CXII. THE METABOLISM OF NAPHTHALENE IN RABBITS.

BY MARGHERITA COTONIO BOURNE AND LESLIE YOUNG¹.

*From the Department of Physiology and Biochemistry,
University College, London.*

(Received April 21st, 1934.)

INTEREST in the metabolism of aromatic hydrocarbons has been stimulated by the recent work on the carcinogenic properties of certain members of this group of substances. But the problem of the metabolism of naphthalene is of especial interest because of the property which this substance possesses of being able to produce, when administered to rabbits in repeated doses, an opacity of the crystalline lens of the eye which, histologically, closely resembles human senile cataract [Bouchard and Charrin, 1886; Adams, 1930].

The work reported in the present paper forms part of a series of investigations directed towards the discovery of the mechanism by which naphthalene acts upon the organism so as to induce degenerative changes in the lens-proteins. At present nothing is known of this mechanism; it is still uncertain whether naphthalene or some derivative of it formed in the body acts directly on the lens, or whether the cataract is a secondary phenomenon resulting from some metabolic disturbance or toxic action upon a tissue remote from the eye. An essential step in the solution of this problem appeared to be to obtain some knowledge of the fate of ingested naphthalene. Therefore an attempt to identify the excretory products of naphthalene in the urine of rabbits has been made.

It is the purpose of the present paper to describe the isolation and identification of one metabolic product of naphthalene, *viz.* *l*- α -naphthylmercapturic acid; and to present evidence for the existence of a second metabolite, which has not been isolated, but which gives rise to naphthalene on acidification.

EXPERIMENTAL.

Buck rabbits weighing between 2 and 3 kg. and feeding on oats and cabbage were used exclusively. Naphthalene was dissolved in warm liquid paraffin and administered by means of a stomach tube. Doses of 1 or 2 g. were given to each rabbit and repeated at intervals of 2 or 3 days. The rabbits were kept in individual metabolism cages of suitable size, and the urine was collected separate from the faeces.

Colour tests for naphthols and glycuronic acid.

Certain authors [Igersheimer and Ruben, 1910; Edelfsen, 1905], on the basis of colour reactions applied to the urine excreted by dogs after naphthalene feeding, concluded that naphthalene was oxidised to naphthol in the body and was excreted in the urine as α - or β -naphthylglycuronate. Lesnick [1888] isolated a small amount of chemically pure α -naphthylglycuronate from dog's urine after the feeding of 12 g. of naphthalene. Penzoldt [1886] was unable to

¹ Bayliss-Starling Memorial Scholar.

obtain positive tests for naphthols, but presented evidence for the presence of naphthoquinone. Baumann and Herter [1877] obtained no naphthol on distillation of acidified naphthalene urine and reported that with steam-distillation only unchanged naphthalene came over.

We have not succeeded in isolating either naphthols or naphthylglycuronate from rabbit urine after dosing with naphthalene, but we have obtained positive diazo and ferric chloride reactions when these tests were applied to the residue from an ether extract of acidified naphthalene urine after hydrolysis. We have also obtained positive naphthoresorcinol tests for glycuronic acid. However, when these tests are applied to so complex a mixture as urine, the results cannot be interpreted with certainty, and we are led to conclude that, although naphthols may be present as ethereal sulphates or glycuronates, the amount is small and they do not appear to be the chief metabolic product of naphthalene in the case of the rabbit.

A compound giving rise to naphthalene on acidification.

The urine of rabbits after naphthalene feeding contains a soluble compound which yields naphthalene on acidification. This was demonstrated in the following manner. The alkaline urine excreted by a rabbit during the 24 hours following a 2 g. dose of naphthalene was collected. It was divided into two equal portions, each portion being placed in a conical flask. One portion was made acid with HCl, the other was kept as control and both were heated on a boiling water-bath. Within a short time colourless crystals began to collect on the cool parts of the flask containing the acidified urine; in the control flask no crystals appeared. The crystals were collected, washed with dilute acid, dilute NaOH and water; they were dried and resublimed. Their melting-point was found to be 79.5°; a mixture of these crystals and naphthalene also melted at 79.5°. A picrate melting at 149.5° was prepared from them. Their identity as naphthalene was thus established.

