5. Partial purification of the enzyme synthesized *in vitro* indicates that there is enzyme synthesis during induction.

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Metabolism of Polycyclic Compounds

23. THE METABOLISM OF PYRENE IN RATS AND RABBITS*

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Elson, Goulden & Warren (1945) showed that when pyrene is given to rats there is an increased urinary excretion of sulphuric acid esters, glucosiduronic acids and neutral sulphur. Harper (1957) found that the hydrocarbon is hydroxylated in rats and mice to 1-hydroxypyrene and 1,6- and 1,8-dihydroxypyrene. A compound that yielded pyrene with mineral acid was also detected. In the present work it has been shown that pyrene is also converted into *trans*-4,5-dihydro-4,5-dihydroxypyrene and into *N*-acetyl-*S*-(4,5-dihydro-4-hydroxy-5-pyrenyl)-*L*-cysteine, and that the latter compound is the main source of the pyrene liberated when the urines of treated animals are acidified. The oxidation products of pyrene with perbenzoic acid gave, on reaction with *N*-acetylcysteine, a small amount of the synthetic mercapturic acid.

* Part 22: Boyland & Sims (1962c).

EXPERIMENTAL

Absorption spectra. These were measured in ethanol solution on a Perkin-Elmer model 137 ultraviolet spectrophotometer.

Melting points. These are uncorrected.

Chromatography. Except where stated paper chromatography was carried out on Whatman no. 1 paper by downward development for 18 hr. in butan-1-ol-propan-1-ol-aq. 2N-NH₃ (2:1:1, by vol.) The dried chromatograms were examined under ultraviolet light and dipped in either the platonic iodide reagent of Toennies & Kolb (1951) or in a 0.2% (w/v) solution of ninhydrin in acetone and heated to 70% for 10 min. Other chromatograms were sprayed with a solution of diazotized *p*-nitroaniline (0.2% in 4N-HCl), heated to 80° for 5 min. and sprayed with aq. Na₂CO₃.

Thin-layer chromatograms were prepared by coating glass plates with a film of silica gel G (E. Merck A.-G., Darmstadt, West Germany) of 0.25 mm. thickness. The chromatograms were developed for 10 cm. with: *A*, hexane-

benzene (19:1, v/v) (in the detection of pyrene); *B*, benzene (in the detection of phenolic compounds); *C*, benzene-ethanol (19:1, v/v) (in the detection of dihydrodihydroxy compounds and quinones); or *D*, benzene-ethanol (9:1, v/v) (in the detection of the methyl esters of mercapturic acids). The developed chromatograms were examined in ultraviolet light while still wet, both before and after exposure to NH_3 . They were then dried and sprayed with 0.5% 2,6-dichloroquinonechloroimide in ethanol either immediately or after being sprayed with HCl and heated to 80° for 10 min. Acid-labile compounds were examined on two-dimensional chromatograms that were developed in the first direction with either solvent *C* or solvent *D*, sprayed with HCl and heated to 80° for 10 min., and developed in the second direction with either solvent *A* or solvent *B*. This method was particularly useful in the identification of dihydrodihydroxy and related compounds, since the phenols produced on treatment with acid could be readily identified. The phenols were characterized by a direct comparison of their properties on thin-layer chromatograms with those of the authentic compounds and by a comparison of the ultraviolet-absorption spectra of the solutions of the phenols, obtained by scraping the appropriate areas of

silica gel (located in ultraviolet light) from the glass plates and eluting the adsorbed material with ethanol, with the ultraviolet-absorption spectra of the authentic phenols measured in ethanol. The properties of the various compounds described below on paper chromatograms and thin-layer chromatograms are recorded in Tables 1 and 2 respectively. The light-absorption results are recorded in Table 3.

Materials. Pyrene was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. *cis*-4,5-Dihydro-4,5-dihydroxypyrene was prepared by the oxidation of pyrene with osmium tetroxide as described by Cook & Schoental (1948). This was oxidized with chromium trioxide in acetic acid to 4,5-pyrenequinone, which was reduced with lithium tetrahydroaluminate in ether by the method of Booth, Boyland & Turner (1950) to yield ($\pm$)-*trans*-4,5-dihydro-4,5-dihydroxypyrene, separating from benzene in colourless needles which rapidly turned pink, m.p. 222° (decomp.) (Found: C, 81.4; H, 5.4. $\text{C}_{16}\text{H}_{12}\text{O}_2$ requires C, 81.3; H, 5.1%). The product was acetylated with acetic anhydride in pyridine to yield a *diacetate* in needles from aq. ethanol, m.p. 218° (Found: C, 74.9; H, 5.0. $\text{C}_{20}\text{H}_{16}\text{O}_4$ requires C, 75.0; H, 5.0%). When either the dihydrodihydroxy compound or its

