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Studies on 4-Hydroxycoumarins. XVI. The Metabolism of 4-Hydroxycoumarin in the Dog[1]

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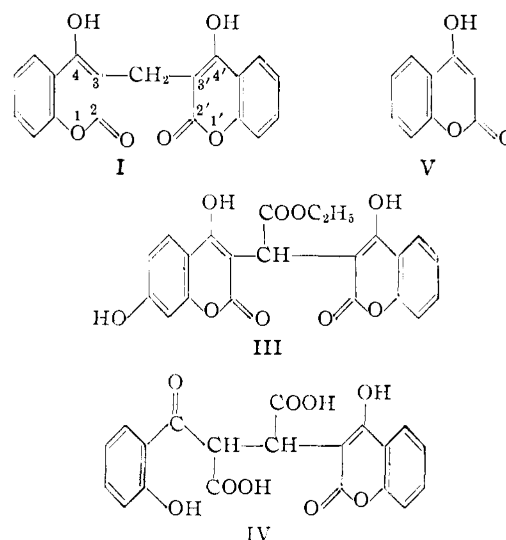
When 4-hydroxycoumarin is injected (0.084 g./kg. body weight) into the blood stream of the dog approximately 75% appears in the urine within 24 hours. The fate of the 25% not recovered is unknown. The urine shows no significant increase in volatile phenols and ethereal sulfates. No salicylic acid was found. Approximately 50% of the 4-hydroxycoumarin injected appears free; the remainder as 4-hydroxycoumarin β-D-glucopyranosiduronic acid. The isolated acid was converted to the methyl uronate, and the methyl uronate to methyl 1-(4-hydroxycoumarin)-2,3,4-tri-O-acetyl-β-D-glucopyranosiduronate. The condensation of 4-hydroxycoumarin with methyl (1-bromo-2,3,4-tri-O-acetyl-α-D-glucuronic acid) uronate gave methyl 1-(4-hydroxycoumarin)-2,3,4-tri-O-acetyl-β-D-glucopyranosiduronate which was identical with the acetylated methyl uronate prepared from the glucuronide isolated from the urine of dogs fed 4-hydroxycoumarin. Dilute acid hydrolysis of 4-hydroxycoumarin β-D-glucopyranosiduronic acid formed *o*-hydroxyacetophenone, identified as its 2,4-dinitrophenylhydrazone. 4-Hydroxycoumarin undergoes a similar degradation under the same conditions.

The primary measurable *in vivo* action of small doses of 3,3'-methylene-bis-(4-hydroxycoumarin)[2] (Dicumarol®) (I) is to cause a reversible hypoprothrombinemia.[3] The postulation that the anticoagulant interferes in some way with the normal synthesis of prothrombin in the body[4] was supported by the experimental work of Lupton,[5] who showed through perfusion experiments with the liver of the rat that this organ was a site of the anticoagulant's action.

Although it had been originally suggested that salicylic acid might be one of the metabolic products of I,[3,6] this has so far not been supported by experiment.[3,7,8] More recently, another possible metabolic course, the loss of the carbon in position 2 of I, was excluded by experiments with Dicumarol-2-C^{14} in mice.[9] Chromatographic analysis of the urine of mice after administration of methylene-labeled I[10,11] indicated that there are at least four unidentified metabolites produced from I. Other workers[12] had evidence that there were at least seven unidentified metabolic products produced by the rat from I. This was deduced from autoradiographs of the filter paper partition chromatograms of urine and plasma from rats given C^{14}-labeled I.



From experiments conducted on the metabolic fate of 3,3'-carboxymethylene-bis-(4-hydroxycoumarin)-ethyl ester (II) Burns, *et al.*,[13] have isolated a metabolite III from the urine of man in which a hydroxyl group was introduced into one of the benzene rings of II. Other workers[14] isolated a metabolite of II from the urine of rabbits in which one of the lactone rings of II had been broken (IV). Compounds III and IV are inactive. It would appear that the bis-3-substituted-4-hydroxycoumarin anticoagulants are metabolized differently by various species of animals.

