Biochemical Studies of Toxic Agents

13. THE METABOLISM OF ACENAPHTHYLENE*

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It is now well established that certain unsaturated cyclic hydrocarbons are converted into dihydrodiols in the animal body. Compounds which undergo this metabolic change include naphthalene (Young, 1947; Booth & Boyland, 1949), anthracene (Boyland & Levi, 1935), phenanthrene (Young, 1947; Boyland & Wolf, 1950), indene (Brooks & Young, 1956) and 2-methylnaphthalene (Grimes & Young, 1956). In the present work it has been shown that in the organism acenaphthylene gives rise to *cis*- and *trans*-acenaphthene-1,2-diol, and that it also undergoes ring fission with the formation of naphthalic acid (naphthalene-1,8-dicarboxylic acid).

Acenaphthylene has been shown to be a constituent of tobacco smoke (Lindsey, 1959). Its metabolism does not appear to have been studied hitherto.

MATERIALS

All melting points are uncorrected. Elementary microanalyses were carried out by Weiler and Strauss, Oxford. Ring Index numbering of compounds has been used throughout this paper.

Acenaphthylene. This compound was purified through the picric acid. A solution of 200 g. of picric acid in 500 ml. of benzene-ethanol (1:1, v/v) at 50° was added to 100 g. of acenaphthylene dissolved in 400 ml. of the same solvent at 50°. The precipitate which formed was separated and heated for 30 min. in 1200 ml. of toluene-ethanol (1:1, v/v). The solid material which formed on cooling, m.p. 206–207° (decomp.), was suspended in 500 ml. of benzene and decomposed by the addition of 100 ml. of aq. 6*N*-NH₃ soln. The benzene layer was separated and to it were added two (200 ml.) benzene washings of the layer containing the picric acid. The benzene solution was washed by shaking with three portions (each 250 ml.) of water and was concentrated to 200 ml. by distillation. The remaining traces of picric acid were removed by passing the benzene solution through a column of 40 g. of alumina (Plattner & Pfau, 1937). The hydrocarbon was then eluted with benzene, the benzene solution was concentrated and addition of light petroleum (b.p. 60–80°) brought about the precipitation of 65.5 g. of acenaphthylene as yellow plates, m.p. 92–92.5°. Kynaston & Jones (1949) give m.p. 92.5–93° and Behr & van Dorp (1873) 92–93°. The red colour typical of picric acid (Feigl, 1954) was not produced when the purified

acenaphthylene was heated with KCN solution. This test detected 0.05% of picric acid added to acenaphthylene.

***cis*-Acenaphthene-1,2-diol.** The *cis*- and *trans*-forms of acenaphthene-1,2-diol were prepared by the reaction of Prévost (1933*a*, *b*). When acenaphthylene reacts with silver benzoate and iodine in dry benzene at room temperature, formation of a benzoate of *cis*-acenaphthene-1,2-diol takes place, whereas at 80° a benzoate of *trans*-acenaphthene-1,2-diol is formed. Hydrolysis of the benzoates with methanolic KOH gives rise to the free diols. To a suspension of 19.86 g. of dry silver benzoate (i.e. 10% excess of 1 mol.prop.) in 480 ml. of benzene (which had been dried over anhydrous CaCl₂ and redistilled) at room temperature were added 6 g. of acenaphthylene and 10.02 g. of iodine (1 mol.prop.). The precipitate of AgI which formed was removed by filtration and the benzene filtrate was washed with water, then with solutions of Na₂S₂O₃ and Na₂CO₃. These were washed with benzene. When the combined benzene solutions and benzene washings were evaporated under reduced pressure to a small volume the monobenzoate of *cis*-acenaphthene-1,2-diol was precipitated and obtained as white needles, m.p. 189–190°, after recrystallization from toluene. It was dissolved in dioxan (45 ml.) and 15 ml. of aqueous 60% (w/v) KOH and 20 ml. of methanol were added. The solution was left overnight and was then neutralized with acetic acid. *cis*-Acenaphthene-1,2-diol was precipitated and dissolved in acetone. After the solution had been treated with charcoal, the compound was obtained as colourless needles, m.p. 207–208°, by the addition of water (Found: C, 77.7; H, 5.5. Calc. for C₁₂H₁₀O₂: C, 77.4; H, 5.4%). The yield was 45.6% of the theoretical amount. The diacetate was obtained as white prisms, m.p. 130–131°, by the method used to prepare the diacetate of 1,2-dihydronaphthalene-1,2-diol (Young, 1947). Ewan & Cohen (1889) reported m.p. 130°.

