but it could have been formed as an artifact during the enzymic hydrolysis of the glucuronic acid conjugate of the *trans*-isomer. 2-Hydroxy-9,10-anthraquinone, anthrone and conjugates of 9-hydroxy-, 9,10-dihydroxy- and 2,9,10-trihydroxy-anthracene, which were shown to be metabolic products of the *trans*-isomer in rats, were also detected in the urine of rats treated with anthracene.

4. 9,10-Anthraquinone and anthrone were metabolized by rats to 2-hydroxy-9,10-anthraquinone and conjugates of 9-hydroxy-, 9,10-dihydroxy- and 2,9,10-trihydroxy-anthracene; 9,10-anthraquinone also yielded anthrone and the sulphuric ester of 2-hydroxy-9,10-anthraquinone.

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Metabolism of Polycyclic Compounds

26. THE HYDROXYLATION OF SOME AROMATIC HYDROCARBONS BY THE ASCORBIC ACID MODEL HYDROXYLATING SYSTEM AND BY RAT-LIVER MICROSOMES

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The ascorbic acid–Fe^{2+} ion–oxygen model hydroxylating system was first described by Udenfriend, Clark, Axelrod & Brodie (1954), and Brodie, Axelrod, Shore & Udenfriend (1954) showed that with many aromatic compounds, e.g. aniline, acetanilide and salicylic acid, phenols are formed. Boyland & Manson (1958), however, found that 2-acetamidonaphthalene was converted by the hydroxylating system into 2-acetamido-5,6-dihydro-5,6-dihydroxy-, 2-acetamido-1-hydroxy- and 2-acetamido-6-hydroxy-naphthalene. In the present work it was found that, of a number of aromatic hydrocarbons examined, most are converted into mixtures of phenols and dihydrodihydroxy compounds. A comparison of the products obtained in the chemical oxidation with those

obtained in the rat-liver-microsomal hydroxylating system showed that, although with some hydrocarbons both systems yield the same products, with others there are marked differences.

EXPERIMENTAL

Oxidations with the ascorbic acid systems. The oxidations of all the hydrocarbons were carried out in the same way. Ascorbic acid (5 g.), $FeSO_4,7H_2O$ (0·75 g.) and EDTA (disodium salt) (5 g.) were dissolved in about 60 ml. of 0·1 M-KH_2PO_4, and 0·1 M-Na_2HPO_4 (about 300 ml.) was added until the pH of the solution was 6·7. The hydrocarbon (0·5 g.) was added, followed by sufficient acetone (about 300 ml.) to bring the hydrocarbon into solution. A brisk current of air was drawn through the solution, which was kept at room temperature, for 24 hr., acetone being added from time to time to replace that lost by evaporation. At the end of this time, the acetone was distilled off under reduced pressure and the precipitate, which consisted of unchanged hydrocarbon, was filtered off. (Examination of the precipitates from the various oxidations on thin-layer chromatograms as described below showed that they contained little or none of the hydroxylated products.) The filtrate was extracted twice with ether (100 ml.) and the ether extracts were dried (over Na_2SO_4) and evaporated, and the residue was examined on thin-layer chromatograms as described below.

Other experiments were carried out in the absence of phosphates, in which case it was necessary to add sufficient $NaHCO_3$ to bring the EDTA into solution (to give solutions of pH 4·5), and in the absence of phosphates and EDTA (to give solutions of pH 2·5). In some experiments $FeSO_4,7H_2O$ was replaced by $CuSO_4,5H_2O$ (0·5 g.). Experiments were also carried out in which either phenanthrene or benz[*a*]anthracene (0·5 g.), ascorbic acid (0·75 g.), either water or phosphate buffer, pH 6·7 (150 ml.), acetone (150 ml.) and either *N*-acetylcysteine (500 mg.) or glutathione (500 mg.) were aerated at room temperature for 24 hr. The solutions were extracted with ether and the extracts examined on thin-layer chromatograms as before. Each aqueous layer was treated with 15 g. of activated charcoal (British Drug Houses Ltd.), and the charcoal was filtered off and washed with water (100 ml.), followed by 250 ml. of methanol–aq. NH_3 (sp.gr. 0·88) (19:1, v/v). The methanol was evaporated and the residue was examined on paper chromatograms for mercapturic acids or glutathione conjugates by the method of Boyland & Sims (1962*a*) in the phenanthrene oxidations and by that of Boyland & Sims (1964*b*) in the benz[*a*]anthracene oxidations. In no case was it possible to detect the presence of mercapturic acids or glutathione conjugates.

