amount of hydrolysis determined by measuring the amount of glucose liberated by the dinitrosalicylic acid method (43). Citrate buffer, at a pH of 5.4, was used for incubation of substrate with β-glucosidase. The incubation temperature was 30°C. for β-glucosidase.

Quantitative protein measurements were made by the method of Folin-Ciocalteu (44). All colorimetric determinations were made using a Beckman Model DB spectrophotometer.

Substrates:

Salicin, used for the determination of β-glucosidase activity, was obtained from the Nutritional Biochemical Corporation. 4-Nitrophenyl-β-D-glucopyranoside was purchased from the California Corporation for Biochemical Research, and used in the determination of the specific activity of β-thioglucosidase.
Preparation of $\beta$-glucosidase:

Purified $\beta$-glucosidase was purchased from the Nutritional Biochemical Corporation. Since $\beta$-glucosidase was extracted from sweet almond, which is a mixture of four carbohydrases, it was necessary to check the purity of the enzyme.

The purified enzyme was passed through a column of N,N-diethylaminoethyl cellulose (DEAE) to see if the purity could be improved. The DEAE was soaked in citrate buffer at a pH of 6.2 for two hours, then allowed to gravity pack in a chromatography column (2 cm. in diameter) to a height of approximately 30 cm. The column was then washed thoroughly with citrate buffer until there was no change in absorption at 280 m\(\mu\). (A flow cell was used in conjunction with the Beckman DB spectrophotometer and a recorder to indicate the appearance of protein). The enzyme, 20 mg., was dissolved in 2.0 ml. of buffer and passed through the DEAE column. Figure 1. represents the elution diagram obtained.

There was obtained only one main peak with a slight shoulder from the elution of the enzyme through the DEAE column. This seemed to indicate only one or two enzymes were present, not the expected four, characteristic of sweet almond emulsin. Attempts were made to resolve this peak, but none were successful. Different buffer solutions, varying the amount of enzyme sample, changing the flow rate, as well as lengthening and shortening the height of the column gave no improvement in separation. The lack of activity of the
Figure 1. Chromatography of partially purified β-glucosidase on a DEAE column. Elution was made with citrate buffer, pH 6.2.
enzyme toward $\alpha$-mannosides and $\alpha$-galactosides seems to indicate that only $\beta$-glucosidase and $\beta$-galactosidase were present. The activities of the various carbohydrases are summarized in Table IV.

Because there was little increase in purity using the DEAE column, the crystalline enzyme was used, since it was thought to be pure enough to check for hydrolysis of the phenyl-thioglucosides.

The effect of $\beta$-glucosidase on the phenyl-thioglucosides:

Beta-glucosidase was incubated with the phenyl-thioglucosides, and was found to have no effect on these compounds. This data is reported in Table V.

The results of the experimentation on the activity of the enzyme $\beta$-glucosidase relative to the phenyl-thioglucosides were as expected, since they correlate well with the findings of other workers (39,45). From all indications, variation of the inductive effect of the aglucone and thus the strength of the glycosidic linkage has no effect on the enzymes ability to cleave the sugar derivative. This strengthens the belief that the enzyme is specific only for oxygen linked glucosides.

Preparation and purification of $\beta$-thioglucosidase:

Beta-thioglucosidase was initially extracted from black mustard seed (Brassica nigra), but because of an extraction technique, the protein was apparently denatured and no appreciable activity was obtained. A similar process was
Table IV

Beta-glycosidase Activity

<table>
<thead>
<tr>
<th>Carbohydrase</th>
<th>Substrate</th>
<th>Micrograms Reducing Sugar Liberated$^1$</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-glucosidase</td>
<td>salicin</td>
<td>3270</td>
<td>20 min.</td>
</tr>
<tr>
<td>$\beta$-galactosidase$^2$</td>
<td>lactose</td>
<td>1030</td>
<td>20 min.</td>
</tr>
<tr>
<td>$\alpha$-mannosidase</td>
<td>$\alpha$-Me-D-mannoside</td>
<td>10</td>
<td>18 hrs.</td>
</tr>
<tr>
<td>$\alpha$-galactosidase</td>
<td>mellibiose</td>
<td>15</td>
<td>10 hrs.</td>
</tr>
</tbody>
</table>

$^1$Substrates for the enzymes were 6.7 mg. substrate in 0.5 ml. buffer. 0.1 mg. enzyme in 0.2 ml. buffer was incubated with each substrate at 37°C.

