KINETICS OF THE ALKALINE HYDROLYSIS OF FLAVONOID GLYCOSIDES

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In the study of the structure of flavonoid glycosides, in addition to acid hydrolysis, alkaline hydrolysis is used [1] which splits off the sugar residue more selectively and enables the structure of the initial substances to be determined with greater probability. However, there is no information in the literature on kinetic investigations of this reaction. In the present paper we give the results of a study of the rate of the alkaline hydrolysis reaction as a function of the degree of hydroxylation and of the position of attachment of the sugar residues in 2-phenylbenzo-γ-pyrone. As the objects of investigation we took the glycosides most frequently found in nature with carbohydrates residues in the 3,3',4', and 7 positions and also compounds with sugar residues are present simultaneously in the 3,7 and 3,4' positions. Some of them were obtained by selective acid hydrolysis with subsequent separation on a column of polyamide.

The substances were hydrolyzed with 0.5% aqueous potassium hydroxide on the boiling water bath [1]. The course of the reaction was analyzed by quantitative paper chromatography by a known method [2]. The kinetic curves were plotted mainly from the results of the dependence of the change in the amount of the starting materials on the time of the reaction, but in individual cases from the substances formed, which are equivalent in amount to the starting material that has disappeared. For a comparative evaluation of the rate of the reaction we used the time during which 50% of the substance is hydrolyzed.

The results obtained (see Fig. 1 and Table 1) show that the rate of the reaction depends on the structure of the flavonoid aglycone, the degree of hydroxylation, and the position of attachment and the properties of the sugar residue. In particular, the degradation of derivatives of 3',4',5,7-tetrahydroxy- and of 3,3',4',5,7-pentahydroxyflavones predominates over the degradation of derivatives of 3,4',5,7-tetrahydroxyflavone. The methoxy derivatives of flavone diglycosides are cleaved with greater difficulty than those mentioned above, and 7-glycosides hydrolyze more rapidly than 3,7-diglycosides. A comparison of the rate of hydrolysis of an isorhamnetin 4'-monoside and an isorhamnetin 3,4'-diglycoside gives grounds for assuming that the introduction of a second residue at C_3 accelerates the splitting out of a glucose molecule at $C_{4'}$ relative to isorhamnetin 4'-glucoside. Thus, for example, isorhamnetin 4'-glucoside is cleaved to the extent of only 15% in 3 hours, while isorhamnetin 3,4'-diglucoside is 50% hydrolyzed in 150 min.

A comparison of the rates of hydrolysis found for the 3,4'- and 3,7'-diglycosylated forms of isorhamnetin showed that the latter is cleaves faster. A comparison of the results from experiments on the hydrolysis of the 7- and 4'-glycosides of isorhamnetin permits the assumption that the difficulty of hydrolysis for the 4'-monoside is due to the influence of the neighboring methoxy group which, apparently, repels the electrons from the glycosidic center, and its effect is equivalent to substitution in the sugar residue at C_2.

The "half-period" of decomposition of the molecule for the monosides is from 2 to 10 min, and for diglycosides and triosides it is more than 100 min.

EXPERIMENTAL

All the glycosides mentioned were dried and weighed before analysis. For chromatography we used Filtrak FN-11 paper and the butan-1-ol–acetic acid–water (4:1:2) system. Extinctions were measured on an FÉK-56 photoelectric colorimeter (filter with its maximum transmission at a wavelength of 490 nm). The calibration curves were plotted according to structural features. Monosides were calculated with respect to the calibration curves of cosmosiin, diglycosides with respect to dactilin, and triosides with respect to robinin. For this purpose, 100 mg of the substance was dissolved in the first case in 100 ml of ethanol, in the second case in 100 ml of a mixture of ethanol and dimethylformamide (7:3), and in the third case in 100 ml of 45% ethanol.