In another experiment, phenanthrene (0·5 g.), ascorbic acid (5 g.) and $FeSO_4,7H_2O$ (0·75 g.) in water (300 ml.) and acetone (300 ml.) were aerated at room temperature as above. The acetone was distilled off under reduced pressure and the residue washed three times with ether (100 ml.). The aqueous phase was divided into three equal portions that were treated separately: (1) one portion was acidified with conc. HCl and treated with charcoal (15 g.) and the charcoal was washed with water (100 ml.), and absorbed material eluted as described above and examined on paper chromatograms for phenanthrene derivatives by using the methods and reagents described by Boyland & Sims (1962*b*): no such derivatives were detected; (2) a second portion was heated to 100° with conc. HCl (30 ml.) for 2 hr. and the cooled solution extracted with ether (50 ml.); (3) a third portion was heated with KOH (20 g.) at 100° for 2 hr. and the cooled solution was acidified with HCl and extracted with ether (50 ml.). In the last two experiments the ether extracts were evaporated and the residues examined on thin-layer chromatograms as described below for hydroxylated derivatives of phenanthrene: none was detected.

trans - 9,10 - Dihydro - 9,10 - dihydroxyphenanthrene (100 mg.), ascorbic acid (1 g.), EDTA (1 g.) and $FeSO_4,7H_2O$ (0·15 g.) in phosphate buffer (60 ml.) and acetone (60 ml.) were aerated at room temperature for 24 hr. At the end of this time the acetone was distilled off and the solid (96 mg.), consisting of unchanged dihydrodihydroxy compound (m.p. and mixed m.p. 186–187°), collected. The filtrate was extracted with ether (100 ml.) and the residue obtained on evaporation of the ether examined on thin-layer chromatograms, when it was seen that it contained mainly unchanged dihydrodihydroxy compound and small amounts of two unidentified substances. 9,10-Phenanthraquinone was not detected.

Experiments with rat-liver microsomes. Microsomes were prepared by the method of Booth & Boyland (1964) except that rat liver was used. Microsomal pellets, which had been stored at −5°, were thawed and homogenized in 0·1 M-phosphate buffer, pH 7·4, prepared from NaH_2PO_4 and Na_2HPO_4, with a Potter & Elvehjem (1936) type of homogenizer with a Teflon pestle. The homogenate from each pellet was divided into two equal portions, each portion containing the microsomes from 1 g. of rat liver. Each oxidation was carried out with one such portion, to which was added nicotinamide (600 μmoles), obtained from Roche Products Ltd., $NADP^+$ (1·5 μmoles) and glucose 6-phosphate (30 μmoles), both as the sodium salts obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and soluble rat-liver fraction (2 ml.), similar to the soluble rabbit-liver fraction described by Booth & Boyland (1964). The volume of the mixture was adjusted to 15 ml. by the addition of phosphate buffer and finally a solution of the hydrocarbon (1 mg.) in ethanol (0·5 ml.) was added. The mixture was incubated in air at 37° for 30 min. by using a metabolic shaker (H. Mickle, Gomshall, Surrey). The mixture was acidified by the addition of conc. HCl (4 ml.) and extracted twice with ethyl acetate (5 ml.). The combined extracts were centrifuged if necessary, dried (over Na_2SO_4) and evaporated, and the residue was examined on thin-layer chromatograms as described below.

Identification of hydroxylated products. With the chemical hydroxylations, portions of the residues from the ether extractions were chromatographed on two-dimensional thin-layer chromatograms prepared by coating glass plates with films of silica gel G (E. Merck A.-G., Darmstadt, West Germany) of 0·25 mm. thickness. The chromatograms were developed for 10 cm. with benzene–ethanol (19:1, v/v) in the first direction, sprayed with conc. HCl and heated to 80° for 10 min. and developed in the second direction with benzene. Dihydrodihydroxy compounds could thus be identified by the phenols produced in the treatment with acid. With the enzymic hydroxylations, residues from the ethyl acetate extractions were applied to the chromatograms which were then developed in the first direction with light petroleum (b.p. 40–60°)–benzene (1:1, v/v). By this means unchanged hydrocarbons and fatty material from the microsomal preparations, which would otherwise interfere with the identi-

fication of the metabolites, were removed from the spot at the origin. The dried chromatograms were then developed again in the same direction with benzene–ethanol (19:1, v/v), treated with acid as above and developed in the second direction with benzene. The methods used in the identification of most of the products have been described previously: for those derived from anthracene see Sims (1964), for those from phenanthrene see Sims (1962) and Boyland & Sims (1962*b*), for those from pyrene see Boyland & Sims (1964*a*) and for those from benz[*a*]anthracene see Boyland & Sims (1964*b*). Of the products derived from naphthalene, 1- and 2-hydroxynaphthalene and *trans*-1,2-dihydro-1,2-dihydroxynaphthalene had R_F 0·65, 0·56 and 0·22 respectively in benzene–ethanol (19:1, v/v) and R_F 0·48, 0·33 and 0·01 respectively in benzene. 1- and 2-Hydroxynaphthalene gave a violet and a green (turning pink) colour respectively on chromatograms sprayed with a 0·5% solution of 2,6-dichloroquinonechloroimide in ethanol followed by aq. 10% (w/v) Na_2CO_3.