***trans*-Acenaphthene-1,2-diol.** The synthesis of this compound was carried out as described for *cis*-acenaphthene-1,2-diol except that acenaphthylene and then iodine were added to a hot suspension of silver benzoate in benzene and the mixture was boiled under reflux for 1 hr. The mixture was then cooled and the AgI was separated by filtration. The filtrate was treated as already described except that the combined benzene filtrate and benzene washings were evaporated to dryness under reduced pressure. The residue was crystallized twice from cyclohexane and yielded almost white crystals of the dibenzoate of *trans*-acenaphthene-1,2-diol. This was hydrolysed as described for the *cis*-isomer. The hydrolysate was neutralized with acetic acid and water was added to the solution, which was then shaken with three equal portions of ether. The combined ether extracts were evaporated to dryness under reduced pressure and gave a crystalline residue which was recrystallized

* Part 12: Grenby & Young (1960).

from benzene-acetone (9:1, v/v). Racemic *trans*-acenaphthene-1,2-diol, m.p. 157–158°, was obtained in 52·6% yield (Found: C, 77·7; H, 5·6. Calc. for $C_{12}H_{10}O_2$: C, 77·4; H, 5·4%).

Acenaphthen-1-one. When either *cis*- or *trans*-acenaphthene-1,2-diol is heated with mineral acid, formation of acenaphthen-1-one takes place. A solution of 800 mg. of *trans*-acenaphthene-1,2-diol in 230 ml. of 15% (v/v) H_2SO_4 was heated under reflux on a boiling-water bath for 2 hr. The yellow-green solution thus obtained was steam-distilled. The precipitate which formed in the distillate was separated by filtration and when crystallized from aqueous ethanol it yielded 293 mg. of acenaphthen-1-one, m.p. 119–120·5°, a yield of 40·5% (Found: C, 85·2; H, 4·8. Calc. for $C_{12}H_8O$: C, 85·6; H, 4·8%).

The oxime, m.p. 174–175°, was prepared by heating acenaphthen-1-one with hydroxylamine hydrochloride and sodium acetate in aqueous ethanol (Found: C, 78·7; H, 5·2; N, 7·3. Calc. for $C_{12}H_9NO$: C, 78·7; H, 5·0; N, 7·7%). Graebe & Gfeller (1893) reported m.p. 175°.

Naphthalic anhydride. This was prepared by the method of Graebe & Gfeller (1892), in which acenaphthene is oxidized by sodium dichromate in acetic acid. When the product of this reaction was crystallized three times from hot conc. HNO_3 to which had been added one-tenth of its volume of water, almost colourless needles in a yield of 47% were obtained with m.p. 273–274°. This and all other measurements of the m.p. of naphthalic anhydride were made on a brass block. Graebe & Gfeller (1892) reported m.p. 274° (corr.). The crystalline material was heated at 110° before microanalysis (Found: C, 72·6; H, 3·0. Calc. for $C_{12}H_6O_3$: C, 72·7; H, 3·1%).

N-Anilidonaphthalimide was prepared by heating 30 mg. of naphthalic anhydride under reflux on a boiling-water bath with 17 mg. of freshly distilled phenylhydrazine in a mixture of 1·2 ml. of benzene and 1·2 ml. of 95% (v/v) ethanol containing 4 drops of acetic acid. After 30 min. 8 drops of hot water were added and the reaction mixture was left at 4° for 5 hr. The precipitate which formed when crystallized from aqueous ethanol gave 24 mg. of yellow needles, m.p. 215–216° (Found: C, 75·4; H, 4·2; N, 9·5. Calc. for $C_{18}H_{12}N_2O_2$: C, 75·0; H, 4·2; N, 9·7%). Chang & Young (1943) reported m.p. 215–216°.

METHODS

Animals and dosing. Male rabbits and rats were used. The rabbits (body wt. 2–3 kg.) were of mixed breed. The rats (body wt. 150–200 g.) were of the Wistar strain or a black-hooded strain. The animals were housed in metabolism cages designed to permit the collection of urine separate from the faeces. The rats were given rat cakes [J. Murray and Sons, (London) Ltd.], and the rabbits were given rat cakes with cabbage in addition. All animals were given water *ad libitum*.