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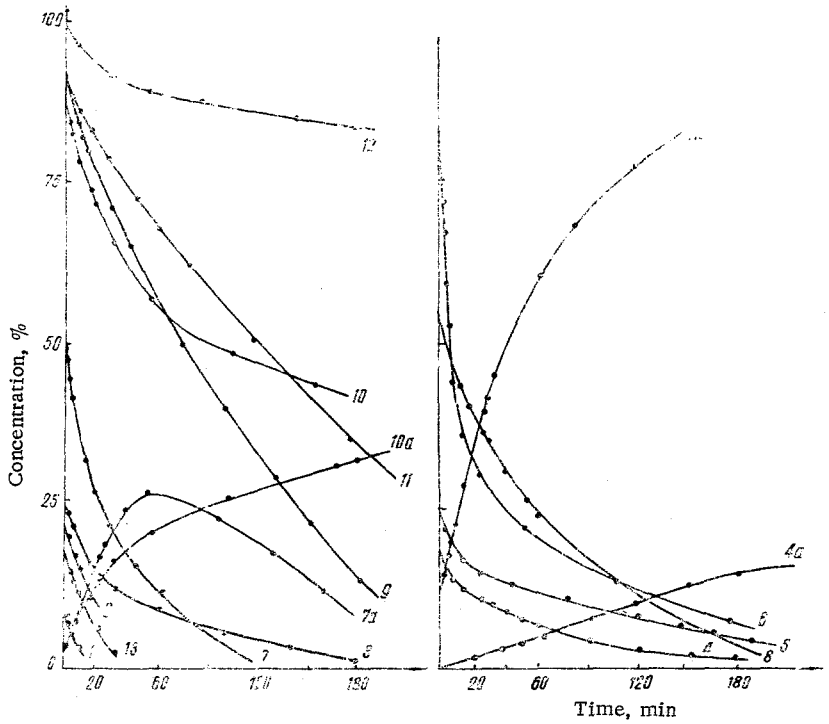

Fig. 1. Kinetic curves of the change in the concentration of flavonoid glycosides during alkaline hydrolysis and of the formation of reaction products: 1) 7-β-D-glucopyranosyloxy-3,3',4',5-tetrahydroxyflavone; 2) 3-β-D-glucopyranosyloxy-3',4',5-trihydroxy-7-α-L-rhamnofuranosyloxyflavone; 3) 3'-β-D-glucopyranosyloxy-4',5,7-trihydroxyflavone; 4) 7-β-D-glucopyranosyloxy-4',5-dihydroxyflavone; 5) 7-β-D-glucopyranosyloxy-3',4',5-trihydroxyflavone; 6) 7-β-D-glucopyranosyloxy-5-hydroxy-4'-methoxyisoflavone; 7) 3,4',5-trihydroxy-7-α-L-rhamnopyranosyloxyflavone; 8) 4',5-dihydroxy-7-α-L-rhamnopyranosyloxy-3-β-D-robinobiosyloxyflavone; 9) 4',5-dihydroxy-3,7-di(α-L-rhamnopyranosyloxy)flavone; 10) 3,4'-di(β-D-glucopyranosyloxy)-5,7-dihydroxy-3'-methoxyflavone; 11) 3,7-di(β-D-glucopyranosyloxy)-4',5-dihydroxy-3'-methoxyflavone; 12) 4'-β-D-glucopyranosyloxy-3,5,7-trihydroxy-3'-methoxyflavone; 13) 7-β-D-glucopyranosyloxy-3,4',5-trihydroxy-3'-methoxyflavone. Reaction products: 4a) 4',5,7-trihydroxyflavone; 7a) 3,4',5,7-tetrahydroxyflavone; 8a) 4',5,7-trihydroxy-3-β-robinobiosyloxyflavone; 10a) 3-β-D-glucopyranosyloxy-4',5,7-trihydroxy-3'-methoxyflavone.

Various volumes of the solutions were transferred by a micropipette to chromatograms, which were cut out in a definite manner; 0.01 ml corresponded to 30 μg of substance.