$^2$$\beta$-glucosidase and $\beta$-galactosidase of almond emulsin have been shown to be a single enzyme (45).
### Table V

Activity of $\beta$-glucosidase on Phenyl-thioglycosides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Micrograms Reducing Sugar Liberated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs.</td>
</tr>
<tr>
<td>2-Aminophenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>4-Aminophenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>2-Chlorophenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>3-Chlorophenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>4-Chlorophenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>2-Carboxyphenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>3-Carboxyphenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>4-Carboxyphenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>2-Methoxyphenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>3-Methoxyphenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>4-Methoxyphenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>2-Methylphenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>3-Methylphenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>4-Methylphenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>2-Nitrophenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>4-Nitrophenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>Phenyl-1-thio-$\beta$-D-glucopyranoside</td>
<td>0</td>
</tr>
</tbody>
</table>

1In the reaction mixtures, 4.0 mg. of substrate were incubated with 0.2 ml. of the purified $\beta$-glucosidase preparation.
used to obtain β-thioglucosidase from nasturtium seeds (*Tropaeolum majus*) which gave a product with increased activity. The procedure used to prepare β-thioglucosidase is described below in general terms for both extractions.

Defatted, finely ground seed, 300 gm., was mixed with 900 ml. of water. The mixture was incubated at 40°C. for about 12 hours and then expressed through several layers of cheesecloth. An equal volume of 80% ethanol was added and the precipitated material removed by centrifugation. The clear supernatant, containing the enzyme, was treated with 2.5 volumes of 95% ethanol, which precipitated the remaining protein. The protein material was collected by centrifugation and the supernatant discarded. The protein was washed with alcohol and ether and finally air dried. This crude enzyme preparation can be stored in a desiccator for several months without appreciable loss of activity.

Two grams of the crude enzyme preparation was dissolved in 100 ml. of water. Appropriate amounts of solid ammonium sulfate were added to increase the saturation of the solution by factors of 10% until no further material appeared to precipitate. The mixture was centrifuged after each addition of ammonium sulfate and the precipitated protein collected, suspended in 10 ml. of water and dialyzed against distilled water for 24 hours. Each fraction obtained by salt precipitation was treated in a similar manner, and the dialyzed protein solution checked for activity on 4-nitrophenyl-β-D-
glucopyranoside. The fraction at 30-40% saturation contained most of the activity (extracted from nasturtium seeds) and was saved for further purification.

A DEAE column (2 cm. in diameter by 15 cm. high) was conditioned with 0.1 M phosphate buffer (pH 7). After equilibration, 10.8 mg. of protein in 2 ml. of buffer were applied to the column. The protein was eluted with phosphate buffer. The greatest activity was obtained from 50-65 ml. in the effluent.

The purification of the enzyme was not as good as had been previously reported (46), but the activity was believed to be strong enough to hydrolyze the phenyl-thioglucosides, if they were going to be hydrolyzed. The activities obtained during purification are summarized in Table VI.

The effect of β-thioglucosidase on the phenyl-thioglucosides:

Due to the high loss of protein during elution through the DEAE column, the ammonium sulfate fraction (30-40%) was used for analysis of enzyme activity on the phenyl-thioglucosides.

Beta-thioglucosidase was incubated at 37°C. with the phenyl-thioglucosides in sodium acetate buffer solution (pH 6.9). From the data reported on Table VII it can be observed that only the nitrophenyl-thioglucosides were hydrolyzed. These data agree with those found by Reese et al (42), although it was hoped that more of the phenyl-thioglucosides would be hydrolyzed than was indicated.
<table>
<thead>
<tr>
<th>Enzyme Fraction</th>
<th>Volume (ml)</th>
<th>Yield (%)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity units/mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water extract</td>
<td>650</td>
<td>100</td>
<td>10,400</td>
<td>0.02</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractionation</td>
<td>55</td>
<td>31.5</td>
<td>93.5</td>
<td>0.72</td>
</tr>
<tr>
<td>DEAE chromatography</td>
<td>14</td>
<td>14.8</td>
<td>1.83</td>
<td>17.15</td>
</tr>
</tbody>
</table>

1 Purified from nasturtium seeds.

2 One unit equals one micromole of 4-nitrophenol liberated in 30 minutes.

Substrate was 1.5 mg. of 1,4-nitrophenol-β-D-glucoside in 1 ml. of buffer (acetate, pH 7.5). One ml. of enzyme solution was incubated with the substrate for 30 minutes at 47.5°C. The reactions were stopped by placing the tubes in boiling water for two minutes. The solutions were diluted to 10 ml. with water and the 1,4-nitrophenol color read at 388 μm on a Beckman Model DB spectrophotometer.
Table VII