Naphthalene was not present in the alkaline urine, since none was sublimed out on heating the control flask, but was rapidly formed on acidification from some soluble precursor present in the urine. Efforts to isolate such a substance have so far been unsuccessful. The observation described might be accounted for by the presence in the urine of some compound such as dihydro- β -naphthol, a soluble compound which is rapidly converted to naphthalene by mineral acids [Bamberger and Lodter, 1895], and which might conceivably be formed in the body as a product of naphthalene metabolism.

1- α -Naphthylmercapturic acid.

This compound is not present in the urine of normal rabbits, nor does it occur after the feeding of liquid paraffin alone. It was found in the urine of rabbits after naphthalene feeding and was isolated in the following manner. The urine excreted by three rabbits during the 48 hours following the administration of 2 g. naphthalene to each rabbit was collected and mixed. It was made just neutral to litmus with HCl and then conc. HCl was added in the proportion of 10 ml. of acid for 100 ml. urine. The acidified urine was allowed to stand for about $\frac{1}{2}$ hour after which it was twice extracted with an equal volume of chloroform. The chloroform layer was collected and transferred to a distillation flask. Most of the chloroform was distilled off on a water-bath, and when the extract had been reduced to a small volume, it was transferred to an evaporating dish and the remainder of the chloroform carefully evaporated off with gentle heating on the water-bath.

The dark-brown, gummy residue so obtained was dissolved in a small volume of absolute ethyl alcohol. The alcohol solution was filtered if necessary and then diluted with 0.5*N* HCl until opalescent. After standing in the ice-chest overnight a considerable bulk of precipitate consisting of colourless crystals mixed with amorphous brown material settled out. After this had been filtered off a second crop of crystals was sometimes obtained by further dilution of the filtrate.

The precipitate, after washing with water, was extracted with saturated barium hydroxide solution. The filtered baryta extract was acidified with HCl, whereupon an impure crystalline product separated out. This material was filtered off, washed with water and dried on a porous plate. The impurities present at this stage, which imparted a pinkish-brown colour to the material, possessed solubilities similar to those of the desired substance; further purification was therefore a matter of some difficulty. For this purpose the dried material was treated with a few ml. of cold 2*N* NaOH; at first all the material went into solution, but upon brisk stirring the sodium salt of the compound separated out. This precipitate was filtered off immediately on a sintered glass filter and washed with a few ml. 2*N* NaOH. The filtrate, which was coloured dark-brown, contained most of the contaminating substances. The sodium salt was dissolved in water; upon acidification of the solution the acid was precipitated as colourless crystals. Treatment with 2*N* NaOH was repeated if the product obtained was more than faintly coloured.

The final stages of the purification consisted in repeated recrystallisations from aqueous alcohol until a colourless product of constant melting-point was obtained.

Amounts of 1- α -naphthylmercapturic acid present in urine. From the 24 hours' urine of a single rabbit after 1 g. dose of naphthalene 52 mg. of the pure compound were obtained; 260 mg. of the pure compound were isolated from the mixed 24 hours' urine of three rabbits after the administration of 1 g. of naphthalene to each rabbit. Yields of between 300 and 400 mg. of the pure compound were obtained from the mixed 48 hours' urine of three rabbits after the administration of 2 g. of naphthalene to each rabbit.

Properties of 1- α -naphthylmercapturic acid. It is an easily crystallisable, colourless, odourless solid; needles are obtained from aqueous alcohol; m.p. 170.5–171° (uncorr.). It is insoluble in water and in dilute mineral acids; soluble in alcohol, chloroform and pyridine; less soluble in ether, and only slightly soluble in hot benzene and toluene; soluble in dilute NaOH, barium hydroxide, sodium bicarbonate and dilute ammonia; completely soluble in conc. H₂SO₄ and when the solution is warmed a clear green colour develops. The compound is an acid and leaves no residue on ashing. When heated with soda-lime ammonia is easily split off. Carbon, nitrogen and sulphur were shown to be present; the test for ethereal sulphates was negative.

Analysis. After drying *in vacuo* at 80° over P₂O₅ the following analytical values were obtained (Schoeller). (Found: C, 62.14, 62.22; H, 5.23, 5.38; S, 11.24, 11.16; N, 4.75, 4.64 %. C₁₅H₁₅O₃NS requires C, 62.28; H, 5.23; S, 11.09; N, 4.84 %.)

