Urinary Metabolites of Coumarin and o-Coumaric Acid

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Coumarin is a naturally occurring constituent of certain forages, especially clover, and is frequently ingested by livestock. It has been widely used as a flavor ingredient in foods and drugs. Hazleton et al. (1) have reported recently on the oral toxicity of coumarin for rats and dogs. However, little was known concerning the metabolic fate of this compound in the animal body until a recent report by Mead et al. (2) appeared. These workers observed hydroxylation and conjugation of coumarin when it was given orally to rats and rabbits. They found no evidence of ring fission (opening of the heterocyclic ring). In their studies approximately 75 per cent of the dose of coumarin was unaccounted for.

The investigations here reported clearly indicate that opening of the heterocyclic ring of coumarin takes place. Evidence of a species difference between rats and rabbits as regards the hydroxylation of coumarin is also presented.

Experimental

Materials and Methods

Coumarin, 4-hydroxycoumarin, and 7-hydroxycoumarin were purchased from commercial sources. A sample of o-hydroxyphenylacetic acid was obtained from Cutter Laboratories. Melilotic acid (o-hydroxyphenylpropionic acid) was prepared from acetyl coumaroyl chloride, glycine, and sodium amalgam, and isolating as the calcium salt from water. All preparations were examined for homogeneity by means of the two-dimensional paper chromatographic system shown in Fig. 1.

The procedures used for the collection of urine and for the extraction and chromatographic separation of its components have been described (5). The rats were maintained on a purified diet containing the following ingredients (in per cent): sucrose 71, vitamin-free casein 18, acetoolein 5, salts (U.S.P. XIV) 4, and a complete vitamin mixture in dextrose 2. The basic diet for the rabbits consisted of corn meal 49, sucrose 10, crude casein 25, corn oil 4, dried brewers’ yeast 5, salts 4, magnesium oxide 0.5, and potassium acetate 2.5.

Results

Rat Experiments—When adult rats were given 100 mg. of coumarin per rat (dissolved in dilute ethanol) by stomach tube, there were at least five major areas appearing on the chromatogram which were not present when control urine was similarly treated. Three of these areas were readily detected and identified as melilotic, o-hydroxyphenylacetic, and o-coumaric acids, (Areas 1, 2, and 5, respectively, Fig. 1). Identification was based on the similarities between $R_F$ values, behavior under ultraviolet light, and color after spraying with diazotized sulfanilic acid (6), when the unknowns were compared with the authentic compounds. Additional evidence for the identity of o-hydroxyphenylacetic acid was based on a comparison of the crystallographic properties of an authentic sample with the material isolated from the urine of rabbits which had received coumarin. The isolation procedure is described later.

An unidentified metabolite of coumarin which gave a blue fluorescence but no color when sprayed with diazotized sulfanilic acid was observed completely surrounding the melilotic acid spot (Area 1).

The fifth spot, Area 3, Fig. 1, contained at least three substances, as became evident after acid treatment (2 n HCl reflux for 1 hour) of the corresponding area cut from an unsprayed chromatogram. Two-dimensional migration of the ether extract of the resulting hydrolysate now produced two spots in addition to a small amount of unchanged material remaining at Area 3. One of the spots corresponded to o-coumaric acid (Area 5) ; the other was identical in behavior with Area 1, corresponding to melilotic acid. The unchanged material was tentatively identified as o-hydroxyphenylactic acid (see under “Rabbit Experiments”). When an unsprayed chromatogram of the ether extract from urine of rats which had received coumarin was set aside for a few weeks, Areas 1 and 3 darkened, probably as a result of oxidative deterioration. A similar darkening also occurs when melilotic acid and melilotoxyglycine are treated in this manner.

The failure to detect glucuronic acid in the hydrolysate of Area 3, suggested the possibility, based on previous work (7), that o-hydroxyphenylhydracrylic acid might be the precursor of the o-coumaric acid. Hydrogenation of 4-hydroxycoumarin with sodium amalgam (with the use of CO$_2$ to control pH) yielded a product whose chromatographic properties were identical with...
those of Area 3. Acid treatment of this compound (2 N KCl reflux for 10 minutes) led to the formation of o-coumaric acid.

Rats, each of which received 100 mg. of o-coumaric acid by stomach tube, excreted melilotic acid, o-hydroxyphenylacetic acid, melilotoylglycine, and unchanged o-coumaric acid in the urine. The unidentified fluorescent component surrounding the melilotic acid (Area 1) observed when rats were given coumarin, was not present when o-coumaric acid was given instead. An additional spot (Area 4, Figure 1) was detected after the ingestion of o-coumaric acid. After hydrolysis of this area, the ether extract of the hydrolysate was chromatographed and o-coumaric acid (Area 5) was identified. The substance in Area 4 was found to have the same chromatographic properties as synthetic o-coumaroylglycine.

When melilotic acid was given to rats (100 mg. each) by stomach tube the urinary metabolites excreted in the urine were identical with those excreted when o-coumaric acid was given. In Area 7, Fig. 1, a substance corresponding to 4-hydroxyo-coumaric acid was isolated. Identification of the two hydroxylated derivatives of coumarin was based not only on paper chromatographic properties but also on the crystallographic properties following their isolation from urine.

