Biochemical Studies of Toxic Agents

THE ISOLATION OF PREMERCAPTURIC ACIDS FROM THE URINE OF ANIMALS DOSED WITH CHLOROBENZENE AND BROMOBENZENE

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1. A premercapturic acid, i.e. a compound that yields a mercapturic acid when decomposed by acid, was isolated as a dicyclohexylammonium salt from the urine of rats and rabbits that had been dosed with bromobenzene. 2. Another premercapturic acid was isolated as its dicyclohexylammonium salt from the urine of rats that had been dosed with chlorobenzene. 3. When decomposed by acid, the premercapturic acid from the urine of animals dosed with bromobenzene gave *p*-bromophenylmercapturic acid, *m*- and *p*-bromophenol and *NN'*-diacetylcystine. 4. The premercapturic acid derived from chlorobenzene gave the corresponding chloro compounds together with *NN'*-diacetylcystine when decomposed by acid. 5. On the basis of these and other observations it is suggested that the premercapturic acid formed in the metabolism of bromobenzene is *N*-acetyl-*S*-(4-bromo-1,2-dihydro-2-hydroxyphenyl)-L-cysteine. 6. It is also suggested that the premercapturic acid derived from chlorobenzene has an analogous structure.

Baumann & Preusse (1879) demonstrated that *p*-bromophenylmercapturic acid could be isolated from the urine of animals to which bromobenzene had been administered. Though these authors found that only after the urine had been treated with hydrochloric acid could the mercapturic acid be isolated in any appreciable quantities, they did not isolate an acid-labile precursor of the mercapturic acid, and later workers tended to view the mercapturic acid, rather than its acid-labile precursor, as a product of the metabolism of bromobenzene. In 1957, a reinvestigation of the problem by Boyland, Sims & Solomon (1957) and by Knight & Young (1957) demonstrated unequivocally that *p*-bromophenylmercapturic acid appears in the urine as an acid-labile precursor. Boyland & Sims (1958) isolated an acid-labile precursor of 1-naphthylmercapturic acid from the urine of rabbits that had been dosed with naphthalene and showed that the most likely structure for this compound was *N*-acetyl-*S*-(1,2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine. Naphthalene is one of several aromatic compounds that Knight & Young (1958) showed to be metabolized by animals to acid-labile mercapturic acid precursors for which they proposed the name 'premercapturic acids'. More recently, from the urine of animals dosed with phenanthrene and benz[*a*]anthracene, Boyland & Sims (1962, 1964) have isolated premercapturic acids derived from these hydrocarbons, namely *N*-acetyl-*S*-(9,10-

dihydro-9-hydroxy-10-phenanthryl)-L-cysteine and *N*-acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)-L-cysteine.

The present paper describes the isolation of *p*-bromophenylpremercapturic acid as its dicyclohexylammonium salt from the urine of rats and rabbits dosed with bromobenzene and gives an account of some of the properties of the isolated compound. A description is also given of the isolation of *p*-chlorophenylpremercapturic acid as its dicyclohexylammonium salt from the urine of rats dosed with chlorobenzene. A preliminary account of the work has already been presented (Gillham & Young, 1967).

MATERIALS

p-Chlorophenylmercapturic acid, m.p. 153–154°, and *p*-bromophenylmercapturic acid, m.p. 153.5–154°, were obtained by the method of Marsden & Young (1958) from the acidified urine of rats that had been dosed with chlorobenzene and bromobenzene. The acids were converted into their dicyclohexylammonium salts by mixing equimolar amounts of acid and base in solution in acetone. A precipitate was obtained in each case and this was filtered and recrystallized from ethanol-acetone. The dicyclohexylammonium salt of *p*-chlorophenylmercapturic acid had m.p. 181–182° (decomp.), $[\alpha]_D^{25} -21 \pm 2^\circ$ (c 1.3 in ethanol) (Found: C, 60.9; H, 7.9; N, 6.7; S, 7.0; $C_{23}H_{35}ClN_2O_3S$ requires: C, 60.7; H, 7.7; N, 6.2; S, 7.0%). The dicyclohexylammonium salt of *p*-bromophenylmercapturic acid had m.p. 186–187° (decomp.), $[\alpha]_D^{21} -17 \pm 2^\circ$ (c 1.1 in ethanol)

Table 1. *Chromatography of p-chlorophenylpremercaptopuric acid and p-bromophenylpremercaptopuric acid and related compounds*

For experimental details see the text.