Table 1. *Properties of compounds related to pyrene on paper chromatograms*

Compound	R_F	Fluorescence	Colour		Reaction with platonic iodide reagent
			With diazotized <i>p</i> -nitroaniline in 4N-HCl, heating at 80° and Na_2CO_3	With ninhydrin	
<i>cis</i> - and <i>trans</i> -4,5-Dihydro-4,5-dihydroxypyrene	0.85	Dark-violet	Green, turning brown	None	None
1-Pyrenyl sulphate, the sulphuric acid esters of 1,6- and 1,8-dihydroxypyrene and 4,5-dihydro-4-hydroxy-5-pyrenyl sulphate*	0.67	Bright-violet	Blue, turning grey	None	None
Compound, probably 4-pyrenyl-mercaptopuric acid	0.44	Violet	None	None	+
<i>N</i> -Acetyl- <i>S</i> -(4,5-dihydro-4-hydroxy-5-pyrenyl)-L-cysteine†	0.38	Dark-violet	None	None	++
Biliary metabolite, probably <i>S</i> -(4,5-dihydro-4-hydroxy-5-pyrenyl)cysteine‡	0.28	Dark-violet	None	Purple	++
Biliary metabolite, probably <i>S</i> -(4,5-dihydro-4-hydroxy-5-pyrenyl)cysteinylglycine‡	0.22	Dark-violet	None	Purple-brown	++
Biliary metabolite, probably <i>S</i> -(4,5-dihydro-4-hydroxy-5-pyrenyl)glutathione‡	0.10	Dark-violet	None	Purple	++
Glucuronic acid conjugate yielding pyrene with HCl, 1-pyrenylglucosiduronic acid, glucuronic acid conjugates of 1,6- and 1,8-dihydroxypyrene and 4,5-dihydro-4-hydroxy-5-pyrenylglucosiduronic acid*	0.25	Bright-violet	Green, turning brown	None	None
Compounds, possibly diglucuronic acid conjugates of 1,6- and 1,8-dihydroxypyrene*	0.12	Violet	Green, turning grey	None	None
Compound yielding pyrene with HCl	0.05	Violet	None	None	None

* The presence of these compounds was detected by eluting the material forming the spots with methanol-aq. NH_3 (sp.gr. 0.88) (19:1), hydrolysing either chemically or enzymically as described in the text, and examining the products on thin-layer chromatograms.

† Metabolites from rat urine and bile and from rabbit urine and the synthetic compound had the same properties.

‡ The structure of these compounds was established by the methods of Boyland, Ramsay & Sims (1961).

diacetate was heated to 100° with 5N-HCl for 15 min. a product was obtained that was indistinguishable on thin-layer chromatograms from 4-hydroxypyrene.

1- and 2-Hydroxypyrene were prepared by the method of Vollmann, Becker, Corell & Streeck (1937), and 4-hydroxypyrene by the action of HCl in acetic acid at 100° on *cis*-4,5-dihydro-4,5-dihydroxypyrene as described by Cook & Schoental (1948). When either the *cis*- or the *trans*-dihydrodihydroxy compound was run on two-dimensional acid-treated thin-layer chromatograms as described above five compounds were detected in addition to 4-hydroxypyrene: these are presumably oxidation products of the phenol.

Pyrene was oxidized to a mixture of 1,6- and 1,8-pyrene-quinone with hydrogen peroxide in acetic acid (Arnold & Larson, 1940), but no attempt was made to separate the isomers. The mixture was reduced with Na₂S₂O₄ as described by Vollmann *et al.* (1937) and the mixture of 1,6- and 1,8-dihydroxypyrene thus obtained used as a standard on thin-layer chromatograms in the identification of the urinary metabolites.

N-Acetyl-L-cysteine and *NN'*-diacetyl-L-cystine were prepared by the methods of Pirie & Hele (1933) and Hollander & du Vigneaud (1931) respectively.

Oxidation of pyrene with perbenzoic acid. In a typical experiment, pyrene (5 g.) and perbenzoic acid (6.8 g.) in

Table 2. *Properties of compounds related to pyrene on thin-layer chromatograms*

Details are given in the text.