Mol. wt. By the camphor method, 311 (Schoeller); theory requires 289.

Neutralisation equivalent. The substance was dissolved in neutral alcohol and titrated with *N*/50 NaOH using phenolphthalein as an indicator. Found: 292; C₁₅H₁₅O₃NS as a monobasic acid requires 289. The compound is therefore a monobasic acid.

Optical activity. The compound is laevorotatory; a 1 % solution in absolute ethyl alcohol has $[\alpha]_D^{25} - 25^\circ$.

Constitution of 1- α -naphthylmercapturic acid.

Decomposition by acid. 0.669 g. of the substance was boiled for $\frac{3}{4}$ hour under reflux with 50 ml. dilute H_2SO_4 (12.5 % by volume). About half of the material remained undissolved and was removed by filtration of the hot solution. As the filtrate cooled slightly a small amount of crystalline material precipitated; this was also removed by filtration. The filtrate, while still warm, was made slightly alkaline to litmus by the cautious addition of concentrated ammonia. Upon cooling a precipitate appeared which separated out in crystalline form when the solution was allowed to stand in the ice-chest overnight. This precipitate was filtered off on a sintered glass filter; washed with water and dried at room temperature in the air; yield 0.183 g. This was recrystallised from hot water and a pure product in poor yield was obtained.

The recrystallised product was dried in air at 37° and had the following properties: insoluble in cold water, cold alcohol, ether, chloroform, and sodium bicarbonate solution; soluble in hot water, hot alcohol, dilute NaOH and dilute H_2SO_4 ; completely soluble in conc. H_2SO_4 and on warming the same green colour as is obtained with the parent substance developed. It had no definite melting-point but decomposed between 160 and 165°.

Analysis (Schoeller). (Found: C, 63.23, 63.08; H, 5.36, 5.36; S, 13.10; N, 5.44 %. $\text{C}_{13}\text{H}_{13}\text{O}_2\text{NS}$ requires C, 63.12; H, 5.30; S, 12.95; N, 5.66 %.)

A comparison of the empirical formula of this derivative with that of the parent substance showed that the two differed by $\text{C}_2\text{H}_2\text{O}$. It was therefore concluded that the effect of acid hydrolysis had been to split off one acetyl group from the molecule according to the equation:

Acetyl estimation. The presence of one acetyl group in the molecule of the parent substance was proved by micro-acetyl estimation. (Found: 14.27, 14.21 % acetyl (Roth); theory requires 14.89 %.)

Decomposition by alkali. When boiled with dilute sodium hydroxide the compound was decomposed with the evolution of ammonia. Upon subsequent acidification of the solution a cloudiness appeared and after standing in the ice-chest, an oil separated out, which was soluble in alcohol and ether, gave a positive test for sulphur and had a characteristic unpleasant odour which suggested naphthylmercaptan. Since its low melting-point made it unsuitable for analysis, it was decided to oxidise it in the hope of obtaining a dinaphthyl-disulphide. Naphthylmercaptans are easily oxidised by the oxygen of the air in the presence of dilute sodium hydroxide.

0.498 g. of the parent substance was suspended in 50 ml. of 6 % NaOH and boiled for $\frac{3}{4}$ hour under reflux. The solution was then cooled, mixed with 50 ml. water and aerated for 48 hours. The solution soon became turbid, and a colourless solid separated out during the aeration period. After aeration was complete the mixture was allowed to stand in the ice-chest overnight to insure complete precipitation; the precipitate was filtered off, washed with water, and recrystallised from aqueous ethyl alcohol. In this way a product of definite melting-point, suitable for purification and analysis, was obtained.

It was a crystalline substance melting at 89°; soluble in ether and hot alcohol. *Analysis* (Schoeller): (Found: C, 75.60; H, 4.26; S, 20.17 %. $\text{C}_{10}\text{H}_7\cdot\text{S}\cdot\text{S}\cdot\text{C}_{10}\text{H}_7$ requires C, 75.44; H, 4.43; S, 20.15 %). This substance is therefore identified as $\alpha\alpha$ -dinaphthyl-disulphide (M.P. 91°), the melting-point of the $\beta\beta$ -form being 139°.