Solvent		R_F				
		Paper chromatogram	Thin-layer chromatograms			
			<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
<i>p</i> -Chlorophenylpremercapturic acid*		—	0.66	0.60	0.10	—
<i>p</i> -Chlorophenylmercapturic acid*		—	0.73	0.65	0.30	—
<i>p</i> -Bromophenylpremercapturic acid*		—	0.55	0.64	0.35	—
<i>p</i> -Bromophenylmercapturic acid*		—	0.64	0.69	0.11	—
<i>N</i> -Acetylcysteine		—	0.58	0.56	0.00	—
<i>NN'</i> -Diacetylcysteine*		—	0.38	0.50	0.00	—
<i>o</i> -Chlorophenol		—	—	—	—	0.68
<i>m</i> -Chlorophenol		—	—	—	—	0.30
<i>p</i> -Chlorophenol		—	—	—	—	0.28
<i>o</i> -Bromophenol		0.39	—	—	—	0.70
<i>m</i> -Bromophenol		0.62	—	—	—	0.34
<i>p</i> -Bromophenol		0.75	—	—	—	0.29

* Applied to the chromatogram as the dicyclohexylammonium salt.

(Found: C, 54.6; H, 6.9; N, 5.7; S, 6.6; $C_{23}H_{35}BrN_2O_3S$ requires: C, 55.2; H, 7.0; N, 5.6; S, 6.4%).

NN'-Diacetylcysteine was prepared by the oxidation of *N*-acetylcysteine with iodine and was isolated as the bis-dicyclohexylammonium salt, which was recrystallized from ethanol-acetone to yield leaf-like crystals, m.p. 191–192° (decomp.) (Found: C, 59.7; H, 9.1; N, 7.5; S, 9.1; $C_{34}H_{62}N_4O_6S_2$ requires: C, 59.4; H, 9.1; N, 8.2; S, 9.3%).

N-Acetylcysteine, chlorobenzene, bromobenzene, *o*-, *m*- and *p*-chlorophenol and *o*- and *p*-bromophenol were purchased from British Drug Houses Ltd., Poole, Dorset, and *m*-bromophenol was bought from Kodak Ltd., Kirkby, Liverpool. All phenols were redistilled under reduced pressure before use.

METHODS

Elementary microanalyses were carried out by Weiler and Strauss, Oxford. Melting points are uncorrected. Ultraviolet extinction coefficients were measured with a Hilger Uvispek spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer model 237 spectrophotometer.

Animals and dosing. Male rabbits, body wt. approx. 2 kg., maintained on SG.1 diet, cabbage and water, were each given 2 g. of bromobenzene as a 20% (v/v) suspension in water by stomach tube. The urine was collected separate from the faeces for 48 hr., after which time the dose was repeated and the urine was collected for a further 48 hr. The urine was filtered through glass wool and stored at 4° until required.

Male black and white hooded rats, average body wt. 180–220 g., were each injected subcutaneously with 0.2 g. of bromobenzene as a 20% (w/v) solution in arachis oil. The rats were housed in metabolism cages suitable for the collection of urine separate from faeces and were fed on Oxoid pasteurized mouse cubes and water. The urine was collected for the 24 hr. immediately after dosing, and the dosing was repeated 48 hr. after the first dosing and the

urine was collected for the next 24 hr. The urines were filtered through glass wool and stored at 4° until required.

Similar experiments were carried out in which chlorobenzene was administered to rats, except that in these experiments the animals were barrier-maintained C.F.E. albino rats, derived from a specific-pathogen-free colony, and each rat was dosed on three occasions.

Chromatography. The bromophenols were separated by paper chromatography by the descending method at room temperature. Whatman no. 4 chromatography paper that had been dipped in 0.2M- Na_2CO_3 was used with solvent mixture A: *n*-hexane-di-isopropyl ether (9:1, v/v) (Gessner & Smith, 1960).

Other compounds were separated by thin-layer chromatography. Glass plates (10 cm. × 20 cm.) were coated with silica gel G (E. Merck A.-G., Darmstadt, Germany) of 0.25 mm. thickness and heated at 105° for 1 hr. For sulphur-containing compounds the chromatograms were developed for 15 cm. with the following solvent mixtures: B, butan-1-ol-acetic acid-water (12:3:5, by vol.); C, chloroform-methanol-aq. 17% (w/w) ammonia (2:2:1, by vol.); D, benzene-acetic acid (3:1, v/v). Solvent mixture E, chloroform-xylene (9:1, v/v), was used for separating phenols. A list of typical R_F values is given in Table 1.

Spraying with $K_2Cr_2O_7$ and $AgNO_3$ solutions (Knight & Young, 1958) was used to detect compounds that contained bivalent sulphur. Phenols were detected by spraying the chromatograms with freshly diazotized sulphanilic acid followed by aq. ammonia (sp.gr. 0.88).