A formidable difficulty in studying the fate of Dicumarol® and the like *in vivo* is that only small continuous doses can be tolerated by laboratory animals (rat, rabbit, dog). 4-Hydroxycoumarin (V) has about $^1/_{20}$th the anticoagulant action of I.[15] In view of the obvious structural relationship to I, V was chosen for this study. The sodium salt of V was first injected (0.084 g. of V/kg. body weight) into the blood stream of dogs for analytical studies on the urine. The urine was analyzed for

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steam-distillable phenols,[16] ethereal sulfate[16] and glucuronic acid[17] for a control period prior to injecting V and after its administration. The free 4-hydroxycoumarin in the urine was determined by its reaction with formaldehyde to form I, which was isolated and weighed.[18]

TABLE I

DAILY GLUCURONIC ACID CONTENT (AS GLUCURONE) OF DOG URINE BEFORE AND AFTER INJECTING[a] 4-HYDROXYCOUMARIN

Days	Glucurone in daily urine output, g. Dog 1	Dog 2	Days	Glucurone in daily urine output, g. Dog 1	Dog 2
1	0.34	0.50	6[a]	0.80	0.87
2	.44	.39	7	.48	.49
3	.40	.45	8	.45	.51
4	.30	.40	9	.42	.39
5	.47	.45			

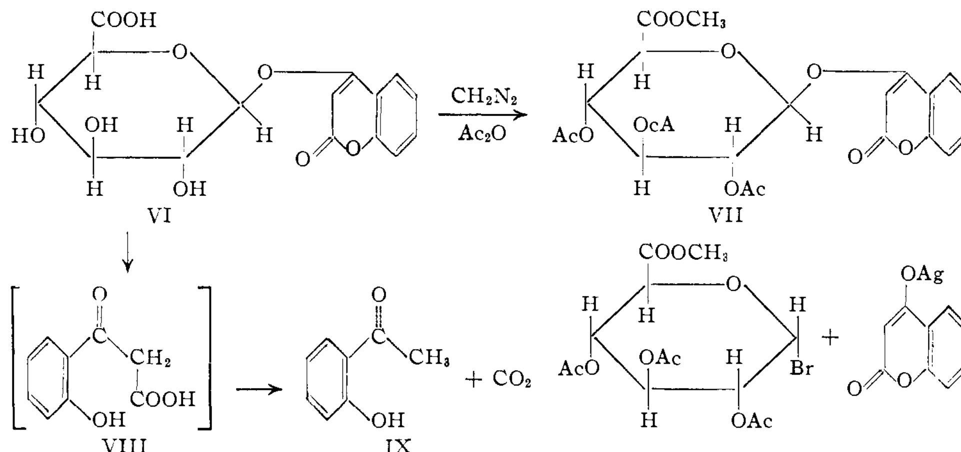
[a] 4-Hydroxycoumarin (1.5 g.) was injected intravenously at the beginning of the sixth day.

The injection of 4-hydroxycoumarin did not cause a significant increase in the urinary output of the steam-distillable phenols or the ethereal sulfate fraction. No salicylic acid was found. However, free 4-hydroxycoumarin appeared in the urine and the glucuronic acid content increased significantly (Table I). For each 1.5 g. of V injected into 18-kg. dogs, approximately 1.2 g. (75%) was detected in the urine. Fifty per cent. was in the free state and 25% was combined as the 4-hydroxycoumarin β-D-glucopyranosiduronic acid (VI). Practically all the free 4-hydroxycoumarin and the glucuronic acid conjugate (VI) excreted, appeared within 24 hours after V was injected (Table I).

When V was fed to dogs it was possible to isolate and characterize both the unchanged 4-hydroxycoumarin and that conjugated as the glucuronide.