Compound	R_F^*		Fluorescence	Colour with the 2,6-dichloroquinone-chloroimide-Na ₂ CO ₃ reagent
	Benzene	Benzene-ethanol (19:1, v/v)		
1-Hydroxypyrene	0.38	0.55	Violet	Blue-green
2-Hydroxypyrene	0.32	0.51	Violet	Pink, then purple
4-Hydroxypyrene	0.42	0.62	Violet†	Green
1,6-Dihydroxypyrene	0.32	0.52	Blue	Olive-green
1,8-Dihydroxypyrene	0.25	0.57	Blue	Olive-green
<i>cis</i> -4,5-Dihydro-4,5-dihydroxypyrene	0.01	0.18	Dark-violet	None
<i>trans</i> -4,5-Dihydro-4,5-dihydroxypyrene	0.01	0.18	Dark-violet	None
1,6-Pyrenequinone	0.02	0.46	Dark-absorption	(Yellow)‡
1,8-Pyrenequinone	0.01	0.42	Dark-absorption	(Red)‡
4,5-Pyrenequinone	0.15	0.65	Dark-absorption	(Orange)‡
Substance, possibly a dihydrohydroxypyrene, yielding pyrene with acid§	—	0.42	—	None
Methyl ester of <i>N</i> -acetyl- <i>S</i> -(4,5-dihydro-4-hydroxy-5-pyrenyl)-L-cysteine	(0.32)		Dark-violet	None
Pyrene	(0.48)¶		Bright-blue	None

* The absolute R_F values varied from one chromatogram to another, although the relative order of the compounds on the chromatograms was the same. The values quoted are those from typical chromatograms.

† Green fluorescence after the chromatogram was exposed to NH₃.

‡ These colours were formed on untreated chromatograms.

§ This compound could only be detected by its decomposition to pyrene when two-dimensional chromatograms were treated with HCl between the first and second developments as described in the text.

|| R_F measured in benzene-ethanol (9:1, v/v). Both the synthetic ester and that obtained from the urine of rats treated with pyrene had the same R_F value.

¶ R_F measured in hexane-benzene (19:1, v/v).

Table 3. *Ultraviolet absorption of some compounds related to pyrene*

Details are given in the text. When measured, the molecular extinction coefficient is shown in parentheses (as log $\epsilon_{\max}$).

Compound	$\lambda_{\max}$ (m μ)
1-Hydroxypyrene	242, 268, 277, 346, 364 and 384
2-Hydroxypyrene	249, 270, 281, 308, 321, 335, 363 and 383
4-Hydroxypyrene	241, 280, 343 and 378
1,6-Dihydroxypyrene	241, 284, 353 and 371
1,8-Dihydroxypyrene	241, 285, 353 and 370
<i>cis</i> -4,5-Dihydro-4,5-dihydroxypyrene	218 (4.63), 257 (4.59), 263 (4.58), 278 (4.13), 286 (4.03) and 297 (4.01)
<i>trans</i> -4,5-Dihydro-4,5-dihydroxypyrene	219 (4.65), 257 (4.60), 263 (4.59), 278 (4.14), 286 (4.07) and 297 (4.04)
<i>trans</i> -4,5-Diacetoxy-4,5-dihydroxy-4,5-dihydroxypyrene	219 (4.62), 256 (4.59), 262 (4.58), 278 (4.14), 286 (4.03) and 297 (3.93)
<i>N</i> -Acetyl- <i>S</i> -(4,5-dihydro-4-hydroxy-5-pyrenyl)-L-cysteine (synthetic)	217 (4.62), 258 (4.52), 262 (4.51), 279 (4.19), 287 (4.14) and 299 (4.03)
Methyl ester of the above mercapturic acid	217, 258, 263, 279, 287 and 299

chloroform (100 ml.) were kept at room temperature for 48 hr. in the dark. At the end of this time, the small amount of brown solid was filtered off and shown on thin-layer chromatograms to contain 1,6- and 1,8-pyrenequinone. The filtrate was washed three times with ice-cold aq. 5% (w/v) KOH (200 ml.). During the first washing a dark-brown flocculent precipitate separated and collected mainly in the chloroform layer. This was filtered off and discarded. The aqueous layers were combined and acidified with HCl and extracted twice with ether (200 ml.). The combined extracts were washed three times with an excess of saturated aq. NaHCO_3 solution to remove benzoic acid and the ether was evaporated. The residue, which on thin-layer chromatograms was found to contain 1,6- and 1,8-pyrenequinone and 1-hydroxypyrene, together with several unidentified coloured substances, was chromatographed in benzene on silica gel (200–300 mesh; L. Light and Co. Ltd.); the fractions that contained 1-hydroxypyrene were combined and evaporated and the residue was methylated with dimethyl sulphate in aq. 2*N*-NaOH to yield 1-methoxypyrene (50 mg.) in plates from aq. ethanol, m.p. and mixed m.p. 91–92°. 2- and 4-Hydroxypyrene were not detected either in the original mixture or in fractions from the column.