The fact that $\alpha\alpha$ -dinaphthylsulphide could be isolated when alkaline decomposition of the compound was followed by aeration proves that α -naphthylmercaptan was one of the products of alkaline hydrolysis of the compound $C_{15}H_{15}O_3NS$.

Formula. On the basis of the information obtained by the acid and alkaline hydrolyses of the compound a probable constitutional formula can be deduced.

One acetyl group is present in the molecule, and the amphoteric character of the deacetylated compound suggests that the acetyl group is combined with nitrogen. Since one of the oxygen atoms is known to belong to an acetyl group, the possibility that the compound is a sulphonic acid is ruled out, and the acidic properties of the substance must be attributed to the presence of a carboxyl group.

That the compound is a naphthalene derivative is proved by the identification of α -naphthylmercaptan as one of the products of alkaline decomposition. The facts that the naphthalene nucleus is linked to the rest of the molecule through sulphur and that the linkage is in the α -position are also established by this means.

The following fragments have therefore been identified:

and of the whole molecule only C_2H_3 is left unaccounted for. On the basis of these facts the following constitutional formula is proposed:

This compound is an acetylated cysteine derivative, analogous to *p*-bromophenylmercapturic acid, which is excreted after bromobenzene feeding. So far as we are aware, it has not been previously described.

DISCUSSION.

It has been shown that naphthalene is detoxicated by the rabbit partly by conjugation with cysteine and excretion as the mercapturic acid and partly by conversion into a soluble compound, not yet identified, which yields naphthalene on acidification. It is possible that other naphthalene derivatives, such as ethereal sulphates or phenolic substances are also excreted.

The evidence presented for the suggested constitutional formula of *l*- α -naphthylmercapturic acid has been confirmed by the synthesis of this compound, which is described in the following paper [Ing *et al.*, 1934].

Two interesting suggestions arise out of the observations here reported. In the first place conversion into the mercapturic acid may be a general method of detoxication of aromatic hydrocarbons in the animal body. Callow and Hele [1926] presented evidence that benzene as well as bromobenzene is excreted as the mercapturic acid, although the phenylmercapturic acid was not isolated. Secondly the fact that naphthalene requires cysteine for its detoxication suggests the possibility that the cataract which naphthalene produces may result from depriving the lens of cysteine. The β -krystallin of the lens is a cysteine-rich protein; glutathione and possibly free cysteine are present in the lens in relatively large amounts; furthermore the lens, which is an avascular tissue and

dependent on the aqueous humour for its supply of oxygen, is thought to have a specialised oxidation mechanism in which the cysteine-containing substances play an important rôle. It therefore seems likely that repeated doses of naphthalene, constituting a drain on the cysteine supplies in the body, might interfere with the metabolism of the lens by depriving it of cysteine; and thus the degenerative changes resulting in cataract might be induced.

SUMMARY.

An organic acid, $C_{15}H_{15}O_3NS$, identified as *l*- α -naphthylmercapturic acid, has been isolated from the urine of rabbits after naphthalene dosing; the isolation and identification are described.

A second compound, a soluble substance yielding naphthalene on acidification, was also found to be present; this has not been isolated.

We wish to thank Prof. J. C. Drummond and Dr H. Raymond Ing for their helpful criticism and valuable advice. One of the authors (M. C. B.) is indebted to the Committee on the Physiology of Vision for a personal grant and for a grant which has defrayed the expense of this research.

REFERENCES.

- Adams (1930). *Brit. J. Ophthalm.* **14**, 49.
Bamberger and Lodter (1895). *Liebig's Ann.* **288**, 74.
Baumann and Herter (1877). *Z. physiol. Chem.* **1**, 244.
Bouchard and Charrin (1886). *Compt. Rend. Soc. Biol.* **8**, 614.
Callow and Hele (1926). *Biochem. J.* **20**, 598.
Edelfsen (1905). *Arch. exp. path. Pharm.* **52**, 429.
Igersheimer and Ruben (1910). *Arch. Ophthalm.* **74**, 467.
Ing, Bourne, and Young (1934). *Biochem. J.* **28**, 809.
Lesnick (1888). *Arch. exp. Path. Pharm.* **24**, 167.
Penzoldt (1886). *Arch. exp. Path. Pharm.* **21**, 34.