The isolated 4-hydroxycoumarin β-D-glucopyranosiduronic acid (VI) was converted to the methyl uronate by reaction with diazomethane, and then to methyl 1-(4-hydroxycoumarin)-2,3,4-tri-O-acetyl-β-D-glucopyranosiduronate (VII). Proof of the structure of VII was obtained by preparing it synthetically by condensing the silver salt of 4-hydroxycoumarin (V) with methyl (1-bromo-2,3,4-tri-O-acetyl-α-D-glucuronic acid) uronate.[19] The physical constants of the natural and synthetic products are identical.

It is interesting to note that when the 4-hydroxycoumarin β-D-glucopyranosiduronic acid (VI) is subjected to a dilute acid hydrolysis, and the hydrolysis is allowed to proceed to completion, no 4-hydroxycoumarin can be isolated. An oil is formed which contains for the most part *o*-hydroxyacetophenone (IX) which is also formed when 4-hydroxycoumarin is subjected to comparable hydrolytic conditions. It was identified and determined by conversion to its 2,4-dinitrophenylhydrazone. Since it is possible to isolate some 4-hydroxycoumarin during the course of the hydrolysis of the glucuronide, it is likely that IX is formed after hydrolysis of the glycosidic bond takes place. The conversion of V to IX probably takes place through the indicated β-keto acid intermediate VIII. This is reminiscent of the decarboxylation of V under alkaline conditions[20] and of the similar degradation of I to 1,3-disalicylylpropane.[21] The mode of synthesis of the glucuronide, VII, suggests that the glycosidic linkage is of the β-type.[19,22]



Experimental

The preliminary quantitative studies for steam-distillable phenols,[16] ethereal sulfate[16] and glucuronic acid[17] were conducted as a guide to the isolation work and were for all practical purposes executed along the well established lines used in the study of the metabolic fate of salicylic acid in man, reported by Kapp and Coburn.[23] We feel that no useful purpose would be served by restating the methods here, and shall therefore give details only for the new work.

Determination of Free 4-Hydroxycoumarin (V).—A one-liter aliquot of urine from an 18-kg. dog into which 1.5 g. of 4-hydroxycoumarin had been injected intravenously 24 hours previously, was adjusted to *p*H 10.0 with dilute potassium hydroxide. The urine was extracted with ethyl ether in a liquid–liquid extractor for four hours. It was then acidified with acetic acid to *p*H 4.0 and extracted for 24 hours to remove V. The ether was evaporated and the resulting light brown oil was taken up in 25 ml. of boiling ethanol. The ethanol solution was filtered while hot, 15

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ml. of 37% formaldehyde solution was added, and the solution was heated on a steam-bath for 15 minutes. The 3,3'-methylene-bis-(4-hydroxycoumarin) (I) formed was collected on a filter, washed and weighed. The melting point was 281–285°. After one recrystallization from cyclohexanone it melted at 288–289° and showed no depression on being mixed with an authentic sample of I.[18] The quantity of I isolated from the first 24-hour urine sample from a dog given 1.5 g. of V was equivalent to 0.784 g. of V. No appreciable quantities of I were obtained from urine samples collected on the second, third and fourth day after V had been injected.

