The formation of hydroxylamine mentioned in paragraph 8 and the positive Feigl-Amaral test [8] for an N—O bond suggests that the hydroxyl group is combined with the nitrogen atom and that the aglucone is a hydroxylamine derivative.

Taking into account these conclusions and the elementary composition of the aglucone, the possibility of an aliphatic side chain in the aglucone is ruled out, and a ring formed of nitrogen, carbon, and oxygen atoms is indicated. Structure II was suggested for the aglucone. This structure was supported by the similarity of the UV-spectra of the aglucone and its reduction product with 2H-1,4-benzoxazin-3-(4H) one synthesized by us (Fig. 2).

The structure of the aglucone could later be confirmed by synthesis by Honkanen and Virtanen [5,6], as already mentioned in this paper.

The support given by the *Rockefeller Foundation* is gratefully acknowledged.

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Received February 25, 1960.

Precursors of Benzoxazolinone in Rye Plants

II. Precursor I, the Glucoside

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The identification and isolation of precursor I (the glucoside). As reported in previous communications [1], the glucoside $C_{14}H_{17}O_9N$ was isolated from rye seedlings. It is a primary compound present in intact rye plants. The aglucone $C_8H_7O_4N$ which is the immediate precursor of benzoxazolinone (BOA) is formed from the glucoside enzymatically.

225 g of 10-day-old seedlings of Pekka rye were put into 1.5 l of boiling water, the solution was decanted, filtered, and evaporated to 250 ml *in vacuo*. Ether-soluble substances were removed by extraction, and a chromatogram was made from the water solution with water-saturated butanol. 150 μl were pipetted on the starting spot. The dark spot, R_F 0.57, visible in UV light, represented the glucoside sought, from which precursor II is formed enzymatically. The spectrum of the substance is very similar to that of the aglucone.

The isolation of larger amounts of the substance was performed by counter-current extraction, using the butanol-water solvent system. In another connection, one of us (H) will give a detailed account of the isolation. The m. p. of the glucoside was 186.5—187°C (uncorr.). R_F 0.57 in water-saturated *n*-butanol. (Found: C 49.04; H 4.98; N 3.81; O 41.04. Calc. for $C_{14}H_{17}O_9N$: C 48.98; H 4.99; N 4.08; O 41.95.) UV spectrum (in ethanol). Glucoside: max. 255 mμ, ε = 7 570, max. 281 mμ, ε = 5 160. Aglucone: max. 254 mμ, ε = 8 500, max. 282 mμ, ε = 5 800. The curves of the UV spectra are presented in the paper of Virtanen and Hietala [1].

The UV spectra of the glucoside and the aglucone are very similar. Already from this it was probable that the structure of the free aglucone was the same as that bound to the sugar glucosidically. This concept was supported by all other observations. Some of these which elucidate the

chemical nature of the glucoside are reported in the following (*cf.* the corresponding observations made on the aglucone [1]).

A potentiometric titration with 0.0200 N NaOH gave equiw.wt. 338 (calc. 343). The p*K* calculated from the curve was 6.67. Titration with acid showed that the structure of the glucoside cannot be betainic. The result was thus the same as with the aglucone.

With $FeCl_3$ the glucoside gave an intensely violet colour, as did the aglucone.

On hydrolysis with 1 N HCl (100°C, 3 h) the largest part of the glucoside remained unchanged. The glucoside is accordingly rather stable on acid hydrolysis. A sugar which gave a weak reaction with aniline phthalate on paper chromatograms was found in the solution. Using three different solvent systems (water-saturated *n*-butanol, *iso*-propanolwater 16:4, phenol-water-NH_3), this sugar travelled as glucose.

Ortho-aminophenol was formed on strong acid hydrolysis (89 mg of glucoside and 1 ml of conc. HCl in a sealed tube for 40 h at 100°C). It was extracted with ether from a solution neutralized with $NaHCO_3$. Hydroxylamine could be found in the hydrolysate, and the qualitative Feigl-Amaral reaction for the N—O bond was positive. The result was thus the same as with the aglucone.

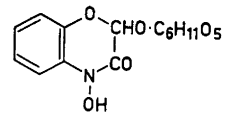
Ortho-aminophenol was formed as the main product on alkali fusion (4.1 mg of glucoside, 371 mg of KOH, and 0.1 ml of water, the temperature was raised to 200°C in 30 min). It was isolated in crystalline form and identified by paper chromatography, mixed m. p., and UV spectrum.

The same products as with the aglucone were also obtained on reduction with HI (*d* 1.96) and red phosphorus at 170—180°C, using reaction times of 2, 4, and 10 h. *Ortho*-aminophenol was identified by the UV spectrum. Glycine was found by amino acid chromatography.

The number of acetylating hydroxyl groups was determined in a pyridine solution by titrating the acetic acid formed from the excess acetanhydride. About five OH groups per mole were found.

The hydrolysis of the glucoside by a homogenate of rye seedlings leads to the formation of the aglucone and glucose. The aglucone can be extracted from the water solution by ether.

The above observations on the glucoside indicate that no rearrangement takes place in the molecule during hydrolysis. Since on the basis of the $FeCl_3$ reaction there is still a free NOH group in the glucoside, the glucose has to be bound to the OH group which is attached to carbon atom 2 in the heterocyclic ring. The structure of the glucoside is accordingly the following.