Separation of the chlorophenols was achieved with a Perkin-Elmer F11 gas chromatograph equipped with a flame-ionization detector and a linear temperature programmer. The column used was similar to that described by Brooks (1959) and consisted of a stainless-steel tube (2 m. long × 2.2 mm. internal diam.) into which had been packed hexamethyldisilazane-Chromosorb W (100–120 B.S. mesh) with a stationary phase of 5% (w/w) tris-(2,4-xylene)l phosphate. The flow rate of the carrier N_2 was 35 ml./min.

The temperature of the injection block corresponded to setting 5, and the temperature of the column was held at 150° for 5 min. after injection of the sample dissolved in ether, and was then raised to 170° at 5°/min.

RESULTS

Isolation of p-bromophenylpremercapturic acid from urine

The pooled urines from either rats or rabbits were brought to pH 4.0 with acetic acid and extracted by shaking for 30 min. with an equal volume of butan-1-ol. The two layers were separated by centrifuging and the urine was restored to its original volume by the addition of water. The diluted urine was readjusted to pH 4.0 with acetic acid and the extraction with butan-1-ol was repeated. The two extracts were combined and adjusted to an apparent pH value of 7.5–8.0 with aq. ammonia (sp.gr. 0.88). The ammoniacal extracts were concentrated to 50 ml. under reduced pressure below 45°.

To the concentrated extract was added 5 ml. of cyclohexane and 0.5 ml. of aq. ammonia (sp.gr. 0.88). The resulting mixture was applied to the top of a column (4.5 cm. diam. × 80 cm.) into which had been packed 1 kg. of cellulose powder (Whatman Chromedia CF 11) as a slurry in the solvent mixture butan-1-ol–cyclohexane–aq. 2N-ammonia (9:2:1, by vol.), and the column was developed by the passage of the same solvent mixture through it. The first 2.5 l. of solvent was discarded and subsequently 200 fractions (each 25 ml.) were collected. From every fifth fraction 10 μ l. was applied to the origin of a 0.25 mm. thin-layer plate, which was developed in solvent mixture *D*. The plate was dried and sprayed with the potassium dichromate–silver nitrate reagent. The fractions that were found to contain premercapturic acid free from the mercapturic acid were combined and evaporated under reduced pressure below 45° to about 3 ml. This gave a white precipitate from which the concentrate was decanted. The precipitate was washed with 2 ml. of butan-1-ol and the washings were added to the 3 ml. of concentrate. The precipitate was shown to be urea and was discarded.

The 5 ml. of butan-1-ol solution was applied as a streak across the base line of 12 1 mm.-thick silica-gel thin-layer plates (20 cm. × 20 cm.), which were then developed in solvent mixture *C*. After the plates had been dried the bands containing the premercapturic acid were detected by spraying two narrow vertical strips of the plate with the potassium dichromate–silver nitrate reagent. The premercapturic acid was eluted by shaking the silica gel for 30 min. with a mixture of 95 ml. of methanol and 5 ml. of aq. ammonia (sp.gr. 0.88). The silica gel was then removed from the suspension by centrifuging and filtering.

The methanol extract was evaporated to a light-brown gum under reduced pressure below 45°, and the gum was dissolved in 50 ml. of acetone. An estimate of the amount of premercapturic acid present was made by measuring by ultraviolet spectrophotometry the mercapturic acid liberated by acid decomposition of a small known volume of the solution. An amount of dicyclohexylamine slightly in excess of an equimolar proportion was then added to the main solution and the mixture was evaporated to a gum, which was dried *in vacuo* over phosphorus pentoxide at 4° for 16 hr. This yielded a fluffy white solid, which was removed from the flask with ice-cold acetone and recrystallized twice from the minimum volume of methanol by the addition of benzene. This yielded needle-like crystals, which were dried *in vacuo* at room temperature.

The amounts of pure dicyclohexylammonium salt isolated in two experiments, in each of which a total of 7.2 g. of bromobenzene was administered to a group of 18 rats, were 0.100 g. and 0.157 g.; and in an experiment in which three rabbits received a total of 12 g. of bromobenzene the amount of the salt isolated was 0.198 g. The weights of the crude dicyclohexylammonium salts that were separated in these experiments were two to three times those of the pure products.