The chloroform extract was mixed with a solution of *N*-acetyl-L-cysteine (1 g.) and NaHCO_3 (1 g.) in water (50 ml.) and the chloroform was removed under reduced pressure. Acetone (50 ml.) was added and the mixture heated under reflux for 30 min. The acetone was distilled off under reduced pressure and the mixture extracted three times with ether (150 ml.). The aqueous layer was acidified to pH 4 with acetic acid and 5 g. of activated charcoal (British Drug Houses Ltd., Poole, Dorset) added. The charcoal was filtered off and washed with water (250 ml.), and the adsorbed material eluted from the charcoal with methanol–aq. NH_3 (sp.gr. 0.88) (19:1, v/v). The methanol was evaporated and the residue chromatographed in butan-1-ol–propan-1-ol–aq. 2*N*- NH_3 (2:1:1, by vol.) on six sheets of Whatman no. 3MM chromatography paper for 18 hr. A number of coloured products seen on the chromatograms were not examined, but a compound of R_F 0.45, which was detected by its dark-violet fluorescence in ultraviolet light and its reaction with the platonic iodide reagent, was eluted with methanol–aq. NH_3 (sp.gr. 0.88) (19:1, v/v) from the appropriate strips cut from the chromatograms. Removal of the solvent yielded a gum which was rechromatographed for 18 hr. on Whatman no. 3MM paper in butan-1-ol–acetic acid–water (2:1:1, by vol.). The product was detected and eluted as described above to yield a solid (30 mg.) which appeared to be a mixture of the diastereoisomers of *N*-acetyl-*S*-(4,5-dihydro-4-hydroxy-5-pyrenyl)-L-cysteine. It was recrystallized with some difficulty from methanol–ether, when it formed a buff powder decomposing at 175–185° (Found: N, 3.5; S, 8.4. Calc. for $\text{C}_{22}\text{H}_{19}\text{NO}_4\text{S}$: N, 3.7; S, 8.4%). The mercapturic acid reacted with diazomethane in ether to yield a gummy neutral product that could not be crystallized. When a solution of the mercapturic acid in water was acidified, a solid separated whose ultraviolet-absorption spectrum and properties on thin-layer chromatograms were identical with those of pyrene. A second product from this reaction detected on paper chromatograms reacted with the platonic iodide reagent and is presumed to be 4-pyrenylmercapturic acid (λ_{max} at 216, 241, 265, 276, 330, 355 and 378 μ).

4-Hydroxypyrene was not detected. The residual liquors from the acidification were shown on paper chromatograms developed with butan-1-ol–acetic acid–water (2:1:1, by vol.) to contain a compound indistinguishable from *NN'*-diacetylcystine. The product from the oxidation was heated to 100° for 2 hr. with aq. 2*N*-NaOH and the solution was acidified with HCl and extracted with ether. The ether extract was shown to contain a product indistinguishable on thin-layer chromatograms from 4-hydroxypyrene. The same phenol was detected in the ether layer when the product was heated under reflux for 6 hr. in methanol with Raney nickel (W-2 grade) and the solution filtered, diluted with water and extracted with ether.

The combined ether extracts described above were dried (over Na_2SO_4) and evaporated and the residue was chromatographed on silica gel (200–300 mesh) (50 g.) by using first light petroleum (b.p. 60–80°) (500 ml.) then benzene (1000 ml.) and finally benzene–ethanol (99:1, v/v) (1300 ml.) as eluting solvents, fractions of 100 ml. being collected and evaporated and the residues examined on thin-layer chromatograms. Fractions 1–5 contained mainly pyrene (1.8 g.), m.p. and mixed m.p. 154–155°, and fractions 8–15 a substance that was recrystallized from benzene–light petroleum (b.p. 80–100°) in light-brown plates (180 mg.), m.p. 275–276° (Found: C, 70.4, 70.0; H, 3.5, 3.5; S, 12.7. Calc. for $\text{C}_{32}\text{H}_{20}\text{O}_3\text{S}_2$: C, 70.0; H, 3.6; S, 11.7%). The compound had R_F 0.39 and 0.51 on thin-layer chromatograms in solvent *B* and solvent *C* respectively, a violet fluorescence in ultraviolet light and gave a feeble purple colour with the 2,6-dichloroquinonechloroimide– Na_2CO_3 reagent. Its ultraviolet-absorption spectrum showed λ_{max} at 221, 245, 254, 267, 282, 346 and 384 μ . It was unaffected by hot 5*N*-HCl or aq. 2*N*-NaOH, and it did not yield an acetyl derivative with acetic anhydride in pyridine at 30°. Since it was not detected as a urinary metabolite of pyrene it was not examined further.

When the elution of the column was continued with benzene–ethanol, fractions 16–20 contained small amounts of unidentified coloured materials, but fractions 21–24, which consisted of the material moving on the column as a dark-coloured band, contained a complex mixture of products that were examined separately. Fractions 25–30 contained only small amounts of dark unidentified substances.