RESULTS

The results are summarized in Table 1. None of the changes in the chemical hydroxylating system described above produced any significant differences in the patterns of the hydroxylated products formed in the oxidations of each hydrocarbon, although the total amounts of the products may have been altered by the changes, since Udenfriend *et al.* (1954) report that the yields of products formed in these hydroxylations depend on many factors including pH, the concentration of EDTA and the nature of the metal ion. In the present work, the amounts of the recovered hydrocarbons show that less than 1% of the hydrocarbons was oxidized in any one reaction. The patterns of the hydroxylated products of phenanthrene and

Table 1. *Comparison of the chemical and the biological hydroxylations of some aromatic hydrocarbons*

The hydroxylations with the ascorbic acid–Fe^{2+} ion system and the rat-liver microsomes were carried out and the products identified as described in the text. The metabolites identified in the urine of animals treated with the hydrocarbons are included for comparison: references are given in the text.

Hydrocarbon	Products of hydroxylations in the ascorbic acid–Fe^{2+} ion system	Products of hydroxylations by rat-liver microsomes	Products excreted as urinary metabolites by animals treated with the hydrocarbons
Naphthalene	1- and 2-Hydroxynaphthalene; 1,2-dihydro-1,2-dihydroxy-naphthalene	1- and 2-Hydroxynaphthalene; 1,2-dihydro-1,2-dihydroxy-naphthalene; an unidentified product	1- and 2-Hydroxynaphthalene; *trans*-1,2-dihydro-1,2-dihydroxynaphthalene; 1,2-dihydroxynaphthalene*
Anthracene	1,2-Dihydro-1,2-dihydroxy-anthracene; 9,10-anthra-quinone†	1,2-Dihydro-1,2-dihydroxy-anthracene	*trans*-1,2-Dihydro-1,2-dihydroxy-anthracene; *cis*- and *trans*-9,10-dihydro-9,10-dihydroxy-anthracene; 9,10-dihydroxy-anthracene*; 2,9,10-tri-hydroxyanthracene; 2-hydroxy-9,10-anthraquinone†; 9,10-anthraquinone†
Phenanthrene	1-, 2-, 3-, 4- and 9‡-Hydroxy-phenanthrene; 1,2-dihydro-1,2-dihydroxy-, 3,4-dihydro-3,4-dihydroxy- and 9,10-dihydro-9,10-dihydroxy‡-phenanthrene; 9,10-phenanthraquinone†‡	1-, 2-, 3- and 4-Hydroxy-phenanthrene; 1,2-dihydro-1,2-dihydroxy-, 3,4-dihydro-3,4-dihydroxy- and 9,10-dihydro-9,10-dihydroxy-phenanthrene	1-, 2-, 3- and 4-Hydroxy-phenanthrene; *trans*-1,2-dihydro-1,2-dihydroxyphenan-threne; 1,2-dihydroxyphenan-threne*; *trans*-3,4-dihydro-3,4-dihydroxyphenanthrene; 3,4-dihydroxyphenanthrene*; *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene; 9,10-dihydroxyphenanthrene*; 9,10-phenanthraquinone†
Pyrene	1-Hydroxypyrene; 4,5-dihydro-4,5-dihydroxypyrene; 1,6- and 1,8-pyrenequinone†	1-Hydroxypyrene; 4,5-dihydro-4,5-dihydroxy-pyrene; 1,6- and 1,8-pyrenequinone†	1-Hydroxypyrene; *trans*-4,5-dihydro-4,5-dihydroxypyrene; 1,6- and 1,8-dihydroxypyrene; 1,6- and 1,8-pyrenequinone†
Benz[*a*]anthracene	1-, 2-, 3- and 4-Hydroxybenz[*a*]-anthracene; 1,2-dihydro-1,2-dihydroxy-, 3,4-dihydro-3,4-dihydroxy-, 5,6-dihydro-5,6-dihydroxy-‡ and 10,11-dihydro-10,11-dihydroxy-benz[*a*]-anthracene‡; 7,12-benz[*a*]-anthraquinone†	1‡-, 3- and 4-Hydroxybenz[*a*]-anthracene; 1,2-dihydro-1,2-dihydroxy-‡ and 8,9-dihydro-8,9-dihydroxy-benz[*a*]-anthracene; 7,12-benz[*a*]-anthraquinone†	3-, 4-, 8- and 9-Hydroxybenz-anthracene; 1,2-dihydro-1,2-dihydroxy-‡, 3,4-dihydro-3,4-dihydroxy-, 5,6-dihydro-5,6-dihydroxy-, 8,9-dihydro-8,9-dihydroxy- and 10,11-dihydro-10,11-dihydroxy-benz[*a*]-anthracene; 7,12-benz[*a*]-anthraquinone†

