parations. The present method is especially applicable to preparing biopsy and other small specimens of tissue for metabolic examination of processes which are controlled by structural factors.

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EXPLANATION OF PLATES 1 AND 2

PLATE 1

Figs. 1 and 2. The chopper. *A*, cutting-arm carrying blade *B*; *H* 1, handwheel on shaft *Sh* 1 which also carries the two cams; *H* 2, handwheel on shaft *Sh* 2 which also carries the leadscrew. *T*, cutting table on the carriage *C* which is moved by the leadscrew. *Bl*, adjustable block whose position in the frame *F* conditions the thickness of section cut.

PLATE 2

Guinea pig tissues prepared with the chopper and photographed, while indirectly illuminated, in a shallow layer of saline in dishes with black background. The whole of the specimens chopped have been transferred for photographing.

Fig. 4. Cerebellum cut at 0.26 mm. intervals. Note the cortical layers and white matter which show little tendency to separate. There is also little debris.

Fig. 5. Cerebral hemisphere cut at 0.21 mm. intervals in two directions at right angles. The specimen cut was a block 5 mm. square taken from about the middle of the hemisphere, extending from its cortical surface to the lateral ventricle. For cutting, it was laid on the sub-cortical white matter. A portion of this is seen at one end of each fragment.

Fig. 6. Liver cut at 0.3 mm. intervals in two directions at right angles.

Fig. 7. Kidney cut at 0.26 mm. intervals.

The Occurrence of *m*-Hydroxybenzoic Acid in Urine

By E. BOYLAND, D. MANSON, J. B. SOLOMON AND G. H. WILTSHIRE*

The Chester Beatty Research Institute, The Royal Cancer Hospital, London

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In a study of the excretion of oxidation products of naphthalene by rabbits (Boyland & Wiltshire, 1952), 1-naphthol was estimated in urine as the blue indophenol formed with 2:6-dichloroquinone chloroimide (phenol reagent). Under the conditions described at pH 9.2, this indophenol was completely extracted into butanol. It was noticed that the urine of rats and rabbits dosed with naphthalene or 1-naphthol contained in addition a phenol which

* Present address: Rothamsted Experimental Station, Harpenden.

reacted to give a blue indophenol which was not extracted by butanol. The compound could not be detected regularly in urine of normal animals by this method, but it could be detected and estimated by chromatography and was isolated from such urine. The amount of the substance appeared to be increased by acid hydrolysis. The compound isolated was shown to be *m*-hydroxybenzoic acid, which Bray, Lake, Thorpe & White (1950) had detected by paper chromatography as a constituent of normal rabbit urine.

EXPERIMENTAL

Reaction of phenols with the phenol reagent. A number of compounds which react with 2:6-dichloroquinone chloroimide has been listed by Fearon (1944). Extraction of the colour with butanol was used in the present work to avoid interference from coloured products in urine. The extraction also separated phenols into four classes (Table 1) as follows:

(1) Monophenols substituted in the position para to the phenolic hydroxyl gave a green, purple or yellow colour or no reaction, in the butanol.

(2) Other para-substituted phenols containing a second hydroxyl or an acidic group gave similar colours in the alkaline buffer or no reaction.

(3) Monophenols not substituted in the para position gave a blue colour in butanol.

(4) Other phenols with a free para position, but with a second hydroxyl or an acidic group, gave a blue, violet or magenta colour in the alkaline buffer.

Anomalous reactions were observed with naphthoresorcinol and 3-hydroxyphthalic acid (Table 1). All speci-

mens of urine from rabbits dosed with naphthalene or 1-naphthol and some specimens of urine from undosed rabbits which were tested gave a blue colour which was not extracted with butanol. This reaction was presumably due to the presence of phenol with no substituent in the position para to the hydroxyl group and a second acidic group (e.g. carboxyl or phenolic hydroxyl).