Acenaphthylene was administered to the animals by stomach tube as a suspension in 1% (w/v) starch solution. The rabbits were given 10–20 ml. of a 10% (w/v) suspension followed by 5–10 ml. of water. Rats were given 1·0–1·5 ml. of a 20% (w/v) suspension. A solution of 20% (w/v) acenaphthylene in arachis oil was used for subcutaneous injections. Rabbits were given 6–7 ml. of this solution and the rats received 1·0 ml.

Urine was collected daily during the period of dosing and

for 2 days after the last dose. It was filtered through glass wool and stored in the refrigerator. Faeces were collected at the same time as the urine and stored over P_2O_5 .

Paper chromatography. Whatman no. 1 paper and the ascending method were used throughout. The solvent or solvent mixture was allowed to ascend the paper at room temperature until the solvent front had moved 30–35 cm. from the origin. It is possible to detect *cis*- and *trans*-acenaphthene-1,2-diol and acenaphthen-1-one by the yellow fluorescence they give in ultraviolet light after they have been sprayed with ethanol-conc. H_2SO_4 -water (18:1:1, by vol.), and heated at 95–100° for 1 min. Under these conditions naphthalic anhydride gives a purple fluorescence. Separation of *cis*- and *trans*-acenaphthene-1,2-diol on paper chromatograms was achieved with the following solvents: A, methanol; B, methanol-carbon tetrachloride (9:1, v/v); C, methanol-cyclohexane (9:1, v/v).

Isolation procedures

cis- and *trans*-Acenaphthene-1,2-diol. If the urine of animals which had been dosed with acenaphthylene was not at pH 7–8 it was adjusted to this pH, and it was then extracted exhaustively with peroxide-free ether or with 'ether anaesthetic' (British Drug Houses Ltd.) until significant amounts of material were no longer being removed. This was accomplished either by shaking the urine with equal volumes of ether on a mechanical shaker for four successive 30 min. periods, or by the use of a continuous extractor. The ether extract was evaporated to dryness under reduced pressure and the residue thus obtained was dried over P_2O_5 . When it was stirred in a few millilitres of ethanol cooled in an ice-salt mixture some material did not dissolve, and this was filtered off and washed three times with small volumes of cold ethanol. This gave a pale-brown crystalline product which after being recrystallized twice from ethanol-cyclohexane (1:1, v/v) yielded silky white needles of *cis*-acenaphthene-1,2-diol.

After separation of the *cis*-acenaphthene-1,2-diol by the procedure already described, the ethanolic filtrate obtained was evaporated to dryness in a current of air. The residue was treated with hot water and the hot solution was filtered to remove a small amount of tar. The filtrate deposited pale-brown needles on cooling to room temperature. These were filtered off and were recrystallized twice from benzene and then once from benzene- $CHCl_3$ (12:1, v/v) to give colourless needles of (+)-*trans*-acenaphthene-1,2-diol.

The aqueous filtrate obtained after separation of the crystalline material was extracted with ether and the ether extract was evaporated to dryness. This yielded a crystalline residue, which, after being recrystallized twice from benzene and twice from benzene- $CHCl_3$ (12:1, v/v), was treated in ethanol solution with charcoal. Evaporation of the ethanol solution to dryness gave a residue which on crystallization from benzene yielded a mixture of needles and plates consisting of (+)- and (±)-*trans*-acenaphthene-1,2-diol.

Acenaphthen-1-one. Urine which had been extracted exhaustively with ether to remove free diols was warmed gently to remove dissolved ether. Sufficient 50% (v/v) H_2SO_4 was then added so that the urine contained 15% (v/v) of H_2SO_4 , and it was heated on a boiling-water bath for 2 hr. It was next steam-distilled and a pale-yellow

solid was obtained in the distillate. This was separated by filtration and when crystallized twice from aqueous ethanol it yielded white prisms of acenaphthen-1-one.

Naphthalic anhydride. Urine which had been exhaustively extracted with ether at pH 7-8 was acidified to pH 2 with conc. HCl and kept overnight at room temperature. It was then extracted with ether. The ether extract was evaporated to dryness under reduced pressure and the gummy, partly crystalline solid was dried over P_2O_5 . On stirring with ethanol cooled in ice-salt mixture, some of the material did not dissolve. This was filtered off and washed twice with cold ethanol. A brown powder was obtained that, when crystallized from aqueous acetone, yielded pale-yellow needles of naphthalic anhydride. In some experiments the brown powder was dissolved in warm 2N-NaOH, and the solution was decolorized with charcoal and acidified with conc. HCl. The precipitate which formed was crystallized from aqueous acetone.