Properties of p-bromophenylpremercapturic acid

The dicyclohexylammonium salt of the *p*-bromophenylpremercapturic acid isolated from rat urine, and also that isolated from rabbit urine, had m.p. 162–163°; a mixture of samples from these two sources also had m.p. 162–163°. The specific optical rotations were as follows: $[\alpha]_D^{25} - 305 \pm 10^\circ$ (from rat urine) and $[\alpha]_D^{18} - 298 \pm 10^\circ$ (from rabbit urine), both in water (*c* 0.2). The u.v.-absorption spectra of the compounds isolated from rat urine and from rabbit urine both showed an absorption maximum, when measured in aqueous solution, of 267 m μ with ϵ 2900. The i.r. spectra of the mercapturate and the premercapturate were measured in potassium bromide disks and found to be quite different from one another. The i.r. spectrum of the premercapturate obtained from rat urine appeared to be identical with that of the premercapturate from rabbit urine.

After they had been dried at 100° at 1 mm. for 30 min. samples of the premercapturates gave the following analytical results: C, 53.1; H, 7.3; N, 5.2; S, 6.2% (from rat urine); C, 53.3; H, 7.0; N, 5.2; S, 6.1% (from rabbit urine). The empirical formula for the dicyclohexylammonium salt of *N*-acetyl-*S*-(bromodihydroxyhydroxyphenyl)-L-cysteine, C₂₃H₃₇BrN₂O₄S, requires C, 53.4; H, 7.2; N, 5.4; S, 6.2%.

When the premercapturate obtained from rat urine was examined by thin-layer chromatography after it had been stored at 4° over phosphorus pentoxide for 6 months, no evidence of decomposition was found.

Decomposition with acid. When 0.5 ml. of conc. hydrochloric acid was added to 21.9 mg. of the dicyclohexylammonium salt dissolved in 1.0 ml. of water a precipitate formed. This was filtered off and dried *in vacuo* over phosphorus pentoxide. The product, alone and when mixed with *p*-bromophenylmercapturic acid, had m.p. 151–152°. It weighed 10.9 mg., and this represented an 80% conversion of the premercapturic acid into the mercapturic acid. The solution that was left after the acid decomposition of the premercapturic acid was extracted four times with equal volumes of ether. After being dried over anhydrous sodium sulphate, the combined ether extracts were evaporated to a gum, which was taken up in 0.25 ml. of ethanol and chromatographed in solvent mixtures *A* and *E*. This revealed traces of both *m*- and *p*-bromophenol. Chromatography of the extract in solvent mixture *B* showed that *NN'*-diacetylcystine was present.

A solution of 0.5 mg. of the dicyclohexylammonium salt of the premercapturic acid in 0.5 ml. of sodium citrate–citric acid buffer, pH 3.0, was left at room temperature for 24 hr., and 0.1 ml. portions were examined by thin-layer chromatography with solvent mixture *D* and the potassium dichromate–silver nitrate detecting reagent. No *p*-bromophenylmercapturic acid was detected. This procedure would have revealed the presence of the mercapturic acid in amounts corresponding to 5% or more of the premercapturic acid used.

Decomposition with Raney nickel. A solution of 50 mg. of the dicyclohexylammonium salt in 0.5 ml. of cyclohexanone was sealed into a tube (0.5 cm. diam. × 5 cm.) with 0.25 g. of Raney nickel (British Drug Houses Ltd.). The tube was rotated in a water bath for 3 hr. at 60–70°. It was then opened and the contents were examined chromatographically in solvent mixtures *A*, *B* and *E*. Most of the sulphur was no longer in solution, but traces of *p*-bromophenylmercapturic acid and *m*-bromophenol were found.

Isolation of p-chlorophenylpremercapturic acid from urine

The procedure used for the isolation of the premercapturic acid excreted after the administration of chlorobenzene to rats closely resembled that described above for the isolation of *p*-bromophenylpremercapturic acid from urine. In an experiment in which 18 rats received a total of 10.8 g. of chlorobenzene, the amount of the crude

dicyclohexylammonium salt of *p*-chlorophenylpremercapturic acid obtained was 0.5 g., from which 0.207 g. of the pure salt was separated.

Properties of p-chlorophenylpremercapturic acid

Dicyclohexylammonium *p*-chlorophenylpremercapturate was obtained as needle-like crystals. This product was dried *in vacuo* at room temperature, m.p. 159–160°, $[\alpha]_D^{25} - 271 \pm 10^\circ$ (c 0.4 in water). In aqueous solution the compound showed an absorption maximum of 263 m μ with ϵ 2800. The i.r. spectrum of the dicyclohexylammonium salt of *p*-chlorophenylpremercapturic acid closely resembled that obtained with the corresponding salt of *p*-bromophenylpremercapturic acid.