The products from fractions 21–24 formed a dark gum (110 mg.) which was examined on two-dimensional acid-treated thin-layer chromatograms. Compounds indistinguishable from 1,6- and 1,8-pyrenequinone were present together with four substances, all of which yielded 4-hydroxypyrene and the five oxidation products described above with acid. In an attempt to identify the compounds, the mixture was applied to the base lines of six thin-layer chromatograms which were developed with solvent *C*. The appropriate areas of the silica gel were scraped off, the adsorbed material was eluted with ethanol and the ultraviolet-absorption spectra of the solutions were measured. The ultraviolet-absorption spectrum of the first compound, of R_F 0.42 and 0.61 in solvent *B* and solvent *C* respectively, was that of 4-hydroxypyrene (λ_{max} at 241, 280, 342 and 378 μ), and the compound was undistinguishable from this phenol on thin-layer chromatograms. The ultraviolet-absorption spectrum of the second compound, of R_F 0.46 in solvent *C*, showed that the compound was related to 4,5-dihydro-4,5-dihydroxypyrene (λ_{max} at 216, 257, 263, 278,

286 and 297 $m\mu$). The ultraviolet-absorption spectrum of the third compound, of R_F 0.37 in solvent C, could not be identified with any compound of known structure (λ_{max} at 246, 268, 288 and 352 $m\mu$), possibly because it was not obtained in a pure enough state. The ultraviolet-absorption spectrum of the fourth compound, of R_F 0.19 in solvent C, was indistinguishable from that of 4,5-dihydro-4,5-dihydroxypyrene (λ_{max} at 218, 257, 262, 278, 285 and 297 $m\mu$). This fraction was evaporated to dryness, the residue dissolved in the minimum of boiling benzene and the solution allowed to crystallize. The solid was recrystallized from benzene to yield *trans*-4,5-dihydro-4,5-dihydroxypyrene (5 mg.) in needles, m.p. 215–216° (decomp.). A mixture with the authentic material had m.p. 217–219° (decomp.).

Enzymic hydrolyses. Hydrolyses with the sulphatases of Takadiastase (Parke Davis Ltd.) were carried out in acetate buffer at pH 5.7, and hydrolyses with β -glucuronidase (Ketodase; Warner-Chilcott Laboratories) in acetate buffer at pH 5.0. The solutions were incubated overnight at 37° and the products extracted from the solutions with ether and examined on thin-layer chromatograms.

Animal experiments. In a typical experiment 12 rats were each given 2 ml. of a solution of pyrene (2%, w/v) in arachis oil by intraperitoneal injection every other day until a total of 12 g. of the hydrocarbon had been administered. The urines were collected daily, combined and stored at 0°. The urine was filtered and the pH of the filtrate adjusted to 4 with acetic acid. Activated charcoal (100 g.) was added and the mixture stirred for a few minutes. The charcoal was filtered off, washed with water (2 l.) and then with methanol-aq. NH_3 (sp.gr. 0.88) (19:1, v/v) (10 l.) followed by methanol-benzene (9:1, v/v) (5 l.). The combined methanol washings were evaporated under reduced pressure to yield a dark gum which was chromatographed on a cellulose-powder column prepared from Whatman standard-grade cellulose powder (750 g.). The column was developed with butan-1-ol-cyclohexane-aq. 2N- NH_3 (9:2:1, by vol.) and fractions of 150 ml. were collected. The fractions were evaporated under reduced pressure and the residues examined on paper chromatograms with the solvent and reagents described above, and fractions containing the same products were combined. In this way five main fractions were obtained.

The first fraction contained small amounts of a compound indistinguishable from 1-hydroxypyrene and the second a compound indistinguishable from 4,5-dihydro-4,5-dihydroxypyrene on thin-layer chromatograms. Although it was not possible to distinguish between the *cis*- and *trans*-isomers of the dihydrodihydroxy compound on thin-layer chromatograms, results described below suggest that the compound was the *trans*-isomer. Other experiments showed that the free phenol and the dihydrodihydroxy compound were not always present in the urine of rats treated with pyrene. Sometimes small amounts of 1,6- and 1,8-pyrenequinone were detected in these fractions, suggesting that 1,6- and 1,8-dihydroxypyrene were originally present. The third fraction contained sulphuric acid esters. A portion was heated with 5N-HCl at 100° for 15 min. and the products were extracted with ether and examined on thin-layer chromatograms. Compounds indistinguishable from 1- and 4-hydroxypyrene and 1,8-dihydroxypyrene were detected. The residual liquors gave a positive test for

inorganic sulphate with aq. $BaCl_2$. In the hydrolysis with sulphatase, compounds indistinguishable from 1-hydroxypyrene and 4,5-dihydro-4,5-dihydroxypyrene were detected, together with small amounts of 1,8-dihydroxypyrene and 1,6- and 1,8-pyrenequinone. 4-Hydroxypyrene was not detected. In both of these hydrolyses 1-hydroxypyrene was the major product.