Activity of β-thioglucosidase on Phenyl-thioglucosides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Micrograms Reducing Sugar Liberated 24 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Aminophenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>4-Aminophenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>2-Chlorophenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>3-Chlorophenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>4-Chlorophenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>2-Carboxyphenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>3-Carboxyphenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>4-Carboxyphenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>2-Methoxyphenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>3-Methoxyphenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>4-Methoxyphenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>2-Methylphenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>3-Methylphenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>4-Methylphenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>2-Nitrophenyl-1-thio-β-D-glucopyranoside</td>
<td>190</td>
</tr>
<tr>
<td>4-Nitrophenyl-1-thio-β-D-glucopyranoside</td>
<td>600</td>
</tr>
<tr>
<td>Phenyl-1-thio-β-D-glucopyranoside</td>
<td>0</td>
</tr>
</tbody>
</table>

1 In the reaction mixtures, 4 mg. of substrate were incubated with 0.2 ml. of the purified β-thioglucosidase preparation.
C. Discussion

The preparation of a systematic series of thioglucosides was in no way as easy as first thought. The difficulty in preparing sufficient 3-aminothiophenol and 3-nitrothiophenol prevented completion of the series.

The condensation of the thiophenols with the aceto-bromoglucose yielded an unexpected result in that there was a difference between the observed and the reported optical rotation for the 4-chlorophenyl-acetylthioglucoside (see Table II). Since the other thiophenols were condensed in the same manner, and their physical properties corresponded with those reported, it seems likely that the chloro-compounds should also be beta sugars, rather than the alpha sugars as might be suspected from their large positive optical rotations. The positive rotation of the chloro-compounds is again observed in the deacetylated sugars.

The following thioglucosides and their tetraacetates were synthesized, and have not yet been reported in the literature:

2-Chlorophenyl-1-thio-β-D-glucopyranoside
3-Chlorophenyl-1-thio-β-D-glucopyranoside
2-Carboxyphenyl-1-thio-β-D-glucopyranoside
3-Carboxyphenyl-1-thio-β-D-glucopyranoside
4-Carboxyphenyl-1-thio-β-D-glucopyranoside
2-Methoxyphenyl-1-thio-β-D-glucopyranoside
3-Methoxyphenyl-1-thio-β-D-glucopyranoside
3-Methylphenyl-1-thio-β-D-glucopyranoside

An elemental analysis of these compounds was not carried out at this time.

The melting points of some of the thioglucosides are
not reported because the compounds could not be obtained in the crystalline state. To examine the enzymatic hydrolysis of the thioglucosides it was unnecessary to have them in a crystalline form, therefore, the syrups of some of the thioglucosides were used.

The sugars were stored over phosphorus pentoxide in a vacuum desiccator under refrigeration, and did not appear to decompose over a six month period. They did decompose readily on heating, when this method was used to obtain the anhydrous products. This instability in some cases prevented adequate drying, and resulted in the appearance of a water peak in the infrared spectrum.

Good activity was obtained from β-glucosidase, and yet when the enzyme was incubated with the thioglucosides, no hydrolysis was observed. These results indicate that the specificity of β-glucosidase is confined to the sugar portion of the molecule. Substitution of sulfur for oxygen in the anomeric position, as done by other workers (39,45), results in no hydrolysis by the enzyme. This specificity does not appear to be absolute, as certain thioglucosides have been hydrolyzed by β-glucosidase (41).

There are several possible explanations for the variance in observed hydrolysis. The change from oxygen to sulfur as the glucosidic bridge, could have affected the formation of the enzyme-substrate intermediate, or it could effect the dissociation of this complex. The first case is unlikely
since most of the aglucone groups used in this study are the same as those used by Nath and Rydon (6) in their work on substituted phenyl-\(\beta\)-D-glucosides. Unless the bridge atom itself is involved in the enzyme-substrate complex, which does not seem likely due to the hydrolysis of certain thio-glucosides, the enzyme attachment to the substrate should be the same. The second situation seems more likely, as it has been shown by Purves (47), Wagner et al (48), and C. Banford et al (49) that most thiophenol-\(\beta\)-D-glucosides are relatively resistant to acid hydrolysis compared to their oxygen counterpart. There has been no direct correlation between acid hydrolysis and enzymatic hydrolysis; however, Nath and Rydon (6) demonstrated that the decomposition of the enzyme-substrate complex resembles alkaline hydrolysis in being facilitated by electron-attracting substituents. Under the test conditions the nitro groups did not deactivate the ring sufficiently to allow enzymatic hydrolysis of the sulfur bond by \(\beta\)-glucosidase.