Enzyme experiments

The urine of animals dosed with acenaphthylene was extracted exhaustively with ether to remove *cis*- and *trans*-acenaphthene-1,2-diol and was then exposed to the action of β -glucuronidase before being extracted again with ether to remove the compound liberated by the enzyme. The β -glucuronidase was introduced either as a component of a rat-liver preparation or as a purified enzyme preparation of bacterial origin (Sigma Chemical Co., St Louis, Mo., U.S.A.).

Liver preparation. The procedure whereby a liver preparation was used for hydrolysing glucuronides was that employed by Corner, Billett & Young (1954) and was based on the glucuronidase preparation described by Kerr & Levvy (1951). The urine from the dosed animals, after being extracted exhaustively at pH 7-8 with ether to remove free *cis*- and *trans*-acenaphthene-1,2-diol, was warmed gently to remove ether and was then adjusted to pH 5.2 with acetic acid. 0.2M-Sodium acetate-acetic acid buffer, pH 5.2, was then added to the extent of one-tenth of the volume of the urine, followed by a liver preparation (1 g. of liver/100 ml. of urine) obtained by comminuting the liver of a freshly killed rat in an Ato-Mix blender with acetate buffer, pH 5.2. The mixture was incubated at 37° for 24 hr. and was then centrifuged to remove liver debris and other solid matter. The supernatant liquid was adjusted to pH 7-8 with 2N-NaOH and was then extracted with peroxide-free ether. The ether extract was examined for the presence of *cis*- and *trans*-acenaphthene-1,2-diol.

Bacterial β -glucuronidase. Experiments with this enzyme were carried out in the same manner as those just described except that the pH of the urine was adjusted with acetate buffer to pH 6.0-6.4 and the liver preparation was replaced by the preparation of bacterial β -glucuronidase, the activity of which was checked with a substrate of phenolphthalein glucuronide (Talalay, Fishman & Huggins, 1946).

Determination of *cis*-acenaphthene-1,2-diol in the presence of the *trans*-isomer

The determination of the *cis*-diol in the presence of the *trans*-isomer depended on the fact that whereas the *trans*-diol reacts very slowly with periodic acid, the *cis*-diol is oxidized with great rapidity by this reagent. Known mixtures of *cis*- and *trans*-acenaphthene-1,2-diol weighing 20 mg. were dissolved in 3-4 ml. of dioxan (AnalaR) and 5 ml. of a solution of periodic acid (0.6 g. of $HIO_4 \cdot 2H_2O$ made to 50 ml. in water) was added, followed by sufficient dioxan to bring the total volume to 10 ml. After 20 hr. at room temperature, portions (2 ml.) of the solution were removed and solid $NaHCO_3$ (0.1-0.2 g.) was added followed by 2 ml. of 0.1N-sodium arsenite solution. Solid KI (0.1 g.) was then added and after 15 min. the excess of arsenite was titrated with 0.01N- I_2 solution. Blank titrations were carried out as just described except that the diol was not present. Table 1 shows the difference between the iodine titre of the blank and the titres obtained for mixtures of *cis*- and *trans*-acenaphthene-1,2-diol. These data when plotted give a straight line which passes sufficiently close to the origin to show that the reaction which occurred between the *trans*-diol and the periodic acid was insignificant.

RESULTS

Metabolism of acenaphthylene in the rabbit

Paper-chromatographic studies. The urine of a rabbit which had received a dose of 1.25 g. of acenaphthylene by stomach tube was examined by means of paper chromatography. Chromatograms were developed after making the following applications to each paper: (i) urine from the dosed rabbit; (ii) normal rabbit urine; (iii) normal rabbit urine, in 50 ml. of which there had been dissolved 15-20 mg. of *cis*-acenaphthene-1,2-diol and 20-30 mg. of the *trans*-isomer. After development of the chromatograms in the solvent systems A, B and C, and treating them with the ethanol-sulphuric acid reagent, no material with yellow fluorescence in ultraviolet light was detected in (ii), whereas corresponding pairs of yellow spots were found in (i) and (iii). In another experiment the urine of a rabbit which had been injected subcutaneously with 1.25 g. of acenaphthylene was examined. The urine was extracted with ether and the ether extract was applied to the paper in position (i). An ether extract of the urine of a rabbit which had been injected subcutaneously with arachis oil was applied to position (ii), and at (iii) there was applied an ether extract of this urine in which had been dissolved *cis*- and *trans*-

Table 1. *Determination of cis-acenaphthene-1,2-diol in the presence of the trans-isomer*

For experimental details see text.