The fourth fraction contained the mercapturic acid. This was purified in the manner described for the synthetic compound when it formed a light-brown gum (220 mg.) that could not be crystallized. The mercapturic acid was indistinguishable from the synthetic compound on paper chromatograms and in its ultraviolet-absorption (λ_{max} at 217, 258, 262, 279, 287 and 299 $m\mu$) and infrared-absorption spectra. It was rapidly decomposed by HCl to yield pyrene, the product believed to be 4-pyrenylmercapturic acid and *NN'*-diacetyl cystine. Its optical rotation could not be determined, but it is presumed to be optically active by analogy with the mercapturic acid derived from phenanthrene (Boyland & Sims, 1962a). The mercapturic acid reacted with diazomethane to yield a gum that could not be crystallized, but which was indistinguishable on thin-layer chromatograms and in its ultraviolet-absorption spectrum (λ_{max} at 217, 258, 263, 279, 287 and 299 $m\mu$) from the ester derived from the synthetic mercapturic acid.

The fifth fraction formed a gum (850 mg.) that contained glucuronic acid conjugates, since a positive naphtharesorcinol test was obtained. A portion was heated to 100° for 15 min. with 5N-HCl, and the ether-soluble products were examined on thin-layer chromatograms. The principal product was 4-hydroxypyrene, together with small amounts of 1,6- and 1,8-dihydroxypyrene and pyrene. 1-Hydroxypyrene was not detected, although small amounts were found in other experiments. In the β -glucuronidase hydrolysis, 4,5-dihydro-4,5-dihydroxypyrene and small amounts of 1,6- and 1,8-dihydroxypyrene, 1,6- and 1,8-pyrenequinone and a substance that yielded pyrene with mineral acid were detected on thin-layer chromatograms. The glucosiduronic acid gum (250 mg.) was hydrolysed with β -glucuronidase and the products were chromatographed on a column of silica gel (10 g.; 200–300 mesh), which was eluted first with benzene (500 ml.) and then with benzene-ethanol (49:1, v/v) (250 ml.). The benzene eluate was discarded, but the benzene-ethanol eluate was evaporated under reduced pressure and the residue dissolved in the minimum amount of boiling benzene and allowed to crystallize. ($\pm$)-*trans*-4,5-Dihydro-4,5-dihydroxypyrene (15 mg.) separated in needles, m.p. and mixed m.p. 218–219°. The residual liquors were evaporated to yield a few milligrams of a solid, m.p. 201–205°, which was indistinguishable from the dihydrodihydroxy compound on thin-layer chromatograms and could contain one of the optically active forms.

The remainder of the gum was chromatographed on six sheets of Whatman no. 1 chromatography paper for 18 hr. with butan-1-ol-propan-1-ol-aq. 2N- NH_3 (2:1:1, by vol.) as developing solvent. Three fluorescent bands were detected when the chromatograms were examined in ultraviolet light, and these were cut out and the adsorbed material was eluted with methanol-aq. NH_3 (sp.gr. 0.88) (19:1, v/v). The solvents were evaporated, and portions of the residues were hydrolysed with acid and with β -glucuronidase and the products examined on thin-layer chromatograms. With acid, fraction 1, of R_F 0.25, yielded

4-hydroxypyrene, 1,6- and 1,8-dihydroxypyrene and small amounts of pyrene; and, with β -glucuronidase, it yielded 4,5-dihydro-4,5-dihydroxypyrene, 1,6- and 1,8-dihydroxypyrene and a substance that yielded pyrene on acidification. 1,6- and 1,8-Pyrenequinone were also formed in these hydrolyses. Fraction 2, of R_F 0.12, yielded 1,6- and 1,8-dihydroxypyrene and the corresponding quinones both with acid and with β -glucuronidase; possibly diglucuronic acid conjugates of the phenols are present. Fraction 3, of R_F 0.05, contained an unidentified substance that was broken down by acid, but not by β -glucuronidase, to pyrene. 1,6- and 1,8-Pyrenequinone, but not the dihydroxy compounds, were detected in the hydrolyses products.

A rabbit was given 10 ml. of a solution of pyrene (2%, w/v) in arachis oil by intraperitoneal injection every other day until 1 g. of the hydrocarbon had been administered. The urine from the animal was collected during this time and treated with charcoal as described above. The material thus obtained was chromatographed on a column prepared from 100 g. of cellulose powder to give five fractions that were examined as described above. No qualitative differences between the metabolism of pyrene in this animal and the metabolism in rats described above could be detected. The mercapturic acid formed a gum whose ultraviolet-absorption ($\lambda_{\max}$ at 217, 258, 262, 279, 287 and 299 $m\mu$) and infrared-absorption spectra and properties on chromatograms were identical with those of the acids obtained synthetically and from rats.