After the running of the chromatograms, they were dried in the air for 30 min and the sections corresponding to markers, determined with the aid of UV light, were cut out and eluted with 5 ml of 0.5% aqueous ammonia in each case. To each eluate was added 1 ml of diazotized sulfanilic acid and the mixtures were left for 5 min, after which 2 ml of 1 N HCl was added to each.

The optical densities of the eluates were measured after 15 min in cells with a layer thickness of 1 cm. The comparison solution was the eluate obtained from a control zone of the chromatogram.

For hydrolysis, 20-30 mg of each flavone glycoside was dissolved in 10 ml of 0.5% KOH and the mixture was heated on the boiling water bath under reflux. The change in the amount of the starting material during the reaction was determined by taking samples for analysis: during the first 10 min, every 2 min, during the next 20 min every 5 min, and then every 10 min for half an hour and every 30 min for another 2 h.

TABLE 1. Rates of Alkaline Hydrolysis of Flavonoid Glycosides (50% cleavage)

Substance	Time, min	Rate relative to cynaroside	Literature
7-β-D-glucopyranosyloxy-3,3',4',5-tetrahydroxyflavone (quercimeritrin)	2	0.57	[3]
3-β-glucopyranosyloxy-3',4',5-trihydroxy-7-α-L-rhamnofuranosyloxyflavone (antoside)	2	1.01	[4]
3'-β-D-glucopyranosyloxy-4',5,7-trihydroxyflavone (dracocephaloside)	2	0.47	[5]
7-β-D-glucopyranosyloxy-4',5-dihydroxyflavone (cosmosiin)	10	0.53	[6]
7-β-D-glucopyranosyloxy-3',4',5-trihydroxyflavone (cynaroside)	1	1.0	[7]
7-β-D-glucopyranosyloxy-5-hydroxy-4'-methoxyflavone (biochanin 7-glucoside)	8	0.086	[8]
3,4',5-trihydroxy-7-α-L-rhamnopyranosyloxyflavone	2	0.32	[9]
4',5-dihydroxy-7-α-L-rhamnopyranosyloxy-3-β-robinobiosyloxyflavone (robinin)	10	0.082	[9]
4',5-dihydroxy-3,7-di(α-L-rhamnopyranosyloxy)flavone (kaempferitrin)	120	0.006	[10]
3,4'-di(β-D-glucopyranosyloxy)-5,7-dihydroxy-3'-methoxyflavone (dactilin)	150	0.0052	[11]
3,7-di(β-D-glucopyranosyloxy)-4',5-dihydroxy-3'-methoxyflavone (isorhamnetin 3,7-diglucoside)	120	0.0051	[11]
4'-β-D-glucopyranosyl-3,5,7-trihydroxy-3'-methoxyflavone	–	Not determined	[11]
7-β-D-glucopyranosyloxy-3,4',5-trihydroxy-3'-methoxyflavone	2	0.55	[11]

Samples of the solutions (0.2 ml) were deposited on strips of paper, and chromatography and photocolorimetric determination were performed under the conditions described above. The amount of substance in the volume of reaction mixture taken for analysis was found from a calibration graph. Then the percentage of the substance present in relation to its amount at the moment of taking the sample for analysis was calculated and a kinetic curve was plotted in the form of the dependence of the amount of substance on the reaction time.

The time from the beginning of the deposition of the sample on the strips of chromatographic paper was 1 min. Assuming the possibility of error because of the occurrence of hydrolysis during the time of deposition of the solution on the paper, we performed a number of experiments in which the reaction was stopped after predetermined intervals of time by the addition of a few drops of acetic acid (for neutralization) to the reaction mixture and the composition was analyzed quantitatively. The results of the analyses were determined only by the first method.

SUMMARY

1. In a study of the kinetics of the alkaline hydrolysis of flavone glycosides it has been found that derivatives of 3,3',4',5,7-pentahydroxyflavone hydrolyze faster than derivatives of 3,4',5,7-tetrahydroxyflavone and of 3,4',5,7-tetrahydroxy-3'-methoxyflavone.