* These products presumably arise by the dehydrogenation of the corresponding dihydrodihydroxy compound.
† These products are probably formed by the oxidation of the corresponding dihydro or dihydrodihydroxy compounds.
‡ These products are either present in small amounts only or are not always detected.

benz[*a*]anthracene were not altered when the oxidations were carried out at either pH 2·5 or 6·7 in the presence of *N*-acetylcysteine or glutathione, and mercapturic acids or glutathione conjugates were not detected among the products of these reactions. The products obtained from the chemical and enzymic hydroxylation of each individual hydrocarbon are discussed below.

Naphthalene. Both the chemical and the enzymic hydroxylations of this hydrocarbon yielded compounds indistinguishable from 1-hydroxy-, 2-hydroxy- and 1,2-dihydro-1,2-dihydroxy-naphthalene, but, in addition, the enzymic hydroxylation yielded a small amount of a metabolite, R_F 0·42 on thin-layer chromatograms developed with benzene–ethanol (19:1, v/v), that yielded 1- but not 2-hydroxynaphthalene after the chromatograms were treated with acid. The structure of this metabolite, which has so far not been recognized as a metabolite of naphthalene in whole animals, could not be determined.

Clark, Downing & Martin (1962), using an ascorbic acid–ferrous sulphate–hydrogen peroxide system, found that naphthalene was oxidized to 1- and 2-hydroxynaphthalene and to two unidentified products, and, in other oxidations of naphthalene with the microsomal enzyme system (Mitoma, Posner, Reitz & Udenfriend, 1956; Booth & Boyland, 1958), 1- but not 2-hydroxynaphthalene, together with 1,2-dihydro-1,2-dihydroxynaphthalene, were detected. The detection of 2-hydroxynaphthalene in the present work is presumably due to the improved chromatographic procedures now available.

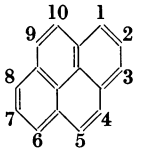
In whole animals, naphthalene is converted into 1-hydroxynaphthalene that is excreted both free and in conjugation with sulphuric acid and glucuronic acid (Lesnik, 1888; Boyland & Wiltshire, 1953; Corner & Young, 1955) and as free 2-hydroxynaphthalene (Corner & Young, 1954; Sims, 1959). It was thought likely that this phenol arose in the urine by the decomposition of conjugates of *trans*-1,2-dihydro-1,2-dihydroxynaphthalene, but it now seems probable that some, at least, arose by the direct hydroxylation of naphthalene in the body. Small amounts of the sulphuric acid and glucuronic acid conjugates of 2-hydroxynaphthalene would be difficult to detect in the presence of large amounts of the same conjugates of 1-hydroxynaphthalene. Naphthalene is also hydroxylated by animals to *trans*-1,2-dihydro-1,2-dihydroxynaphthalene, which is excreted either free (Young, 1947; Booth & Boyland, 1949) or in conjugation with glucuronic acid (Corner, Billett & Young, 1954; Sims, 1959), and it is dehydrogenated in the body to 1,2-dihydroxynaphthalene (Corner & Young, 1954, 1955; Boyland & Sims, 1957).

Anthracene. The chemical and the enzymic hydroxylations of anthracene were similar in that both yielded a compound indistinguishable from 1,2-dihydro-1,2-dihydroxyanthracene, but not 1- or 2-hydroxyanthracene. 9,10-Anthraquinone was detected in the chemical oxidation and the quinone is also a product of the oxidation of anthracene with ascorbic acid and air (Warren, 1943). 9,10-Anthraquinone was not detected in the microsomal hydroxylation, possibly because the method used in its detection (see Sims, 1964) was not sufficiently sensitive. Neither hydroxylation yielded 9-hydroxyanthracene or *trans*-9,10-dihydro-9,10-dihydroxyanthracene. The latter compound, which is a metabolite of anthracene in whole animals (Sims, 1964), is readily oxidized so that its absence in both the chemical and enzymic hydroxylations is not unexpected. Anthracene is also converted into 9,10-dihydroxyanthracene, 2,9,10-trihydroxyanthracene and 2-hydroxy-9,10-anthraquinone by animals (Sims, 1964) and into *trans*-1,2-dihydro-1,2-dihydroxyanthracene (Boyland & Levi, 1935). 1- and 2-Hydroxyanthracene are not formed in the body.