<i>cis</i> -Diol present in 20 mg. of mixed diols (%)	Vol. of 0.01N- I_2 equivalent to mixed diols (ml.)
0	0.21
20.7	0.95
40.2	1.80
56.4	2.73
79.4	3.61
100.0	4.73

acenaphthene-1,2-diol. The results were similar to those of the experiment in which the acenaphthylene was administered by stomach tube (Table 2).

cis-Acenaphthene-1,2-diol. Five rabbits were dosed by stomach tube with a 10% (w/v) suspension of acenaphthylene in starch solution on 2 successive days so that at each dosing the animals received 0.5 g. of hydrocarbon/kg. body wt. The animals received 13.8 g. of acenaphthylene and from their urine 33 mg. of *cis*-acenaphthene-1,2-diol was isolated, m.p. and mixed m.p. 207–208° (Found: C, 77.5; H, 5.0. Calc. for $C_{12}H_{10}O_2$: C, 77.4; H, 5.4%).

Further evidence supporting the identity of the compound was obtained as follows. The compound isolated in this and similar experiments had $[\alpha]_{D}^{24} 0^\circ$ (c 0.15 in $CHCl_3$, 0.29 in acetone). From 30 mg. of the compound 16 mg. of diacetate was prepared, m.p. and mixed m.p. 130–131° (Found: C, 71.2; H, 5.1. Calc. for $C_{16}H_{14}O_4$: C, 71.1; H, 5.2%). The rates of reaction of the isolated compound with lead tetra-acetate and periodic acid corresponded closely to those observed with synthetic *cis*-acenaphthene-1,2-diol. The ultra-violet-light absorption of the isolated compound in 95% (v/v) ethanol showed maxima at 225, 247, 275, 285.5 and 297 $m\mu$, $\log \epsilon$ 4.9, 3.2, 3.8, 3.9 and 3.7 respectively and was not significantly different from that of synthetic *cis*-acenaphthene-1,2-diol over the range 210–300 $m\mu$.

trans-Acenaphthene-1,2-diol. This diol was isolated after separation of *cis*-acenaphthene-1,2-diol from the ether extract of the urine of rabbits dosed with acenaphthylene by stomach tube. Colourless needles of (+)-*trans*-acenaphthene-1,2-diol weighing 251 mg. were obtained, m.p. 158–159° (Found: C, 77.3; H, 5.3. Calc. for $C_{12}H_{10}O_2$: C, 77.4; H, 5.4%). In addition 222 mg. of a mixture of needles and plates consisting of (+)- and (±)-*trans*-acenaphthene-1,2-diol was obtained, m.p. 145–149° (Found: C, 76.9; H, 5.4%). Attempts to separate the needles from the plates were unsuccessful.

It was concluded that the needles, m.p. 158–159°, were (+)-*trans*-acenaphthene-1,2-diol on the basis of the following evidence. Jack & Rule (1938) prepared the (+)-isomer by resolution of the synthetic racemate and described it as colourless needles, m.p. 158–158.5°. They reported that the m.p. of (+)-*trans*-acenaphthene-1,2-diol was depressed to 149–152° on admixture with the (±)-diol (m.p. 159.5°). In the present work the m.p. of the isolated (+)-diol was depressed to 146–152° on admixture with synthetic (±)-diol. The specific optical rotations of the isolated compound in six different solvents corresponded closely to those reported by Jack & Rule for the (+)-isomer (see Table 3).

The mixture of needles and plates consisting of (+)- and (±)-*trans*-acenaphthene-1,2-diol in general showed smaller rotations (see Table 3) than those of the (+)-diol. When 40 mg. of the mixture was heated in 25 ml. of 15% (v/v) H_2SO_4 under reflux on a boiling-water bath for 45 min. and then steam-distilled, a precipitate (11 mg.) formed in the distillate, m.p. 119–120°, not depressed on admixture with synthetic acenaphthene-1-one, m.p. 119–120.5°.

Synthetic *trans*-acenaphthene-1,2-diol reacted very slowly with lead tetra-acetate in accordance with the observations of Criegee, Kraft & Rank (1933), who found k_{20} 0.03 l./mole/min. Samples of (+)-*trans*-diol and the mixed (+)- and (±)-*trans*-diol isolated from urine in the present work reacted with lead tetra-acetate at the same slow rate as the synthetic compound, namely k_{20} 0.027 l./mole/min. A similar situation was encountered with respect to the interaction of synthetic and biosynthetic *trans*-acenaphthene-1,2-diol with periodic acid, for only 0.04–0.05 mol.prop. of this reagent was consumed in 29 hr.