Isolation of m-hydroxybenzoic acid from urine of rabbits dosed with naphthalene. (All melting points are uncorrected.) Five rabbits were dosed daily, each with 0.25 g. naphthalene, and the collected urine centrifuged to remove insoluble material. It was acidified to pH 2 by addition of HCl and warmed to 90°. When effervescence had ceased it was cooled and extracted three times with 0.2 vol. ether. Colorimetric tests with the phenol reagent showed that transfer of the required substance was practically complete. The ether extract was washed repeatedly with water and the acidic compounds extracted with 2% NaHCO₃ solution. The NaHCO₃ extract was washed with ether, concentrated to 100 ml. and acidified to pH 4 with HCl. On standing at 0° for 12 hr., crystalline material separated which did not

Table 1. *Colour reactions of phenols with 2:6-dichloroquinone chloroimide at pH 9.2*

(The colour was developed in 0.1 M-Na₂B₄O₇ and extracted with an equal volume of *n*-butanol.)

	Colour in Na ₂ B ₄ O ₇	Colour in butanol
Monophenols		
(a) Substituted in para position		
<i>p</i> -Cresol	None	None
2-Naphthol	None	Green
Di-2-naphthol	None	Purple
(b) Not substituted in para position		
Phenol	None	Blue
1-Naphthol	None	Blue
2-Nitroso-1-naphthol	None	Green
Polyphenols		
(a) Substituted in para position		
Quinol	Yellow	Yellow
(b) Not substituted in para position		
Catechol	Violet	Blue
Resorcinol	Magenta	Blue
Orcinol	Magenta	Blue
Resorcylic aldehyde	None	Blue-green
Naphthoresorcinol	Orange	None
Monophenols with acidic group		
(a) Substituted in para position		
<i>p</i> -Hydroxybenzoic acid	None	None
Sulphosalicylic acid	None	None
2-Hydroxy-1-naphthoic acid	Green	Purple
(b) Not substituted in para position		
Salicylic acid	Blue	None
<i>m</i> -Hydroxybenzoic acid	Blue	None
3-Hydroxyphthalic acid	None	None
Polyphenols with acidic group		
(a) Substituted in para position		
Gentisic acid	None	None
Homogentisic acid	None	None
(b) Not substituted in para position		
γ-Resorcylic acid	Magenta	None
3:4-Dihydroxyphenylalanine	Pink	Violet

react with phenol reagent. This deposit was purified and found to contain naphthalene mercapturic acid (m.p. 169°) and benzoic acid (m.p. 122°). The filtrate was further acidified to pH 2 and extracted with benzene. This removed the remaining benzoic acid, but very little of the phenolic acid. The aqueous solution was concentrated to 5 ml. and filtered while hot. On cooling, there were deposited colourless needles, which gave the appropriate reaction with phenol reagent. These were recrystallized once from hot water and once from benzene, m.p. (alone and mixed with an authentic sample of *m*-hydroxybenzoic acid) 199–200°. Neutralization equivalent found, 148; calc. for hydroxybenzoic acid, 138. Analysis (Found: C, 60.6; H, 4.3. Calc. for $C_7H_6O_3$: C, 60.9; H, 4.4%). The hydroxy acid (0.02 g.) set aside in pyridine with acetyl chloride for 12 hr. gave the *O*-acetyl derivative m.p. (alone and with authentic *m*-acetoxybenzoic acid) 130–131° after crystallization from xylene. (Found: C, 60.0; H, 4.7. Calc. for $C_9H_8O_4$: C, 60.0; H, 4.5%.)