The isolation of o-hydroxyphenylacetic acid, 3-hydroxycoumarin, and 7-hydroxycoumarin (Areas 8 and 6, respectively, Fig. 1) was accomplished by Soxhlet extraction with alcohol of lyophilized unacidified urine from rabbits receiving coumarin orally. The alcohol was removed by

Estimates of the amounts of urinary metabolites excreted by rats were based on comparisons of spot sizes on chromatograms containing appropriate volumes of urine with known amounts (5 to 10 µg) of the authentic compounds. Of a total dose of 200 mg. of coumarin 10 to 15 mg. of o-hydroxyphenylacetic acid and 6 to 7 mg. of melilotic acid were accounted for in the urine. A minimum of 4 to 6 mg. of o-coumaric acid was detected in the urine after acid hydrolysis, which included the o-coumaric acid obtained by conversion of o-hydroxyphenylhydracrylic acid to o-coumaric acid.

Rabbit Experiments—Rabbits were allowed to adjust to the semipurified diet for several days before the addition of coumarin at a level of 0.5 per cent. Urine was collected, acidified, subjected to extraction with ether, and chromatographed in the same manner as described for rat urine. A major metabolite was o-hydroxyphenylacetic acid the identity of which was confirmed by crystallographic comparison of the isolated material with an authentic sample. Melilotic acid was also present surrounding the unidentified blue fluorescing material previously observed in rat urine. Very little o-coumaric acid was detected until after Area 3 was refluxed with acid, suggesting that o-hydroxyphenylhydracrylic acid was the precursor. Also present on the chromatogram of the ether extract of Area 3 was melilotic acid, indicating the presence of melilotoylglycine at Area 3. Some of the material at Area 3 was resistant to acid hydrolysis (2 N HCl reflux for 1 hour). Synthetic o-hydroxyphenylacetic acid was found to be stable to acid hydrolysis and had the same Rf values and color with diazotized sulfanilic acid as this acid resistant urinary component.

Relatively large amounts of two metabolites not detected in rat urine were 3-hydroxycoumarin and 7-hydroxycoumarin (Areas 8 and 6, respectively, Fig. 1). Identification of the two hydroxylated derivatives of coumarin was based not only on paper chromatographic properties but also on the crystallographic properties following their isolation from urine.

The isolation of o-hydroxyphenylacetic acid, 3-hydroxycoumarin, and 7-hydroxycoumarin was accomplished by Soxhlet extraction with alcohol of lyophilized unacidified urine from rabbits receiving coumarin orally. The alcohol was removed by
evaporation and the residue dissolved in aqueous HCl; this solution was subjected to extraction successively with carbon tetrachloride and chloroform. The carbon tetrachloride solution was decolorized with charcoal and the 3-hydroxycoumarin and 7-hydroxycoumarin were separated by fractional crystallization from water. The chloroform extract which contained the o-hydroxyphenylacetic acid was chromatographed on a cellulose column with the use of chloroform-acetic acid-water (2:1:1). The o-hydroxyphenylacetic acid was crystallized from the appropriate cellulose column eluate.

When rabbits received o-coumaric acid in the diet (1 per cent) an examination of the urine by means of paper chromatography revealed results almost identical to those obtained for the rats. Thus, in addition to the compound fed, melilotic acid, o-coumaroylglycine, melilotoylglycine, 4-hydroxycoumarin, o-hydroxyphenyllactic acid, and o-hydroxyphenylacetic acid were detected in the urine. Neither 3-hydroxycoumarin nor 7-hydroxycoumarin was present. 3-Hydroxyphenyllactic acid was crystallized from the urine of rats to which o-hydroxyphenyllactic acid had been administered.

Our findings pertaining to the opening of the heterocyclic ring of coumarin in the animal body and the absence of 3-hydroxycoumarin and 7-hydroxycoumarin in rat urine after ingestion of coumarin are at variance with the report of Mead et al. (2). An explanation of these differences is not readily apparent. These workers also suggested that o-hydroxyphenylhydracrylic acid was an intermediate in the formation of 4-hydroxycoumarin. Our work clearly indicates the presence of appreciable amounts of o-hydroxyphenylhydracrylic acid in rat and rabbit urine as well as smaller amounts of 4-hydroxycoumarin.

The formation of o-hydroxyphenylhydracrylic acid from o-coumaric acid may be visualized to proceed simply by the addition of water across the double bond, the hydroxyl group going on the a carbon of the side chain. However, if the hydroxyl group goes on the a carbon of o-coumaric acid a lactic acid derivative would be formed.

The metabolism of m- and p-coumaric acids has already been shown to proceed via beta oxidation to produce the corresponding benzoic acid derivatives (7, 8). The absence of salicylic acid in the urine after the ingestion of o-coumaric acid suggests that the presence of a hydroxyl group ortho to the 3 carbon side chain may either inhibit beta oxidation, or favor decarboxylation to yield o-hydroxyphenylacetic acid. Mead et al. (2) also reported the absence of salicylic acid as a metabolite of coumarin.

The interconversion of o-coumaric acid and melilotic acid appears to take place readily. A similar condition has been observed for ferulic and dihydroferulic acids (5).

SUMMARY

The finding of o-hydroxyphenylacetic acid in the urine of rats or rabbits receiving coumarin orally indicated that opening of the lactone ring and decarboxylation had occurred, though by an undetermined route. An alternate pathway for the formation of o-hydroxyphenylacetic acid involves o-coumaric acid and o-hydroxyphenyllactic acid as intermediates. Some of the o-coumaric acid was converted to o-hydroxyphenylhydracrylic acid and 4-hydroxycoumarin. A species difference was encountered in that rabbits excreted 3-hydroxycoumarin and 7-hydroxycoumarin after ingestion of coumarin whereas rats did not excrete either of these compounds in detectable amounts.

REFERENCES

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