The infrared-absorption spectra of biosynthetic (+)-*trans*-acenaphthene-1,2-diol and the mixture of the (+)- and (±)-*trans*-diol were indistinguishable from that of the synthetic (±)-*trans*-diol, as also were the ultraviolet-absorption spectra. The

Table 2. *Excretion of cis- and trans-acenaphthene-1,2-diol in the urine of rabbits dosed with acenaphthylene*

The urine of the dosed animals, or an ether extract of it, was examined by paper chromatography. The ascending method was used, and the solvent systems were: A, methanol; B, methanol–carbon tetrachloride (9:1, v/v); C, methanol–cyclohexane (9:1, v/v). For method of detecting compounds see text. Animals were dosed by stomach tube (ST) or subcutaneous injection (SC).

Solvent system	Acenaphthene-1,2-diol	R_F			
		Reference diol	ST dosing	Reference diol	SC dosing
A	<i>cis</i> -	0.65	0.62	0.64	0.65
	<i>trans</i> -	0.72	0.70	0.72	0.71
B	<i>cis</i> -	0.70	0.67	0.70	0.68
	<i>trans</i> -	0.76	0.76	0.77	0.75
C	<i>cis</i> -	0.72	0.72	0.68	0.68
	<i>trans</i> -	0.80	0.79	0.76	0.75

Table 3. *Specific optical rotations of (+)-trans-acenaphthene-1,2-diol in different solvents and of trans-diols obtained from rabbit urine*

The specific optical rotations reported by Jack & Rule (1938) for synthetic (+)-*trans*-acenaphthene-1,2-diol are compared below with those of the *trans*-diols obtained from the urine of rabbits dosed with acenaphthylene. All rotations were determined in a 1 dm. tube (*c* ranged from 0.30 to 0.42), except when benzene was the solvent, in which case a 2 dm. tube was used (*c* ranged from 0.065 to 0.080). The solvents are listed in order of increasing polarity. Jack & Rule (1938) described the compound as dextrorotatory on the basis of its positive rotation in the non-polar solvent benzene.

Solvent	[α] _D ²⁰ ₄₆₁			
	Synthetic diol (Jack & Rule, 1938)	Diol isolated from urine	Mixture of (+)- and (±)-diol from urine	Diol isolated from enzyme-hydrolysed urine
Benzene	+66°	+77°	+37°	+67°
Chloroform	+52	+66	+41	+53
Ethanol	0	-3	-3	-3
Methanol	0	+3	+5	-3
Acetone	-25	-25	-13	-26
Nitrobenzene	-76	-70	-47	-61

latter were determined in 95% (v/v) ethanol over the range 210–300 m μ , and showed maxima at 226, 244.5 and 287 m μ , log ϵ 4.9, 3.2 and 3.9 respectively.

Five rabbits were dosed by subcutaneous injection with a 20% (w/v) solution of acenaphthylene in arachis oil on 2 successive days, so that each rabbit received 0.5 g. of hydrocarbon/kg. body wt., and the rabbits received a total of 13.3 g. of acenaphthylene. From the urine at pH 7–8 14 mg. of pure *cis*-acenaphthene-1,2-diol was isolated, m.p. 206–207°, not depressed by admixture with synthetic *cis*-acenaphthene-1,2-diol. In addition, 56 mg. of a mixture of (+)- and (±)-*trans*-acenaphthene-1,2-diol, m.p. 141–144°, was obtained, [α]_D²³₄₆₁ +41° (*c* 0.062 in benzene) and -12° (*c* 0.490 in nitrobenzene). Pure (+)-*trans*-acenaphthene-1,2-diol was not obtained in this experiment.

Conjugates of the acenaphthene-1,2-diols. The first indication of the excretion of conjugated acenaphthene-1,2-diol was obtained when the formation of acenaphthen-1-one was observed on steam-distilling acidified urine from which the free acenaphthene-1,2-diols had been extracted before acidification. Half the urine from five rabbits which had been dosed with 13.8 g. of acenaphthylene by stomach tube was extracted with ether to remove free acenaphthene-1,2-diols. It was warmed to remove dissolved ether and was then acidified with H₂SO₄, heated under reflux and steam-distilled as already described. The distillate contained a pale-yellow solid, which, when crystallized twice from aqueous ethanol, yielded 347 mg. of white crystals, m.p. and mixed m.p. with synthetic acenaphthen-1-one 119–120.5° (Found: C, 85.6; H, 4.8. Calc. for C₁₂H₈O: C, 85.6; H, 4.8%). From 50 mg. of the ketone 35 mg. of the oxime was prepared, m.p. and mixed m.p. 174–175° (Found: C, 78.2; H, 5.1; N, 7.8. Calc. for C₁₂H₉NO:

C, 78.7; H, 5.0; N, 7.7%). The amount of acenaphthen-1-one isolated in this experiment corresponded to 4.8% of the acenaphthylene administered. When the other half of the urine of the rabbits dosed with acenaphthylene was examined as just described (except that the urine was not acidified) no acenaphthen-1-one was obtained. Moreover, when the untreated urine of the dosed animals was examined by paper chromatography for the presence of acenaphthen-1-one, none could be detected. As free *cis*- and *trans*-acenaphthene-1,2-diol had been extracted from the urine from which the acenaphthen-1-one was separated it appeared probable that the ketone had been formed by the breakdown of conjugated acenaphthene-1,2-diols. This was supported by the results of the following experiments in which the urine of rabbits dosed with acenaphthylene was subjected to enzymic hydrolysis. Two types of experiment were carried out. In one the hydrolysis of diol conjugates in the urine of the dosed animals was brought about by incubation with a commercial preparation of β -glucuronidase, and in the other a suspension of rat liver was used.

In an experiment of the first type, five rabbits were dosed with acenaphthylene by subcutaneous injection so that they received a total of 13.3 g. of the hydrocarbon. The urine of the dosed animals was extracted exhaustively with ether and was then incubated at pH 6.0–6.4 with 1.60 g. (61700 Fishman units) of β -glucuronidase for 24 hr. at 37°. After the incubation the urine was extracted at pH 7–8 with ether. When this ether extract was examined by paper chromatography in the solvent systems A, B and C, it was found to contain *cis*- and *trans*-acenaphthene-1,2-diol. No evidence for the presence of compounds with *R_f* values corresponding to those of *cis*- and *trans*-acenaphthene-1,2-diol was obtained when an examination was

made of the ether extract of normal rabbit urine which had been incubated with β -glucuronidase. The ether extract of the enzyme-hydrolysed urine of the dosed animals yielded 46 mg. of *cis*-acenaphthene-1,2-diol, m.p. and mixed m.p. 207–208° (Found: C, 76.9; H, 5.5. Calc. for $C_{12}H_{10}O_2$: C, 77.4; H, 5.4%). From 30 mg. of the compound 18 mg. of diacetate was prepared, m.p. and mixed m.p. 130–131° (Found: C, 70.8; H, 5.2. Calc. for $C_{16}H_{14}O_4$: C, 71.1; H, 5.2%). The ether extract of the hydrolysed urine also yielded 447 mg. of (+)-*trans*-acenaphthene-1,2-diol, m.p. 158–159° (Found: C, 76.9; H, 5.6%). The m.p. of the product was depressed to 147–151° by admixture with synthetic ($\pm$)-*trans*-acenaphthene-1,2-diol. The specific optical rotations (Table 3) were close to those reported by Jack & Rule (1938) for (+)-*trans*-acenaphthene-1,2-diol. A mixture of (+)- and ($\pm$)-*trans*-acenaphthene-1,2-diol was also obtained as needles and plates (490 mg.), m.p. 147–150° (Found: C, 77.2; H, 5.2%). The specific optical rotations of the product, $[\alpha]_{D}^{24}$, ranged from +37° (c 0.067 in benzene) to –38° (c 0.345 in nitrobenzene). Further evidence in support of the identity of the separated material was obtained by its conversion into acenaphthen-1-one, m.p. 119–121°, which was not depressed by admixture with synthetic acenaphthen-1-one (Found: C, 86.0; H, 5.3. Calc. for $C_{12}H_8O$: C, 85.6; H, 4.8%).

Another enzymic experiment was carried out in which five rabbits were dosed by stomach tube with acenaphthylene so that they received a total of 8.6 g. of the hydrocarbon. The urine of the dosed animals was extracted exhaustively with ether and was incubated for 24 hr. with a rat-liver suspension as already described. It was then extracted with ether and the ether extract yielded 60 mg. of *cis*-acenaphthene-1,2-diol, m.p. 205–206°, 65 mg. of ($\pm$)-*trans*-acenaphthene-1,2-diol, m.p. 157–158°, and 66 mg. of a mixture of (+)- and ($\pm$)-*trans*-acenaphthene-1,2-diol, m.p. 147–151°. The biosynthetic *cis*- and ($\pm$)-*trans*-acenaphthene-1,2-diol showed the same R_f values as their synthetic counterparts when examined by paper chromatography in the solvent systems A, B and C. It was concluded that the urine of rabbits dosed with acenaphthylene contained glucuronic acid conjugates of *cis*-, (+)-*trans*- and (–)-*trans*-acenaphthene-1,2-diol.