The ability of \(\beta\)-glucosidase to hydrolyze certain thio-glucosides appears to depend on the aglycone substituent effects on the sulfur linkage and on the proximity of the substituted group to the glycosidic bond. These effects also seem to apply for \(\beta\)-thioglucosidase since it was able to hydrolyze only the nitrophenyl-D-thioglucosides, although old seeds, partial denaturation, or chemical contamination could explain the low specific activity of the enzyme and
lack of measurable hydrolysis of the other thioglucosides.

Group effects may also be a reason for the unmeasurable hydrolysis. Groups that possess strong ionic character may favor formation of the enzyme-substrate complex, but decrease the dissociation of the complex to such an extent that the overall hydrolysis rate is decreased. It is difficult to evaluate this effect and its influence on the system. A study of the inhibitory effects of these thioglucosides may yield insight into such behavior.

The hydrolysis of some glucosides by $\beta$-thioglucosidase (46) indicates that this enzyme has the same specificity as $\beta$-glucosidase for the glycone moiety, but no absolute specificity for the atom involved in the glucosidic linkage. If one accepts the fact that the sulfur-glucosidic bond in mustard oil glucosides, the natural substrates for $\beta$-thioglucosidase, is rather weak, as has been observed by Reese et al (42), then the chemical or configurational nature of the aglucone can slow down or halt hydrolysis.

The absence of hydrolysis in most of the thioglucosides prevented the application of the Hammett equation in testing the quantitative electronic effects of the substituents on the rate of bond hydrolysis. The hydrolysis of these compounds would have been especially helpful in correlating the electron attracting character of the substituents with the formation and dissociation of the enzyme-substrate complex.
These results indicate many similarities between $\beta$-glucosidase and $\beta$-thioglucosidase. Their dual activities toward both glucosides and thioglucosides indicate an apparent lack of specificity for the glucosidic linkage. Yet, the inductive and group positioning effects cannot be the entire answer, since there are many glucosides and thioglucosides that neither enzyme will cleave at the expected rate of hydrolysis. This still leaves the dilemma as to the difference between the two enzymes, and will have to be answered by future experimentation.
Part III

Summary

1. Phenyl-thioglucosides were prepared by reacting substituted thiophenols with α-acetobromoglucose and de-acetylation of the resulting products. The compounds were characterized by optical rotation, melting point, and infrared spectrum. Most of the thioglucosides had physical properties comparable, within experimental error, to those reported in the literature, with the exception of the chlorophenyl-thioglucosides. The following unreported thioglucosides and their tetraacetates were synthesized;

2-Chlorophenyl-1-thio-β-D-glucopyranoside
3-Chlorophenyl-1-thio-β-D-glucopyranoside
2-Carboxyphenyl-1-thio-β-D-glucopyranoside
3-Carboxyphenyl-1-thio-β-D-glucopyranoside
4-Carboxyphenyl-1-thio-β-D-glucopyranoside
2-Methoxyphenyl-1-thio-β-D-glucopyranoside
3-Methoxyphenyl-1-thio-β-D-glucopyranoside
3-Methylphenyl-1-thio-β-D-glucopyranoside

The thioglucopyranosides were used to determine the specificity of β-glucosidase and β-thioglucosidase with respect to the sulfur linkage.

2. Beta-glucosidase was obtained from the Nutritional Biochemical Corporation. Attempts to further purify the enzyme by chromatography on N,N-diethylaminoethylcellulose resulted in no increase in activity.

The β-glucosidase failed to show hydrolysis of the thioglucosides. This indicates an extreme relative specificity for the glucosidic bond.
3. Beta-thioglucosidase was finally isolated from nasturtium seeds by alcohol precipitation and purified by ammonium sulfate fractionation.

The enzyme did not have strong activity, yet it was able to hydrolyze ortho and para nitrophenyl-thioglucosides. The lack of hydrolysis of the other thioglucosides indicated that the activity of the enzyme maybe affected by the relative strength of the linkage and the binding energy between the enzyme and its substrate, as in agreement with previous findings (38).
Part IV

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