After it had been dried at 100° at 1 mm. for 30 min. a sample of dicyclohexylammonium *p*-chlorophenylpremercapturate gave the following analytical results: C, 58.6; H, 8.1; N, 6.0; S, 7.0; C₂₃H₃₇ClN₂O₄S requires: C, 58.4; H, 7.9; N, 5.9; S, 6.7%.

Decomposition with acid. To 25 mg. of the dicyclohexylammonium salt of the premercapturic acid in 1 ml. of water was added 0.5 ml. of conc. hydrochloric acid. The mixture was left overnight at 4° and the crystalline precipitate that formed was separated by centrifuging and dried *in vacuo* over phosphorus pentoxide; the m.p. and mixed m.p. with *p*-chlorophenylmercapturic acid was 152–154°. The crystals weighed 7.5 mg. and this corresponded to 52% of the premercapturic acid.

The supernatant liquid obtained after separating the mercapturic acid was found to contain *NN'*-diacetylcystine when examined by thin-layer chromatography with solvent mixture *B*. The supernatant liquid was extracted with ether, and when the ether extract was examined by thin-layer chromatography with solvent mixture *E*, and also by gas–liquid chromatography, it was found to contain both *m*- and *p*-chlorophenol.

Decomposition with Raney nickel. Only *m*-chlorophenol was found when thin-layer chromatography and gas–liquid chromatography were used to examine the products obtained by decomposing *p*-chlorophenylpremercapturic acid with Raney nickel in cyclohexanone by the procedure used for *p*-bromophenylpremercapturic acid.

DISCUSSION

Early work (e.g. Baumann & Preusse, 1879) on the metabolism of bromobenzene gave indications that the mercapturic acid that was obtained from the urine of the dosed animals was formed by the breakdown of a precursor when the urine was acidified. This precursor, i.e. the premercapturic acid (Knight & Young, 1958), has only recently been

isolated (Gillham & Young, 1967). The first premercapturic acid to be isolated from urine was obtained by Boyland & Sims (1958) from the urine of rabbits that had been dosed with naphthalene. The properties of the compound, and in particular the fact that it gave 1-naphthylmercapturic acid and 2-naphthol when decomposed by alkali, and that it gave these compounds together with 1-naphthol and *NN'*-diacetylcystine when decomposed by acid, led Boyland & Sims (1958) to suggest that it was *N*-acetyl-*S*-(1,2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine.

In the present investigation the empirical formula of the dicyclohexylammonium salt of *p*-bromophenylpremercaptopuric acid corresponded to that of the dicyclohexylammonium salt of a dihydromonohydroxy derivative of *p*-bromophenylmercapturic acid, and on acid decomposition the compound yielded *p*-bromophenylmercapturic acid, *m*- and *p*-bromophenol and *NN'*-diacetylcystine. The absorption spectrum showed an absorption maximum at $267\text{m}\mu$ with ϵ 2900. (The values for the dicyclohexylammonium *p*-chlorophenylpremercapturate were $263\text{m}\mu$ with ϵ 2800.) For the compound that they isolated from the urine of rabbits dosed with chlorobenzene, and that they identified as a cyclohexa-1,3-diene derivative, namely 1-chloro-3,4-dihydro-3,4-dihydroxybenzene, Smith, Spencer & Williams (1950) found a single band with an absorption maximum at $264\text{m}\mu$ and ϵ 3030. These various considerations lead to two possible structures, (I) and (II), for the premercaptopuric acid.

Acid decomposition of both compounds (I) and (II) would be expected to yield *p*-bromophenylmercapturic acid (III) by the loss of the elements of water. The detection of *m*- and *p*-bromophenol and *NN'*-diacetylcystine among the acid-decomposition products of the premercapturic acid shows that breakdown of the bond between the sulphur atom and the ring carbon took place. If this gave rise to the intermediate formation of the corresponding dihydrodihydroxy derivative, it is to be expected that, whereas the compound derived from compound (II) would give only *p*-bromophenol, that from compound (I) would give *m*- and *p*-bromophenol, as was observed to occur.

Further support for structure (I) is provided by the experiment in which *m*-bromophenol was formed when Raney nickel acted on the premercapturic acid, for any bromophenol formed under these conditions would be expected to reflect the position of the hydroxyl group attached to the ring of the premercapturic acid.

The evidence at present available is consistent with the belief that the premercapturic acid isolated from the urine of rats and rabbits dosed with bromobenzene had structure (I), namely *N*-acetyl-*S*-(4-bromo-1,2-dihydro-2-hydroxyphenyl)-L-cysteine.

The properties of *p*-chlorophenylpremercapturic acid corresponded closely to those of *p*-bromophenylpremercapturic acid, and the two compounds appear to have analogous structures.

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