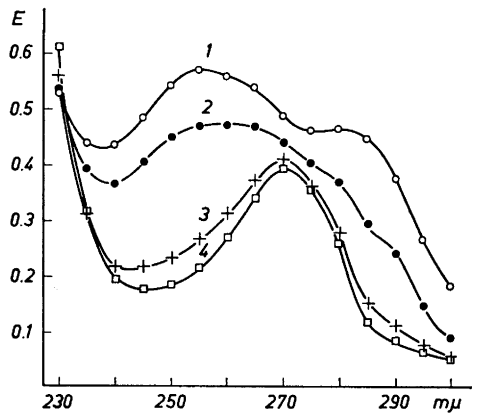


Fig. 1. UV spectra of the raw aglucone preparation from rye seedlings. A dilute solution was heated in a water bath for 2 min (2), 6 min (3), and 60 min (4). 1 was not heated.

The quantitative determination of the glucoside in rye seedlings. Since the glucoside is rapidly hydrolysed to the aglucone in crushed rye seedlings, it can be determined by the UV spectrum of the aglucone extracted by ether. A quantitative method has not, however, been developed yet for the estimation of the aglucone. On the other hand, a method has been developed by the present authors [2] for the determination of BOA formed rapidly from the aglucone at 100°C. The transfer of the aglucone into BOA in very dilute solutions is an extremely rapid reaction as shown in Fig. 1. Already at 50°C a slow formation of BOA can be observed. The kinetics of this complicated reaction has not been investigated closely, but it seems that the relative amount of BOA formed from the aglucone

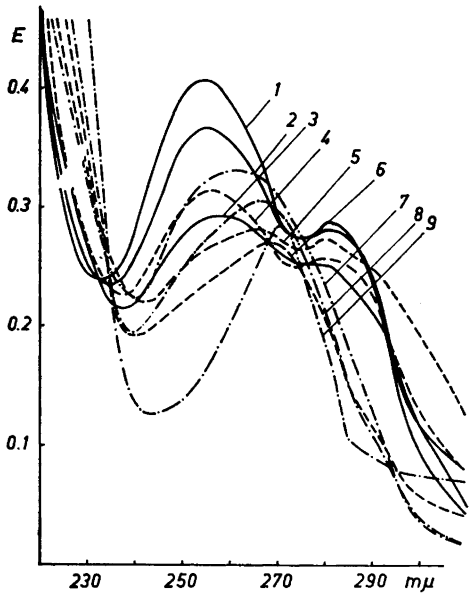


Fig. 2. UV spectra of water solutions of the aglucone of different concentrations heated at 100°C. Aglucone, max. 254 mμ, 282 mμ; BOA max. 270 mμ.

1. 10 mg/ml 5 min, *2.* 15 min, *3.* 60 min, *4.* 300μg/ml 5 min, *5.* 15 min, *6.* 60 min, *7.* 10 μg/ml 5 min, *8.* 15 min, *9.* 60 min.

decreases when the aglucone concentration rises. This is shown by the following test.

The aglucone was heated in a test tube in a boiling water bath at concentrations of 10 mg/ml, 300 μg/ml, and 10 μg/ml for 5 min, 15 min, and 1 h. For the measurement of spectra the first solution was diluted 1:1 000 and the second 1:30, and the most dilute solution was measured as such. Fig. 2 shows the changes occurring in the spectra of the different solutions.

When a relatively short reaction time is used, the formation of BOA can therefore be followed by spectrophotometry only in very dilute solutions of the aglucone.

The highest values found in this laboratory for BOA in rye seedlings have been about 1.1 mg of BOA per g fresh weight[3], corresponding to 2.8 mg of the glucoside if the yield is quantitative. The quantitativeness of the yield depends decisively on how thoroughly the plant material is crushed. A 90 % yield of BOA is more probable when plant material (0.5—1 g) is crushed in a mortar. The hydrolysis of the glucoside in a homogenate of rye seedlings is rapid. For the sake of safety it is best to allow the homogenate to stand at room temperature for 30 min before heating.

The support given by the *Rockefeller Foundation* is gratefully acknowledged.

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Received February 25, 1960.

The Synthesis of Precursor II of Benzoxazolinone Formed in Rye Plants, and the Enzymic Hydrolysis of Precursor I, the Glucoside

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In earlier papers Virtanen *et al.*[1,2] have presented the structure 2,4-dihydroxy-1,4-benzoxazin-3-one (I) for the aglucone enzymatically formed in crushed rye seedlings. The structure is now confirmed by the synthesis of the aglucone itself and its reduction product (II), which is obtained by reduction with zinc dust in boiling acetic acid[3].

The starting material for the synthesis of the aglucone was *o*-(methoxymethoxy)-nitrobenzene (III), from which the corresponding phenylhydroxylamine derivative (IV) is prepared in the usual way by reduction with zinc dust in a neutral medium. By allowing this compound to react with dichloroacetylchloride, N-(*o*-methoxymethoxyphenyl)-dichloroacethydroxamic acid (V) is obtained. The methoxymethoxy group is then hydrolysed by heating in a dilute methanolic hydrochloric acid solution. Finally the compound formed (VI) is