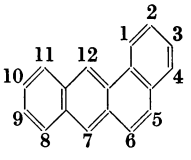
Phenanthrene. Both the chemical and the enzymic hydroxylations yielded compounds indistinguishable on thin-layer chromatograms from 1-, 2-, 3- and 4-hydroxyphenanthrene and 1,2-dihydro-1,2-dihydroxy-, 3,4-dihydro-3,4-dihydroxy- and 9,10-dihydro-9,10-dihydroxy-phenanthrene. In addition, the chemical oxidations sometimes yielded small amounts of compounds indistinguishable from 9-hydroxyphenanthrene and 9,10-phenanthraquinone. A comparison of the chromatograms of the products from the two hydroxylations showed that much less 9,10-dihydro-9,10-dihydroxyphenanthrene was formed in the chemical than in the enzymic hydroxylations, and that, in the former, products formed by hydroxylations at the 9- and 10-positions constituted only a small proportion of the total.

In whole animals, phenanthrene is converted into all the phenols and dihydrodihydroxy compounds (with the exception of 9-hydroxyphenanthrene) described above, which are excreted free and in conjugation with sulphuric acid and glucuronic acid (Young, 1947; Boyland & Wolf, 1950; Sims, 1962; Boyland & Sims, 1962*b*). In addition, conjugates of 1,2-dihydroxy-, 3,4-dihydroxy- and 9,10-dihydroxy-phenanthrene are formed in the body, presumably arising from the dehydrogenation of the corresponding dihydrodihydroxy compounds.

Pyrene (*I*). The chemical and enzymic hydroxylations of pyrene were similar in that compounds indistinguishable on thin-layer chromatograms from 1-hydroxy- and 4,5-dihydro-4,5-dihydroxy-pyrene were formed in each case, but relatively less of the dihydrodihydroxy compound was formed in



(I)



(II)

the chemical oxidation. Neither 2-hydroxy- nor 1,2-dihydro-1,2-dihydroxy-pyrene was detected in either oxidation. Both oxidations yielded 1,6- and 1,8-pyrenequinone, which could have arisen by oxidation of the corresponding dihydroxy compounds. Dewhurst & Calcutt (1961) have reported the formation of 1-hydroxypyrene in the ascorbic acid oxidation, and Harper & Calcutt (1961) the formation of 1-hydroxypyrene in the microsomal system, the formation of the phenol being inhibited by the addition of riboflavin to the system.

In whole animals pyrene is converted into 1-hydroxypyrene, 1,6- and 1,8-dihydroxypyrene (Harper, 1957) and 4,5-dihydro-4,5-dihydroxypyrene (Boyland & Sims, 1964*a*). 2-Hydroxypyrene and 1,2-dihydro-1,2-dihydroxypyrene were not detected.

Benz[a]*anthracene* (*II*). The products obtained from the chemical and the enzymic oxidations differed in many respects. In the chemical oxidation the principal products were compounds indistinguishable from 1-, 2-, 3- and 4-hydroxybenz[*a*]anthracene, two compounds that, because they yielded 1- and 2-hydroxybenz[*a*]anthracene and 3- and 4-hydroxybenz[*a*]anthracene respectively on two-dimensional acid-treated thin-layer chromatograms, are presumed to be 1,2-dihydro-1,2-dihydroxy- and 3,4-dihydro-3,4-dihydroxy-benz[*a*]anthracene, and a compound indistinguishable from 7,12-benz[*a*]anthracenequinone. Small amounts of 5,6-dihydro-5,6-dihydroxybenz[*a*]anthracene were also formed. On one occasion, when the complete oxidizing system was used, the presence of 10,11-dihydro-10,11-dihydroxybenz[*a*]anthracene in the oxidation products was suspected, but this was not confirmed in later experiments. 5- and 7-Hydroxybenz[*a*]anthracene were not detected in the oxidation products.

In the enzymic hydroxylations, the major product was 8,9-dihydro-8,9-dihydroxybenz[*a*]anthracene, but it is possible that 1,2-dihydro-1,2-dihydroxy- and 10,11-dihydro-10,11-dihydroxybenz[*a*]anthracene were also present since it is difficult to detect small amounts of the two latter in the presence of large amounts of the former in the chromatographic procedures used. Small amounts of compounds indistinguishable from 3- and 4-hydroxybenz[*a*]anthracene, 5,6-dihydro-5,6-dihydroxybenz[*a*]anthracene and 7,12-benz[*a*]anthracenequinone were also detected among the hydroxylation products, but 5- and 7-hydroxybenz[*a*]anthracene were not found. In some of these hydroxylations, trace amounts of a compound with the properties on thin-layer chromatograms of 1-hydroxybenz[*a*]anthracene were also detected. On two-dimensional acid-treated thin-layer chromatograms, two spots of this compound were formed and their positions on the chromatograms indicated that one was that of the phenol present in the oxidation mixture as such, whereas the other was that of the phenol derived from an acid-labile compound present in the oxidation mixture; this precursor was indistinguishable on thin-layer chromatograms from 1,2-dihydro-1,2-dihydroxybenz[*a*]anthracene described above. 2-Hydroxybenz[*a*]anthracene, which was expected both as the free phenol and as the second product of the acid decomposition of the dihydrodihydroxy compound, was not detected on these chromatograms, possibly because the spots were masked by the spots of other compounds.