Isolation of 4-Hydroxycoumarin β-D-Glucopyranosiduronic Acid (VI) as the Sodium Salt.—Six 15-kg. dogs were fed 3.25 g. of V daily for three days. The collected urine was filtered through a layer of Filter-cel on a büchner funnel and adjusted to *p*H 6.7. Neutral lead acetate (saturated solution) was added dropwise with vigorous stirring until precipitation ceased.[24] While the lead solution was being added the *p*H of the solution was constantly adjusted to 6.7 with concentrated sodium hydroxide. After a slight excess of lead ion had been added, the *p*H was adjusted to 7.0 and the solution was filtered through Filter-cel. The filtrate and water washings were collected and adjusted to *p*H 7.3. An excess of lead subacetate[25] was then added with vigorous stirring. The lead precipitate was collected on a filter and washed thoroughly with water. The daily urine output was worked up to this stage and then the lead subacetate precipitates from the urine of the three dogs were pooled, and stirred into 1.5 liters of water. With a *p*H control of 6–7 (adjustment with concentrated sodium hydroxide), hydrogen sulfide was bubbled in under stirring to convert all the lead to lead sulfide. The *p*H was then adjusted to 6.8–7.0. The solution was filtered, and the filtrate was concentrated to a volume of about 50 ml. under reduced pressure, from which 7.7 g. of crude sodium salt of the 4-hydroxycoumarin β-D-glucopyranosiduronic acid crystallized. The crude salt was collected and redissolved in 100 ml. of water, the solution was filtered to remove the inorganic sludge and concentrated to about 50 ml. Upon standing, 6.2 g. of nearly pure 4-hydroxycoumarin β-D-glucopyranosiduronic acid, sodium salt separated. After recrystallization from 90% ethanol, it was dried at 100° *in vacuo* for four hours, and showed a constant $[\alpha]^{23}D$ −81.4 (*c* 5, water).

Anal. Calcd. for $C_{15}H_{13}O_9Na$: ash, 6.1. Found: ash, 6.0.

Conversion of the Sodium Salt of VI to the Free Acid.—One and two-tenths grams of the sodium salt was dissolved in slightly less than one equivalent of 0.2 *N* sulfuric acid. Fine, colorless needles of the free acid separated within a few hours. The product, filtered, washed carefully with cold ethanol and dried, weighed 1.0 g. After recrystallization from water it melted at 184–185°; $[\alpha]^{23}D$ −82.8 (*c* 2.0, water). The free acid is fairly soluble in cold water, and very soluble in hot water and ethanol. It gives a positive test with boiling Benedict solution in about a minute, and the Tollens uronide test is strongly positive. The neutralization equivalent was 357.2; the calculated value for $C_{15}H_{14}O_9 \cdot H_2O$ is 356. After drying for eight hours to constant weight at 80° over phosphorus pentoxide *in vacuo*, the acid lost the equivalent of 1/2 mole of water.

Anal. Calcd. for $C_{15}H_{14}O_9 \cdot 1/2H_2O$: C, 51.90; H, 4.32; neut. equiv., 347. Found: C, 51.81; H, 4.28; neut. equiv., 350.

Preparation of Methyl 1-(4-Hydroxycoumarin)-2,3,4-tri-O-acetyl-β-D-glucopyranosiduronate (VII).—One and two-tenths grams of VI was dissolved in 10 ml. of methanol, cooled in ice, and an excess of diazomethane in ether was distilled into this solution following the usual procedure.[26] After standing for several hours, the mixture was concentrated *in vacuo*, and 1.0 g. of crystals (needles) separated. After a preliminary softening they melted at 105°. Three recrystallizations from water and four from ethanol gave m.p. 126–128°, $[\alpha]^{26}D$ −92.7 (*c* 1.5, methanol). The direct yield of the pure methyl uronate was 65%, and 25% more was recovered from the mother liquors. The methyl uronate is readily soluble in methanol and hot ethanol, moderately soluble in cold ethanol and hot water, and practically insoluble in cold water. For analysis the uronate was dried to constant weight at 65° over phosphorus pentoxide *in vacuo*.

Anal. Calcd. for $C_{16}H_{16}O_9 \cdot H_2O$: C, 51.90; H, 4.87. Found: C, 51.60; H, 4.81.

The methyl uronate (0.13 g.) was dissolved in a mixture of 0.5 ml. of pyridine and 4 ml. of acetic anhydride at 0°. The reaction mixture was first maintained at 0° for four hours, and was then allowed to come to room temperature slowly. It was poured cautiously, with vigorous stirring, into a mixture of 10 g. of ice and water. The acetylated methyl uronate (0.17 g.), m.p. 120–123°, precipitated. After two recrystallizations from ethanol the product formed glistening plates, m.p. 125–126°, $[\alpha]^{28}D$ −49.4 (*c* 2, chloroform). The acetylated methyl uronate is soluble in hot methanol and ethanol, and practically insoluble in water. For analysis it was dried for four hours at 80° over phosphorus pentoxide *in vacuo*.

