Preliminary Notes

Urinary products of scopoletin in laboratory rats

The discovery by YANG *et al.*[1] that the mainstream smoke from cigarettes commonly used in the United States contained scopoletin (6-methoxy-7-hydroxycoumarin) has stimulated interest in the metabolic fate of this compound in animals. This preliminary note reports the conversion of some of the scopoletin to esculetin (6,7-dihydroxycoumarin) and the conjugation of these two compounds with glucuronic acid, glycine, and sulfate in the rat. The conversion of scopoletin to esculetin is particularly interesting because a demethylation reaction is indicated.

The urine from three female albino rats (Sprague-Dawley), weighing 250–300 g each and fed a vitamin B complex test diet with complete vitamin fortification (Nutritional Biochemicals Corp., Cleveland, Ohio) for 3 weeks, was collected under toluene and spotted on S & S No. 589 red-ribbon chromatography paper (11½ in × 12 in). Each chromatogram was developed in the first direction in chloroform–acetic acid–water (2:1:1, v/v/v, lower layer) and then in the second direction in 20 % KCl–water. When observed under long-wavelength ultraviolet light (3660 Å), none of the compounds described below was found on the paper chromatograms. Then the same animals were fed the complete diet as previously mentioned, with the addition, however, of scopoletin (1 %). Upon analysis in like manner as the control, paper chromatograms of the urine collected after administration of the latter diet now contained at least three blue-fluorescing zones which were not found on the chromatograms of urine from the animals before they were fed scopoletin.

The separation of each of the individual fluorescent compounds was undertaken by chromatography on 7½ in × 21 in papers, using *n*-butanol–pyridine–benzene–water (5:3:1:3, v/v/v/v upper layer) as the solvent system. In this solvent system, using only one-dimensional chromatography, three major fluorescent zones, with R_F values of approx. 0.15, 0.55, and 0.89, were found on the chromatograms. Two other zones, with R_F values of 0.20 and 0.68, were present, but exhibited no fluorescence.

The farthest moving zone (R_F, 0.89) in this *n*-butanol–pyridine–benzene–water system was purified further by cutting the zone out of twelve such chromatograms, sewing each onto a new sheet of paper and developing them in *n*-butanol–acetic acid–water (6:1:2, v/v/v). The resulting major zone in each (R_F, 0.92) was cut out, eluted with 75 % ethanol–water, and the combined eluates were reduced in volume *in vacuo*. On comparison by paper chromatography in all the solvent systems mentioned in this note and by ultraviolet-absorption spectrophotometry, the purified compound in the concentrate was found to be identical with the reference scopoletin synthesized by BRAYMER *et al.*[2].

The zone with R_F 0.68 in *n*-butanol–pyridine–benzene–water was not visible under the ultraviolet light before spraying, but it produced a red color when sprayed with a diazotized sulfanilic acid solution[3]. Identification studies on this compound are now in progress.

The third zone (R_F, 0.55 in the *n*-butanol–pyridine–benzene–water) from each chromatogram was cut out, sewed onto new sheets of paper, and developed a second time in the same solvent system. After spraying, first with 3 *N* HCl, and secondly with a solution of 100 mg $BaCl_2$ in 50 ml 70 % ethanol, followed by a solution of sodium rhodizonate (10 mg in 20 ml of 50 % ethanol)[4], the part of the zone with lesser R_F value gave a positive test for sulfate. That portion of the zone with larger R_F value gave a positive test for hippuric acid using *p*-dimethylaminobenzaldehyde as the reagent[5]. A procedure suitable for separating completely the two or more compounds present in this third zone was not found. Therefore, the entire zone was cut out, eluted with 75 % ethanol–water, concentrated, and then hydrolyzed with 6 *N* HCl. The concentrated ethyl acetate extract of the hydrolysate contained scopoletin and esculetin, as determined by comparison with authentic samples of each, using paper chromatography in all the solvent systems already listed and also in 15 % acetic acid–water. The correct linkage and combinations of esculetin and scopoletin with the glycine and sulfate have not yet been established.

The combined eluates in 50 % methanol of the slowest moving zone (R_F, 0.15) from twenty chromatograms developed in the *n*-butanol–pyridine–benzene–water were evaporated to dryness *in vacuo*, and then dissolved in 25 ml phosphate buffer, pH 6. The solution was then incubated at 37° for 24 h with 50 mg β-glucuronidase, and then extracted with ethyl acetate. The ethyl acetate fraction contained mainly scopoletin, but a relatively much smaller amount of esculetin was also found by paper chromatographic analysis. Another aliquot of the eluate from this zone which was treated identically except that no enzyme was added contained neither free scopoletin nor esculetin. Thus, this zone contained mainly scopoletin glucuronide, but a relatively small amount of a glucuronide of esculetin also was present.

This work is part of a larger investigation supported by grants from the National Institutes of Health and from the Brown-Hazen Fund of Research Corporation.

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Received October 7th, 1960