In whole animals, benz[*a*]anthracene is converted into 4-hydroxybenz[*a*]anthracene (Berenblum & Schoental, 1943) and into 3-, 8- and 9-hydroxybenz[*a*]anthracene and 3,4-dihydro-3,4-dihydroxy-, 5,6-dihydro-5,6-dihydroxy-, 8,9-dihydro-8,9-dihydroxy- and 10,11-dihydro-10,11-dihydroxybenz[*a*]anthracene (Boyland & Sims, 1964*b*), A compound present as a sulphuric acid and a glucuronic acid conjugate in the urines of animals treated with benz[*a*]anthracene yields 7,12-benz[*a*]anthracenequinone when the conjugates are hydrolysed enzymically. The compound is probably either 7,12-dihydro-7,12-dihydroxy- or 7,12-dihydroxy-benz[*a*]anthracene. 1- and 2-Hydroxybenz[*a*]anthracene were not detected as metabolites of the hydrocarbon, and 1,2-dihydro-1,2-dihydroxybenz[*a*]anthracene was detected in very small amounts in one experiment only.

DISCUSSION

In experiments with whole animals, even with the simpler hydrocarbons, it is difficult to estimate the amounts of the various metabolites formed, partly because of losses during the isolation procedures and partly because of the further metabolic processes that some of the first-formed metabolites undergo. If it is assumed that the mercapturic acids and related compounds, the phenols, the dihydrodihydroxy compounds and the dihydroxy compounds are all derived from some common intermediate, then, with most hydrocarbons, there appears to be some relationship between the amounts of metabolites formed on the various bonds in the molecule and the bond orders (see, for example, Pullman & Pullman, 1952), those bonds with the highest bond order being the most reactive

biologically. Thus, for example, in benz[*a*]anthracene, the 5,6-bond has the highest bond order and is the most reactive, and the 8,9- and 10,11-bonds are next both in bond order and in reactivity (Boyland & Sims, 1964*b*).

In the present work it has not been possible to devise methods for the estimation of the ratio of the hydroxylated products formed in the hydroxylations, but comparisons of the thin-layer chromatograms of the products of the hydroxylations of each hydrocarbon in the two systems showed a number of interesting features. With the simpler hydrocarbons, naphthalene and anthracene, the products of the chemical and the enzymic hydroxylations were similar, whereas those of the more complex hydrocarbons, phenanthrene, pyrene and benz[*a*]anthracene, showed a number of differences. These appeared to involve the amounts of the products formed at the so-called '*K* regions' of phenanthrene, pyrene and benz[*a*]anthracene and also at the 8,9- and 10,11-bonds of the latter hydrocarbon, there being apparently little or often no reaction at these bonds in the chemical oxidations.

One possible explanation for these apparent anomalies is that different mechanisms are involved in the two hydroxylating systems. It has been suggested (see for example, Boyland & Sims, 1962*b*) that the metabolism of aromatic hydrocarbons in the body involves the addition of oxygen across double bonds to form epoxides, and that the excreted metabolites are formed by the further reaction of these intermediates, the phenols by the rearrangement of the epoxides, the dihydrodihydroxy compounds by the reaction of the epoxides with water, and the mercapturic acids by the reaction of the epoxides with glutathione, followed by removal of glutamic acid and glycine and the acetylation of the *S*-substituted cysteine thus formed. The products obtained in the microsomal hydroxylations could have arisen from intermediates of this type. In the hydroxylations of 1,2-dihydronaphthalene (Booth, Boyland, Sato & Sims, 1960) and of naphthalene and phenanthrene (Booth, Boyland & Sims, 1961) in the rat-liver microsomal system, it was found that, if the hydroxylations were carried out in the presence of the soluble rat-liver fraction and glutathione, then glutathione conjugates, as well as phenols and dihydrodihydroxy compounds, were formed, the reactions of the intermediates with glutathione being catalysed by an enzyme present in the soluble fraction. Epoxides formed on bonds of high bond order should be more stable than those on other bonds in the same molecule and would therefore be more likely to yield dihydrodihydroxy compounds and glutathione conjugates than phenols. This would explain the absence of 1- and 2-hydroxyanthracene, 9-hydroxyphenanthrene, 4-hydroxypyrene and 5-hydroxybenz[*a*]anthracene in the enzymic hydroxylations of the respective parent hydrocarbons.

