Study of Hydrolysis of Urinary Metabolites of 2-Methyl-1,4-Naphthoquinone. (23897)

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Several investigators have measured the concentration of urinary metabolites of 2-methyl-1,4-naphthoquinone after administration of both radioactive(1-4) and non-radioactive(5-7) forms of this quinone. Since these metabolites have been detected only in conjugated forms, hydrolysis has been a necessary prerequisite to quantitative determination of the non-radioactive compound. Agents used for hydrolysis of these conjugates include enzymes, ceric sulfate, hydrochloric acid, and a combination of stannous chloride and hydrochloric acid. Different recoveries of administered 2-methyl-1,4-naphthoquinone have been obtained by investigators. These variations may be due to differences in hydrolytic procedures employed or in species studied. Richert reported that rabbits excrete 31-42% in forms hydrolyzed by hydrochloric acid(5), and an additional 25-30% liberated by ceric sulfate(6). Rat urine collected 24 hours following injection of 2-methyl-1,4-naphthoquinone was studied for its content of metabolites. Canady(7) demonstrated 50% in a form hydrolyzed with hydrochloric acid alone or in presence of stannous chloride. Li(1) found 13-20% after treatment with hydrochloric acid or bacterial β-glucuronidase. Forty-five % of radioactivity of C^{14} labeled 2-methyl-1,4-naphthoquinone was recovered by Jaques(4). Three conjugates, phosphate, diglucuronide, and monosulfate have been separated by chromatographic methods from urine collected following administration of radioactive 2-methyl-1,4-naphthoquinone(3).

In our investigation the conjugates of 2-methyl-1,4-naphthohydroquinone present in urine of rats were separated by paper chromatography. Two types of urine containing these conjugates were chromatographed: 1) untreated urine and 2) urine remaining after treatment with specific hydrolytic agent. After their chromatographic separation they were hydrolyzed with ceric sulfate and with hydrochloric acid. These procedures permitted estimation of relative concentration of each conjugate and the response of conjugates to different hydrolytic procedures.

Methods. Urine collection. Urine was collected under petroleum ether (B.P. 40-50°C) at 0°C for 24-hour periods from 6 groups of 3 rats each. During these periods, rats were housed in metabolism cages with raised wire floors and subfloors to prevent contamination of urine with feces. They had access to water and ground rat chow *ad libitum*. Examination of such urine for its content of metabolites of 2-methyl-1,4-naphthoquinone was conducted in the following sequence: 1) Urine was collected from normal rats since such urine contains products which influence colorimetric determination of 2-methyl-1,4-naphthoquinone. They were chromatographed, hydrolyzed and measured quantitatively. Values obtained were considered as blanks for each individual rat, and were subtracted to give a true value for 2-methyl-1,4-naphthoquinone present in experimental urine. 2) Urine was collected following administration of 2-methyl-1,4-naphthoquinone. To obtain high urinary concentrations of metabolites this quinone was administered at 11 mg/kg to normal male rats weighing 300-400 g. Vitamin, dissolved in 1 ml of dibutyl succinate was injected subcutaneously into the left hind leg. Urine was examined qualitatively and quantitatively for content of metabolites of 2-methyl-1,4-naphthoquinone. Qualitatively, the conjugates in urine were separated by paper chromatography and isolated conjugates hydrolyzed by hydrochloric acid and by ceric sulfate. Chromatograms were prepared from untreated urine as well as from urine remaining after hydrolysis by procedures indicated below. Quantitatively, a determination was made of 2-methyl-1,4-naphthoquinone liberated following hydrolysis of urine with 1. hydrochloric acid, 2. ceric sulfate, 3. hydrochloric