Table 2. Amounts of 3-hydroxybenzoic acid isolated from urine

Source of urine	Volume of urine (l.)	Amount isolated (mg.)	Amount (mg./l.)
Five rabbits each of which received 0.25 g. naphthalene daily for 3 consecutive days	2	31	15
Five rabbits each of which received 0.25 g. naphthalene daily for 3 consecutive days	2	58	29
Five normal rabbits	3	19	6
Five normal rabbits	4.7	Nil	—
Five normal rabbits	10	29	3

Isolation of m-hydroxybenzoic acid from urine of normal rabbits. Urine of normal rabbits was treated as described for the urine of rabbits dosed with naphthalene. Concentration of the acidified $NaHCO_3$ extract gave no crystalline material but only a black tar. This was extracted with 50% $CHCl_3$: 50% methanol. (Preliminary trial showed that *m*-hydroxybenzoic acid could be adsorbed on alumina and recovered in 90% yield by elution with NH_3 .) The extract of the tar was added drop by drop to an alumina column (Savory & Moore) (3×6 cm.), prepared in benzene. After adsorption the column was eluted with the following solvents: $CHCl_3$; 50%

$CHCl_3$ /50% methanol; 25% $CHCl_3$ /75% methanol; methanol and finally 300 ml. NH_3 , sp.gr. 0.88. The ammoniacal eluate was acidified and extracted with ether. The ether was evaporated to yield a gummy residue which was sublimed at 150° (0.2 mm. Hg). The sublimate crystallized from benzene to give *m*-hydroxybenzoic acid, m.p. (alone and with an authentic specimen) 197–198°.

The yields of acid isolated are shown in Table 2.

Acetylation of the product isolated from urine of undosed rabbits with acetyl chloride in pyridine gave the acetate, m.p. (alone and mixed with an authentic specimen) 129–130°. (Found: C, 60.0; H, 4.9. Calc. for $C_9H_8O_4$: C, 60.0; H, 4.5%.)

Properties of the compound. The compound gave a blue colour with the 2,6-dichloroquinone chloroimide phenol reagent and the colour was not extracted by butanol. It gave no colour with $FeCl_3$ solution (salicylic acid gave a characteristic violet colour) and a red colour with diazotized *p*-nitroaniline. It did not reduce ammoniacal $AgNO_3$ solution. It was more soluble in hot water than in cold, very soluble in methanol, ethanol, butanol and ether, but only slightly soluble in benzene and light petroleum.

Separation and estimation of m-hydroxybenzoic acid by paper partition chromatography. Urinary phenolic acids were separated by ascending development on sheets of Whatman no. 1 (special chromatographic) paper hung in a tank so that the bottom of the paper was in contact with butanol saturated with 0.005 *N*- NH_4OH . After development for approximately 40 hr. at room temperature the papers were removed and dried at room temperature. The phenolic acids were detected by spraying successively with freshly diazotized 0.0005 *M*-*p*-nitroaniline solution and then with *M*- Na_2CO_3 , when the *m*- and *p*-hydroxybenzoic acids were revealed as red spots.

Typical R_f values under these conditions were: *o*-hydroxybenzoic acid 0.46, *p*-hydroxybenzoic acid 0.33, *m*-hydroxybenzoic acid 0.26, *m*-hydroxyhippuric acid 0.22 and *p*-hydroxyhippuric acid 0.19.

Analyses for free *m*-hydroxybenzoic acid were carried out on ether extracts obtained by continuous extraction of the urines at pH 2 for 20 hr. Ether-insoluble *m*-hydroxybenzoic acid conjugates were hydrolysed by adjusting the acid content of the extracted urine to 2*N* with H_2SO_4 and boiling under reflux for 1 hr. The hydrolysed urines were adjusted to pH 6 and continuously extracted with ether for 20 hr. The ether extract was then evaporated to dryness and the residue dissolved in 40% (v/v) aqueous ethanol (1–5 ml.).