The intermediate formation of epoxides in the chemical oxidation could account for the observed formation of phenols and dihydrodihydroxy compounds but it is also possible that the phenols and dihydrodihydroxy compounds are formed by separate mechanisms, the former compounds arising by direct hydroxylations of the aromatic nucleus. If this is the case, however, it is then difficult to explain the absence of phenols formed at the 1-, 2- and 9-positions of anthracene, the 4-position of pyrene and the 5-, 6- and 7-positions of benz[*a*]anthracene, where hydroxylations would be expected to occur most readily. If epoxides are intermediates it is then difficult to explain the low yields or the absence of dihydrodihydroxy compounds that should have been formed readily at the chemically reactive bonds. One possibility is that if epoxides are formed at these bonds they react preferentially with a constituent of the oxidizing system, the most likely being ascorbic acid itself. Attempts to find such a product in the reaction were, however, unsuccessful. Attempts to convert any epoxides formed during the oxidations into mercapturic acids or glutathione conjugates by the addition of *N*-acetylcysteine or glutathione respectively to the oxidizing systems were also unsuccessful, but this may be because the thiols were more readily oxidized in the system than the hydrocarbons. Another possibility is that products formed at the more active sites of the molecules were more readily oxidized than those formed elsewhere. This could be true of dihydrodihydroxy compounds formed at the '*meso*' positions of anthracene and benz[*a*]anthracene, since 9,10-anthraquinone and 7,12-benz[*a*]anthracenequinone respectively were products of the oxidations, but very little oxidation of *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene occurred in the chemical system and in the oxidations of phenanthrene, 9,10-phenanthraquinone was only occasionally detected. In the oxidations of pyrene or benz[*a*]anthracene in this system, 4,5-pyrenequinone or 5,6-benz[*a*]anthracenequinone were not detected in the products. On the other hand, the products from the oxidations of all the hydrocarbons contained a number of unidentified substances, which were recognized on thin-layer chromatograms either by their colour, by their fluorescence in u.v. light or by the colour they gave with the 2,6-dichloroquinonechloroimide–sodium carbonate reagent.

The evidence for the formation of epoxides, particularly at the '*K* regions' and at the 7- and 12-positions of benz[*a*]anthracene in the oxidation of phenanthrene, pyrene and benz[*a*]anthracene with perbenzoic acid (Boyland & Sims, 1961, 1964*a*, *b*), is much stronger than that obtained in the ascorbic

acid oxidations, and the products differ in many respects from those of the latter oxidations. With phenanthrene, the principal reaction was at the 9,10-bond to give an intermediate that appeared to be an epoxide since it yielded *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene with water and *N*-acetyl-*S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)-cysteine with *N*-acetylcysteine. 9-Hydroxyphenanthrene and small amounts of 1-, 2-, 3- and 4-hydroxyphenanthrene were also formed, but 1,2-dihydro-1,2-dihydroxy- and 3,4-dihydro-3,4-dihydroxy-phenanthrene were not detected. Pyrene yielded an intermediate that reacted with water to form *trans*-4,5-dihydro-4,5-dihydroxypyrene and with *N*-acetylcysteine to form *N*-acetyl-*S*-(4,5-dihydro-4-hydroxy-5-pyrenyl)cysteine. Large amounts of 1-hydroxypyrene were formed, but 2-hydroxypyrene, 4-hydroxypyrene and 1,2-dihydro-1,2-dihydroxypyrene were not detected. Benz[*a*]-anthracene formed two intermediates, one of which yielded 7,12-dihydro-7,12-dihydroxybenz[*a*]anthracene with water and the other *trans*-5,6-dihydro-5,6-dihydroxybenz[*a*]anthracene with water and *N*-acetyl-*S*-(5,6-dihydro-5-hydroxy-6-benz[*a*]anthracenyl)cysteine with *N*-acetylcysteine. No phenols or other dihydrodihydroxy compounds were detected. Since the perbenzoic acid system differs from the ascorbic acid system in that in the former water is introduced only at the end of the oxidation, the formation of phenols and not dihydrodihydroxy compounds in the perbenzoic acid oxidation at the less reactive bonds of phenanthrene and pyrene can be explained if it is assumed that epoxidation on these bonds occurs and that these products rearrange to the phenols. It is difficult to explain why phenolic products of benz[*a*]anthracene are not formed in the perbenzoic acid oxidation, but in the microsomal oxidations and in whole-animal experiments the hydroxylated derivatives at the 3,4-bond of benz[*a*]anthracene are possibly formed, at least in part, through the intermediate formation of 7,12-dihydro-7,12-dihydroxybenz[*a*]anthracene (Boyland & Sims, 1964*b*) and not by the direct hydroxylation of the aromatic nucleus.