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acid followed by ceric sulfate, and 4. Ketodase.† It has been demonstrated previously (8) that treatment of normal rat urine and aqueous solutions containing added 2-methyl-1,4-naphthoquinone by hydrolytic and extraction procedures described here permitted a recovery of 82-88%. *Chromatography.* Conjugates of 2-methyl-1,4-naphthohydroquinone were separated by the ascending technic of Hoskin(3) on Whatman No. 1 paper, 17″ x 22½″. A 1 ml aliquot of urine was deposited on a line 9 inches long and 2 such bands were placed on each sheet, one from untreated urine, the other from urine after hydrolysis. Chromatograms were developed in the solvent system containing butanol, methanol, water and acetic acid until the solvent front traveled about 35 cm from origin. After drying in air, the papers were examined with an ultraviolet lamp and from areas of fluorescence and absorption, the positions of conjugates of 2-methyl-1,4-naphthohydroquinone were determined. In each experiment 2 sheets were developed simultaneously. After separation, all conjugates on one sheet were treated with hydrochloric acid, all on the other with ceric sulfate. Hydrolytic procedures were applied either to urine or to paper containing conjugates previously separated by chromatography. When urine was used a volume was selected which would yield approximately 100 μg of 2-methyl-1,4-naphthoquinone following hydrolysis. and this quantity was diluted to 10 ml. Hydrolysis of compounds separated by paper chromatography was effected by cutting the filter paper containing a particular conjugate into 10 ml of water. Urinary conjugates were hydrolyzed by the following agents: A. *Hydrochloric acid.* Ten ml of urine and 1 ml of concentrated hydrochloric acid were placed in flask attached to water-cooled West condenser and the solution boiled 30 minutes. The condenser and its fittings were then washed with petroleum ether and the washings added to the reaction mixture. B. *Ceric Sulfate.* To 10 ml of urine overlaid with petroleum ether were aded 2 ml of glacial acetic acid and 5 ml of a solution of 0.1 N ceric sulfate in 1 N H_2SO_4. This mixture was allowed to stand 5 minutes at room temperature and was then diluted and extracted. C. *Hydrochloric acid followed by ceric sulfate.* Ten ml of urine were hydrolyzed with hydrochloric acid as described above. Hydrolysis with ceric sulfate was then carried out by adding 2 ml of glacial acetic acid and 5 ml of ceric sulfate solution and allowing the mixture to stand 5 minutes at room temperature. D. *Ketodase.* Ten ml of urine at pH 7.0 were placed in flask attached to water-cooled West condenser and heated 30 minutes in boiling water to inactivate urinary enzymes. The solution was cooled to room temperature and the condenser and its fittings were washed with petroleum ether and the petroleum ether washings added to the solution in the flask. pH was adjusted to 4.5 and Ketodase, assayed by the method of Talalay(9), was added at a level of 400 units of β-glucuronidase/ml followed by 0.2 ml of an 80 mg % solution of 8-hydroxyquinoline. Urine was incubated 24 hours at 37°C. *Extraction.* After hydrolysis contents of flask were transferred to a separatory funnel using such volumes of water and petroleum ether that final volumes for extraction were 50 ml of each solvent. The aqueous mixture was extracted twice with 50 ml portions of petroleum ether. The combined petroleum ether extract was washed once with 2 ml of 0.5% $NaHCO_3$ solution and once with 1 ml of water. The volume of petroleum ether was reduced under diminished pressure and last traces were carefully removed by evaporation under stream of nitrogen. The residue was immediately taken up in 1 ml of absolute ethanol. *Assay.* 2-methyl-1,4-naphthoquinone was determined by modification of the procedure of Richert(5). The ethanol solution was transferred to test tube calibrated to contain 6 ml using borate buffer pH 10.5 to complete the transfer. Three drops of ethyl cyanoacetate were added and 10 minutes allowed for color development. Pigments which were extracted from urine with petroleum ether were in this solution and could be almost completely removed by successive extractions with 2 ml portions of petroleum ether and diethyl ether. The solution obtained by application of these technics

† Obtained from Warner-Chilcott Laboratories, N. Y.

TABLE I. Urinary Excretion of 2-Methyl-1,4-naphthoquinone by the Normal Rat.

Method of hydrolysis	Conc. of 2-methyl-1,4-naphthoquinone, μg/ml
HCl	109
$Ce(SO_4)_2$	95
Ketodase	102
HCl followed by $Ce(SO_4)_2$	123

These values represent conc. of this quinone in urine collected for 24 hr following administration of 2-methyl-1,4-naphthoquinone at level of 11 mg/kg.

to paper chromatograms was filtered through a sintered glass funnel. Optical density of final solution was read in Beckman D.U. spectrophotometer at 570 mμ.

Results. Hydrolysis of urine containing metabolites of 2-methyl-1,4-naphthoquinone release this quinone in amounts which were determined by method employed. As indicated in Table I treatment with hydrochloric acid yielded greater amounts (109 μg/ml) than did hydrolysis with ceric sulfate (95 μg/ml). When hydrochloric acid treatment was followed by ceric sulfate 123 μg/ml were released.

Phosphate, glucuronide and sulfate were separated chromatographically from rat urine containing metabolites of 2-methyl-1,4-naphthoquinone. As seen in Table II ceric sulfate and hydrochloric acid were not equally effective in hydrolyzing these conjugates. Boiling in hydrochloric acid liberated more 2-methyl-1,4-naphthoquinone from sulfate than was released after treatment with ceric sulfate, the latter being about 78% as effective as acid in hydrolyzing the sulfate. Ceric sulfate liberated more 2-methyl-1,4-naphthoquinone from the glucuronide and phosphate than was obtained after treatment of these conjugates with hydrochloric acid. In hydrolysis of the glucuronide, hydrochloric acid was 89% as effective as ceric sulfate. A greater difference existed in ability of these reagents to split phosphate, with acid only about 55% as effective as ceric sulfate.