Quantitative estimations of *m*-hydroxybenzoic acid were made using a relationship between spot area and the

Table 3. The *m*-hydroxybenzoic acid content of some animal and human urine estimated by paper chromatography

	Free <i>m</i> -hydroxybenzoic acid		Conjugated <i>m</i> -hydroxybenzoic acid		Total <i>m</i> -hydroxybenzoic acid Total daily excretion (mg.)
	mg./100 ml. urine	Daily excretion (mg.)	mg./100 ml. urine	Daily excretion (mg.)	
Rabbits (F.)	6.9	15	4.7	10	25
Rabbits (F.)	7.0	15	4.9	10	25
Rabbits dosed with naphthalene	4.7	10	10.1	20	30
Rats (F.)	Nil	Nil	Nil	Nil	Nil
Humans (M.)	0.6	8.8	0.08	1.2	10.0
Humans (F.)	1.0	15.0	0.04	0.6	15.6

logarithm of its acid content (Fisher, Parsons & Holmes, 1949). Two standard solutions of *m*-hydroxybenzoic acid and two dilutions of an ether extract were applied to the paper serially by Exelo pipettes (5 μ l.). Five applications of each solution were made on each sheet of paper, two sheets of paper being used for each determination. After chromatographic development in the same tank and detection of the acid with diazotized *p*-nitroaniline reagent the papers were dried at room temperature. The spots of the *m*-hydroxybenzoic acid dye were traced on graph paper (ruled in 1 mm. squares) from which accurate estimations of areas were obtained. From the mean area the concentration of *m*-hydroxybenzoic acid in a urine extract was found from the formula used by Fisher *et al.* (1949) with errors of $\pm 2-7\%$.

The *m*-hydroxybenzoic acid content of female rats and rabbits and adult male and female human urines have been determined by this method (Table 3). No *m*-hydroxyhippuric acid could be detected in any of the rat urines examined.

DISCUSSION

The isolation of *m*-hydroxybenzoic acid has been reported from the urine of rabbits dosed with β -ionone (Bielig & Hayasida, 1940), urine of pregnant mares (Lederer & Polonsky, 1948) and the scent gland of the beaver (Lederer, 1941, 1949) and from the chemical degradation of juglone (Bernthsen & Semper, 1886). Fischer & Bielig (1940) found that rabbits fed with a number of unsaturated aromatic compounds excreted *m*-hydroxybenzoic acid (which was not present in normal rabbit urine examined by them) and that there was a concurrent increase in the excretion of benzoic acid.

Quick (1932), Williams (1938) and Bray, Ryman & Thorpe (1948) investigated the fate of *m*-hydroxybenzoic acid administered to animals. In men, *m*-hydroxyhippuric acid is the main product; in the dog, a glucuronide. In rabbits 70-97% is excreted as free acid, 6-11% as ester glucuronide, 2-19% as ether glucuronide and 2-4% as ethereal sulphate. Gentisic acid may also be formed.

It was suggested by Fischer & Bielig (1940) that

hydroxybenzoic acids might be formed by oxidation of benzoic acid. However, Smith & Williams (1950) were unable to detect any of the three mono-hydroxybenzoic acids after dosing rabbits with benzoic acid. Lederer (1949) mentioned *m*-tyrosine and *m*-hydroxyanthranilic acid as possible precursors. The latter compound is known to be converted to quinolinic acid in liver (Henderson & Ramasarma, 1949), and it was suggested it might be deaminated by intestinal bacteria. *m*-Tyrosine has not so far been found in animals.

The isolation of the *m*-hydroxybenzoic acid from urine of rabbits which were dosed with naphthalene and from urine of undosed rabbits indicates that it may be the metabolic product of some food constituent as yet unidentified. The acid was isolated in a pure form more easily from the urine of naphthalene-treated rabbits than from untreated rabbits. It was also detected more easily in naphthalene urine by the 2:6-dichloroquinone chloroimide reagent, but the estimation by the paper-chromatographic method indicated no difference in the concentration of the acid in the two types of urine.

SUMMARY

m-Hydroxybenzoic acid was estimated in and isolated from urine of rabbits injected with naphthalene and from untreated rabbits.

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