No evidence was obtained for the hydroxylating species involved in either the enzymic or the chemical hydroxylations. Mason (1957) has suggested that, in the enzymic hydroxylations of aromatic compounds, a protein–Fe^{2+} ion–oxygen complex, of the type Enzyme·$Fe^{2+}O_2$ or Enzyme·$Fe^{2+}O$, is involved and that an enzyme–oxygen–hydrocarbon complex is formed which would yield either a phenol, an epoxide or a *trans*-dihydrodihydroxy compound according to its nature. Although this is not inconsistent with the present work, the postulated formation of free epoxides, which then yield phenols, dihydrodihydroxy compounds and mercapturic acids as described above, provides an equally satisfactory explanation for some of the experimental results, particularly for the absence of phenolic products at sites on the molecules where these might be expected.

The hydroxylating species involved in the ascorbic acid oxidations is also unknown, but free hydroxyl radicals are probably not involved since in the oxidation of naphthalene with Fenton's reagent or with hydrogen peroxide irradiated with u.v. light (both systems that yield free hydroxyl radicals), 1- and 2-hydroxynaphthalene but not 1,2-dihydro-1,2-dihydroxynaphthalene were formed (Boyland & Sims, 1953). Norman & Radda (1962) also conclude that free hydroxyl radicals are not involved in these oxidations and they suggest an electrophilic radical, such as the perhydroxyl radical, $HO_2\cdot$, as the hydroxylating species. Grinstead (1960), as a result of studies on the hydroxylation of salicylic acid in the complete system, has suggested that either $HO\cdot$ or $HO_2\cdot$ radicals are the hydroxylating species.

In the metabolism of polycyclic aromatic hydrocarbons, the dihydrodihydroxy compounds excreted normally have the *trans*-configuration, a configuration that would be expected if they arose from epoxides by the action of water. In the present work, the dihydrodihydroxy compounds detected in both the enzymic and chemical oxidations had the same R_F values as the corresponding compounds isolated from or detected in the urines of treated animals, but in most cases the corresponding *cis*-isomers were not available for comparison. In cases where both isomers of a dihydrodihydroxy compound were available it was not always possible to distinguish between them on thin-layer chromatograms.

A third class of hydroxylated metabolites of aromatic hydrocarbons has sometimes been recognized, that yield the parent hydrocarbon on treatment with mineral acid (Chang & Young, 1943). The naphthalene metabolite 1,2-dihydro-1-hydroxynaphthalene has been isolated and characterized as a glucuronic acid derivative (Boyland & Solomon, 1955). There was no evidence for the formation of such derivatives in the present work.

SUMMARY

1. The products of the hydroxylations of some aromatic hydrocarbons in the rat-liver-microsomal enzyme system and the ascorbic acid–Fe^{2+} ion–oxygen model system have been compared. With the simpler hydrocarbons the products were similar, whereas with the more complex hydrocarbons less reaction appeared to have occurred at the more reactive bonds of the molecules in the chemical than in the enzymic reactions.

2. Naphthalene was converted into 1-hydroxy-, 2-hydroxy- and 1,2-dihydro-1,2-dihydroxy-naph-

thalene by both systems. An unidentified metabolite that yielded 1-hydroxynaphthalene with acid was detected with the enzyme.

3. Anthracene was hydroxylated to 1,2-dihydro-1,2-dihydroxyanthracene in both systems, but 1- and 2-hydroxyanthracene were not formed. 9,10-Anthraquinone was detected in the chemical but not in the enzymic system.

4. Phenanthrene was converted into 1-, 2-, 3- and 4-hydroxyphenanthrene and 1,2-dihydro-1,2-dihydroxy- and 3,4-dihydro-3,4-dihydroxy-phenanthrene in both systems. 9,10-Dihydro-9,10-dihydroxyphenanthrene was also a product of both hydroxylations, but the amount formed in the chemical was much less than that formed in the enzymic hydroxylation. Small amounts of 9-hydroxyphenanthrene were formed in the chemical oxidation.

5. Pyrene was converted into 1-hydroxypyrene, 4,5-dihydro-4,5-dihydroxypyrene and 1,6- and 1,8-pyrenequinone in both hydroxylations, but the amount of the dihydrodihydroxy compound formed in the chemical oxidation was small.

6. Benz[*a*]anthracene was oxidized in the chemical system to 1-, 2-, 3- and 4-benz[*a*]anthracene and to 1,2-dihydro-1,2-dihydroxy- and 3,4-dihydro-3,4-dihydroxy-benz[*a*]anthracene, whereas the enzymic system yielded mainly 8,9-dihydro-8,9-dihydroxybenz[*a*]anthracene. Small amounts of 3- and 4-hydroxybenz[*a*]anthracene were formed in the enzymic system together with trace amounts of 1-hydroxybenz[*a*]anthracene and a compound that appeared to be 1,2-dihydro-1,2-dihydroxybenz[*a*]anthracene. Both systems yielded 7,12-benz[*a*]anthracenequinone and small amounts of 5,6-dihydro-5,6-dihydroxybenz[*a*]anthracene.

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