Two rats in which biliary fistulae had been established (Boyland, Ramsay & Sims, 1961) were each given 2 ml. of a solution of pyrene (2%, w/v) in arachis oil on 2 successive days, and the bile was collected to the end of the third day and stored at 0°. Examination of the bile by the methods described by Boyland *et al.* (1961) showed the presence of four acid-labile compounds that were identified as the mercapturic acid described above, together with *S*-(4,5-

dihydro-4-hydroxy-5-pyrenyl)cysteine and the corresponding cysteinylglycine and glutathione derivatives. All yielded pyrene and the oxidized form of the corresponding side chain with mineral acid and all had similar ultraviolet-absorption spectra ($\lambda_{\max}$ at 217, 238, 262, 279, 287 and 299 $m\mu$). The sulphuric acid and glucuronic acid conjugates of 4,5-dihydro-4,5-dihydroxypyrene were also present, together with 1-pyrenylglucosiduronic acid and 6- and 8-hydroxy-1-pyrenylglucosiduronic acid. 1-Pyrenyl sulphate was not detected.

DISCUSSION

The oxidation of pyrene with perbenzoic acid is in many ways similar to the oxidation of the hydrocarbon in both rats and rabbits (see Scheme 1) and it is possible that the same intermediates are involved in both cases. It is generally accepted (see, for example, Swern, 1953) that perbenzoic acid is an electrophilic reagent and would be expected to react most readily with hydrocarbons at the regions of highest electron density. On theoretical considerations (see, for example, Pullman & Pullman, 1952; Badger, 1954), the 1-2 and 4-5 bonds and the 1 and 4 positions of pyrene are most likely to be involved in the oxidations, and the present work has shown this to be the case. The reactions on the 4-5 bond can be explained if it is assumed that the product first formed in solution is 4,5-epoxy-4,5-dihydroxypyrene, which reacts either with water to form *trans*-4,5-dihydro-4,5-dihydroxypyrene or with *N*-acetylcysteine to form *N*-acetyl-*S*-(4,5-dihydro-4-hydroxy-5-pyrenyl)cys-

Scheme 1. Probable pathways in pyrene metabolism.

teine. The intermediate, whatever its structure, appears to be fairly stable in chloroform solution, but all attempts to isolate it have proved unsuccessful. Although the dihydrodihydroxy compound was always obtained as a product of the reaction, the formation of the mercapturic acid was less certain, presumably because the intermediate had decomposed during the working-up procedures. The structures of the two other compounds formed in the oxidation, in which reaction had evidently occurred about the 4-5 bond since they both yielded 4-hydroxypyrene after being heated with acid, could not be established, but it is possible that one is the monobenzoate of the dihydrodihydroxy compound, arising from the reaction of the epoxide with benzoic acid. There was no evidence of direct hydroxylation of the 4 position since the alkali extracts of the reaction mixture did not contain 4-hydroxypyrene. The small amount of 4-hydroxypyrene detected in the later stages of the working-up procedure could have arisen either by rearrangement of the epoxide intermediate or by the alkaline hydrolysis of the mercapturic acid. The latter reaction seems the more likely.

The reactions with perbenzoic acid about the 1-2 bond differ from those about the 4-5 bond. With the 1-2 bond, the only product identified, apart from the expected 1,6- and 1,8-pyrenequinone, is 1-hydroxypyrene. This could have arisen either by direct hydroxylation by cationoid hydroxyl groups or by the formation of an unstable epoxide intermediate that rapidly rearranged to the phenol. There is, however, no evidence in favour of either of these mechanisms. In other oxidations, 1-hydroxypyrene is formed when pyrene is oxidized in the Fe^{2+} ion-ascorbic acid system (Dewhurst & Calcutt, 1961), and both ozone (see, for example, Dessy & Newman, 1958) and osmium tetroxide (Cook & Schoental, 1948) attack the 4-5 bond of the hydrocarbon. The 1 position is the site of attack by free benzyl and phenyl radicals (Norman, Thompson & Waters, 1958), but it is generally accepted that free radicals are not formed in perbenzoic acid oxidations, although Cohen & Edwards (1960) suggest that free radicals are formed from perbenzoic acid in benzene solution.

The metabolic products of pyrene can be divided into two groups, arising from oxidation on the 1-2 and 4-5 bonds respectively. The principal phenolic product is 1-hydroxypyrene which is excreted in the urine mainly as a sulphuric acid conjugate. In contrast it is present in the bile mainly as a glucuronic acid conjugate, but the significance of these observations is not clear.