Anal. Calcd. for $C_{22}H_{22}O_{12} \cdot H_2O$: C, 53.50; H, 4.84. Found: C, 53.40; H, 4.53.

Independent Synthesis of Methyl 1-(4-Hydroxycoumarin)-2,3,4-tri-O-acetyl-β-D-glucopyranosiduronate (VII).—One gram of the silver salt of V,[22] 0.9 equivalent of methyl (1-bromo-2,3,4-tri-O-acetyl-α-D-glucuronic acid) uronate[19] and 1.0 g. of Drierite were added to 25 ml. of benzene in a black bottle. The mixture was shaken four days at room temperature. The reaction mixture was then filtered to remove the Drierite and silver salts. These solids were washed with hot ethanol whereupon the ethanol solution was concentrated *in vacuo* at 40° to a sirup. This was dissolved in 10 ml. of hot ethanol, water was added to turbidity and the solution was allowed to cool. Glistening plates, m.p. 125–126°, were obtained; yield 40%. Two recrystallizations from ethanol did not change the m.p., and the $[\alpha]^{28}D$ was −49.4° (*c* 2, chloroform). The melting point of a mixture of the synthetic product and the triacetyl methyl uronate prepared from the excreted 4-hydroxycoumarin β-D-glucopyranosiduronic acid showed no depression. For analysis the product was dried as indicated above.

Anal. Calcd. for $C_{22}H_{22}O_{12} \cdot H_2O$: C, 53.50; H, 4.84. Found: C, 53.20; H, 4.70.

Acid Hydrolysis of 4-Hydroxycoumarin β-D-Glucopyranosiduronic Acid (VI).—Two grams of the sodium salt of VI and 30 ml. of 0.45 *N* sulfuric acid were refluxed for six hours, at which time no glucuronide could be detected. When the hydrolysis was interrupted earlier, some of the original glucuronide and a small amount of free 4-hydroxycoumarin were isolated. The sulfuric acid was removed from the hydrolysate quantitatively with barium hydroxide and, after the solution was concentrated *in vacuo*, an oil remained. The oil was taken up in a small amount of ethyl ether. The ether solution was washed with 5% sodium bicarbonate and then with water. After removing the ether and drying *in vacuo*, 0.45 g. of a fragrant purified oil which exhibited phenolic properties was obtained. One and two-tenths grams of 2,4-dinitrophenylhydrazine and 0.5 ml. of concd. hydrochloric acid were added to a mixture of the oil and water. After warming on a steam-bath, 0.30 g. of the 2,4-dinitrophenylhydrazone of *o*-hydroxyacetophenone, m.p. 207–210°, yield 28%, was isolated. When pure *o*-hydroxyacetophenone was treated in the same manner, the product melted at 210–211°, and the melting point of a mixture of the two showed no depression. For analysis it was recrystallized from ethanol and dried at 80° for six hours *in vacuo*.

Anal. Calcd. for $C_{14}H_{12}O_5N_4$: C, 53.15; H, 3.80. Found: C, 52.86; H, 3.85.

Acid Hydrolysis of 4-Hydroxycoumarin (V).—Compound V (0.25 g.) was refluxed in 15 ml. of 0.45 *N* sulfuric acid for six hours. When the hydrolysis was terminated, 0.011 g. of V remained. This was isolated by conversion to I by condensation with formaldehyde as indicated above.[18] The oil remaining after the removal of V was treated with 2,4-dinitrophenylhydrazine, and gave 0.44 g. of the 2,4-dinitrophenylhydrazone of *o*-hydroxyacetophenone, m.p. 210–211°. Allowing for the recovered 4-hydroxycoumarin, the yield of the *o*-hydroxyacetophenone, as the hydrazone, was 99%.

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