Using data obtained from hydrolyses of these conjugates, estimates were made of their distribution in urine. Hydrolysis with ceric sulfate suggested the following: 22% phosphate, 46% glucuronide, 32% sulfate. After hydrolysis with hydrochloric acid 12% phosphate, 44% glucuronide and 44% sulfate were obtained. These figures indicate that 2-methyl-1,4-naphthohydroquinone was conjugated primarily as glucuronide and to least extent as phosphate.

Chromatograms were also prepared from urine remaining after treatment with the hydrolytic agents. Examination of these chromatograms indicated that conjugated 2-methyl-1,4-naphthohydroquinone was still present. In data summarized on Table III it can be seen that following hydrolysis with hydrochloric acid or with ceric sulfate significant quantities were present in the compound having the R_f of sulfate. Little phosphate and glucuronide remained after treatment with either of these agents. When hydrolysis was accomplished by a combination of hydrochloric acid and ceric sulfate almost all of conjugated hydroquinone was removed. The source of this additional quinone might be inferred from examination of values in Table III. These data suggest that sulfate, present in urine after its hydrolysis with hydrochloric acid was removed from urine which had been hydrolyzed with both hydrochloric acid and ceric sulfate. Doubling the hydrolysis time or concentration of hydrochloric acid did not result in an increased release of 2-methyl-1,4-naphthoquinone from urine. Incubation of

TABLE II. Concentration of 2-Methyl-1,4-naphthoquinone in Conjugates Separated from Normal Rat Urine.

	Conjugate	Method of hydrolysis: $Ce(SO_4)_2$ (μg/ml)	Method of hydrolysis: HCl (μg/ml)
	Sulfate	13.1	18.9
	Glucuronide	21.1	19.4
	Phosphate	11.2	3.9
	Sulfate	13.4	18.8
	Glucuronide	20.5	19.1
	Phosphate	8.7	4.4
	Sulfate	15.3	17.9
	Glucuronide	20.5	18.5
	Phosphate	10.6	6.1
	Sulfate	15.9	18.4
	Glucuronide	21.7	16.8
	Phosphate	8.6	7.3
Avg	Sulfate	14.4	18.5
	Glucuronide	20.9	18.5
	Phosphate	9.8	5.4

Each value above represents avg of 3 determinations.

TABLE III. Effect of Various Hydrolytic Procedures on Urinary Metabolites of 2-Methyl-1,4-naphthoquinone.

Method of hydrolysis	Conjugate	Original urine: Method of hydrolysis $Ce(SO_4)_2$ (μg/ml)	Original urine: Method of hydrolysis HCl (μg/ml)	Urine after hydrolysis: Method of hydrolysis $Ce(SO_4)_2$ (μg/ml)	Urine after hydrolysis: Method of hydrolysis HCl (μg/ml)
HCl	Sulfate	12.9	16.2	17.9	23.9
	Glucuronide	24.4	25.0	.8	.4
	Phosphate	5.5	2.7	.8	.6
$Ce(SO_4)_2$	Sulfate	14.5	19.7	8.2	12.3
	Glucuronide	21.2	20.0	1.5	0
	Phosphate	7.7	4.8	3.3	1.1
Ketodase	Sulfate	17.0	19.5	6.5	7.0
	Glucuronide	21.3	21.2	15.0	11.4
	Phosphate	10.5	7.7	13.5	7.3
HCl followed by $Ce(SO_4)_2$	Sulfate	13.2	14.5	4.7	2.1
	Glucuronide	16.2	14.4	3.7	0
	Phosphate	10.1	7.8	1.9	1.7

urine with Ketodase resulted in removal of small quantities of glucuronide and greater quantities of sulfate. This enzyme preparation did not hydrolyze the phosphate although it contains both an acid and alkaline phosphatase.

Summary. Urinary metabolites of 2-methyl-1,4-naphthoquinone have been separated by paper chromatography and their response to various hydrolytic procedures studied. Three conjugates, phosphate, glucuronide and sulfate were hydrolyzed both by hydrochloric acid and by ceric sulfate. The sulfate was hydrolyzed to a greater degree by hydrochloric acid and this reagent was less effective than ceric sulfate in splitting phosphate and glucuronide. Following treatment of rat urine with hydrochloric acid or with ceric sulfate, significant quantities of sulfate remained unhydrolyzed. A combination of these procedures hydrolyzed the sulfate. 2-methyl-1, 4-naphthoquinone was released from glucuronide and sulfate after incubation in the presence of Ketodase. The conjugate present in lowest relative concentration was phosphate, that present in largest amount was glucuronide.

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