1,6- and 1,8-Dihydroxypyrene presumably arise by further hydroxylation of 1-hydroxypyrene, although phenols are not usually metabolized in this way. 1,6- and 1,8-Pyrenequinone must arise

from atmospheric oxidation of the corresponding dihydroxy compound since they are only obtained when conjugates of the dihydroxy compounds are hydrolysed. 2-Hydroxypyrene, 1,2-dihydro-1,2-dihydroxypyrene and the related mercapturic acid could not be detected in the urine.

The metabolites arising from oxidation on the 4-5 bond of pyrene resemble those previously found in the metabolism of phenanthrene (Boyland & Sims, 1962*a, b*; Sims, 1962), except that 4,5-dihydroxypyrene (which could arise by the dehydrogenation of 4,5-dihydro-4,5-dihydroxypyrene) or its oxidation product, 4,5-pyrenequinone, were not found. The dihydrodihydroxy compound was present in small amounts, however, as compared with the corresponding mercapturic acid, so that the amounts of any dehydrogenation product might be small.

The presence of glutathione, cysteinylglycine and cysteine conjugates as well as the mercapturic acid in the bile suggests that the initial reaction in the formation of the mercapturic acid is one between glutathione and 4,5-epoxy-4,5-dihydro-pyrene in a manner analogous to that suggested in the metabolism of phenanthrene (Boyland & Sims, 1962*a*). Though the mercapturic acid is the precursor of most of the pyrene that is liberated when the urine of treated animals is acidified there appear to be two other compounds which yield pyrene with acid. One is present as a glucuronic acid conjugate and could have a dihydrohydroxypyrene type of structure analogous to that reported in naphthalene metabolism (Boyland & Solomon, 1955). No evidence for the structure of the second substance, which is not a glucuronic acid conjugate, could be obtained.

It is impossible to say whether or not 1-hydroxypyrene arises in the body by direct hydroxylation or by epoxidation followed by rearrangement, but the fact that direct hydroxylation in the 4 position does not occur since no 4-hydroxypyrene has been detected suggests that the second alternative is the more likely.

SUMMARY

1. The sulphuric acid and glucuronic acid conjugates of 1-hydroxypyrene, 1,6- and 1,8-dihydroxypyrene and *trans*-4,5-dihydro-4,5-dihydroxypyrene, together with *N*-acetyl-*S*-(4,5-dihydro-4-hydroxy-5-pyrenyl)-L-cysteine, were found in the urine of both rats and rabbits treated with pyrene. Two compounds giving rise to pyrene with mineral acid were also detected, but neither 4-hydroxypyrene nor its conjugates was present.

2. *N*-Acetyl-*S*-(4,5-dihydro-4-hydroxy-5-pyrenyl)-L-cysteine and the corresponding cysteine, cysteinylglycine and glutathione derivatives, as well as glucuronic acid conjugates of 1-hydroxy-

pyrene and *trans*-4,5-dihydro-4,5-dihydroxypyrene, were present in the bile of rats treated with pyrene.

3. In the oxidation of pyrene with perbenzoic acid an intermediate was formed, believed to be 4,5-epoxy-4,5-dihdropyrene, which reacted with water to yield *trans*-4,5-dihydro-4,5-dihydroxypyrene and with *N*-acetyl-L-cysteine to form *N*-acetyl-*S*-(4,5-dihydro-4-hydroxy-5-pyrenyl)-L-cysteine. 1-Hydroxypyrene and 1,6- and 1,8-pyrenequinone were also formed in the oxidation.

4. The *N*-acetylcysteine derivative obtained both synthetically and from treated animals yielded pyrene and *NN'*-diacetylcystine and a compound believed to be 4-pyrenylmercapturic acid with mineral acid and 4-hydroxypyrene with aqueous alkali.

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Degradation of Starch Granules by some Amylolytic Bacteria from the Rumen of Sheep

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During an investigation into the digestion of starch by sheep MacPherson (1953) obtained several strains of a *Streptococcus*, probably related to *S. bovis*, from the rumen. The extracellular α -amylase produced by one of these strains was described by Hobson & MacPherson (1952), who also studied the extracellular α -amylase of *Clostridium butyricum* from sheep rumen. The limit of hydrolysis of starch solution and of starch fractions was determined, and also the nature of the main end products of the hydrolysis. The action of these enzymes on starch granules was not reported.

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Baker, Nasr, Morrice & Bruce (1950) concluded that in pig caecum *C. butyricum* is the main agent of breakdown of starches, and they showed that amylase was present in cell-free culture filtrates when the organism was grown on starch. Whelan & Nasr (1951) purified the amylase, and showed that it belonged to the group of α -amylases, but did not determine its ability to degrade starch granules. Nasr (1950*a*) found that both pig-caecal filtrate containing amylase and filtrates of pure cultures of a strain of *C. butyricum* isolated from the same source failed to digest starch granules. Cell-free rumen filtrate from sheep also produced no change in the appearance of maize- and