2. In the hydrolysis of diglycosides of 3,3',4',5,7-pentahydroxyflavones the maximum amount of intermediate product is formed after 2 min (3,4',5,7-tetrahydroxyflavone glycoside), and in the case of 3,4',5,7-tetrahydroxy-3'-methoxyflavone glycosides after 120-150 min.

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SYLPIN – A NEW C-METHYLATED FLAVONOID FROM *Pinus sylvestris*

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Among natural flavonoids, C-methylated derivatives form a fairly small group [1]. In the family Pinaceae they are characteristic for species of *Pinus* and are found specifically in the wood. From the wood of some species of *Pinus* have been isolated cryptostrobin (5,7-dihydroxy-6-methylflavanone), strobopinin (5,7-dihydroxy-8-methylflavanone), strobochrysen (5,7-dihydroxy-6-methylflavone), strobobanksin (3,5,7-trihydroxy-6-methylflavone), and from the bark pinoquercetin (6-methylquercetin) and pinomyricetin (6-methylmyricetin) [2-7].

The C-methyl group in these compounds is present in position 6 or 8. Similar methylation of the flavone molecule has been reported for eucalyptus compounds [8, 9].

From needles of a representative of the genus *Pinus* – Scotch pine (*Pinus sylvestris* L.) – we have isolated a new C-methylated flavonoid which we have called sylpin. This is the first case of the detection of C-methylated derivatives in conifer needles. Sylpin (I) was isolated from an ethereal solution of a methanolic extract of the needles by chromatography on polyamide in the chloroform–methanol (95 : 5) system.

The maxima in the UV spectrum (271 and 338 nm) permit sylpin to be assigned to flavones or flavonols with a substituted 3-OH group. According to elementary analysis and mass and NMR spectra, sylpin contains one C-methyl group, one methoxy group, and three hydroxy groups. The presence of three hydroxy groups was confirmed by the preparation of a triacetate. The nature of the substitution of the aromatic nucleus can be judged from the PMR spectrum (Fig. 1), which contains the signals of five aromatic protons: two two-proton doublets at 7.96 ppm and 6.96 ppm with J = 9 Hz (4′-substituted side ring) and a singlet at 6.52 ppm belonging either to H-3 or to one of the protons of the trisubstituted ring A (H-6, H-7, or H-8).

One of these three hydroxyls is in position 5, as is shown by the presence of a singlet at 13.0 ppm in the PMR spectrum. On the basis of the UV and mass spectra (ion C with m/e 121) it may be considered that another hydroxy group is in position 4′. The third hydroxy group may occupy position 6, 7, or 8. The last case is excluded by the negative gossypetone test. The location of an OH group in position 7 does not agree with the UV spectrum (sodium acetate does not lead to a bathochromic shift of the short-wave band). At the same time, the presence of a 6-OH group is shown by characteristic fragmentation in the mass spectrum: an intense $(M-1)^+$ ion is formed by the splitting off of the radical (in this case – H) from the 6-OR group [10]. Furthermore, the UV spectrum in the presence of $AlCl_3$/HCl is also characteristic for 5-hydroxyflavones in which position 6 has the hydroxyl substituent [11].

The remaining methyl and methoxy groups can be located in positions 3 and 8 or 7 and 8. The variant of 3- and 7-substitution is excluded by the Gibbs reaction, which shows that one of the groups is present in position 8.

We made an unambiguous choice of one of the two variants listed on the basis of analysis of the results of the demethylation of sylpin. The fact that demethylation forms flavonol (UV spectrum) corresponds to an initial position of the CH_3O group at C-3 and, consequently, of the methyl group at C-8.

Thus, sylpin has the structure of 4′,5,6-trihydroxy-3-methoxy-8-methylflavone (I). The triacetate (II) and the demethylation product – 3,4′,5,6-tetrahydroxy-8-methylflavone (III) – that we obtained have not been described in the literature, either.

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