Naphthalic anhydride. The urine of four rabbits which had received 10.5 g. of acenaphthylene (0.5 g./kg. body wt.) by subcutaneous injection yielded 29 mg. of naphthalic anhydride, m.p. 268–269°, unchanged by admixture with synthetic naphthalic anhydride. The pure naphthalic anhydride corresponded to 0.2% of the acenaphthylene administered. The identity of the biosynthetic compound was confirmed by the fact that it

had the same R_f values as the authentic compound, and by its conversion into *N*-anilidonaphthalimide (Found: C, 74.9; H, 3.9; N, 9.6. Calc. for $C_{18}H_{12}N_2O_2$: C, 75.0; H, 4.2; N, 9.7%). The m.p. of the derivative, 215–216°, was not depressed on admixture with synthetic *N*-anilidonaphthalimide, m.p. 215–216°.

Absence of an acid-labile precursor of acenaphthylene from the urine. Certain polycyclic hydrocarbons are known to be converted in the animal body into derivatives which are excreted in the urine and yield the parent hydrocarbon when decomposed by acid. Among the hydrocarbons which undergo this reaction *in vivo* is naphthalene (Bourne & Young, 1934), and the compound which breaks down to give this hydrocarbon has been identified by Boyland & Solomon (1955) as 1,2-dihydro-1-naphthyl glucosiduronic acid. In an attempt to discover whether a compound of this type is formed from acenaphthylene in the animal body an examination was made of the urine of four rabbits which had been dosed with acenaphthylene by subcutaneous injection on 2 successive days so that they had received a total of 10.5 g. of the hydrocarbon. No evidence for the excretion of an acid-decomposable acenaphthylene precursor was found in this experiment or in a similar experiment in which the acenaphthylene was administered by stomach tube.

Faecal excretion of acenaphthylene. The method of Chang (1943) was used to separate acenaphthylene from the faeces of five rabbits which had been dosed with a total of 9.6 g. of acenaphthylene by stomach tube. The total amount of acenaphthylene isolated was 6 mg., with m.p. 90–92°; the mixed m.p. with authentic acenaphthylene (m.p. 92–93.5°) was 90–92°.

Metabolism of acenaphthylene in the rat

Paper chromatograms were developed for the urine of rats which had received acenaphthylene by stomach tube or by subcutaneous injection. In ultraviolet light the chromatograms showed faint spots corresponding to *cis*- and *trans*-acenaphthene-1,2-diol. There was also present a third spot with purple fluorescence. The urine of the rats which had received subcutaneous injections of acenaphthylene in arachis oil was extracted at pH 7–8 with ether and the ether extract was shaken with 2N-NaOH. Under these conditions only two faint spots were visible on exposure of the chromatograms to ultraviolet light and these corresponded in colour and R_f values to *cis*- and *trans*-acenaphthene-1,2-diol. These compounds were present in very low concentration, however, for when an attempt was made to isolate them from the urine of rats which had received a total of 18 g. of acenaphthylene by stomach tube, only a small amount of product was

obtained and attempts to fractionate this into the *cis*- and *trans*-isomers were unsuccessful.

The urine of rats which had received a total of 9 g. of acenaphthylene by stomach tube was extracted exhaustively with ether and was then made strongly acid with H_2SO_4 , heated under reflux and finally steam-distilled. The distillate yielded 45 mg. of acenaphthen-1-one, m.p. and mixed m.p. 119–120°. The ketone was converted into its oxime, m.p. and mixed m.p. 173–175° (Found: C, 78.1; H, 4.9; N, 7.8. Calc. for $\text{C}_{12}\text{H}_9\text{NO}$: C, 78.7; H, 5.0; N, 7.7%). The separation of acenaphthen-1-one from the urine under these conditions and a failure to detect it in urine which had not been heated with acid suggested that the urine of the dosed animals contained conjugated diols. This was supported by the finding that when the urine of rats which had been dosed with a total of 13.5 g. of acenaphthylene by stomach tube was extracted exhaustively with ether and then incubated with rat-liver-suspension, it was possible to separate 32 mg. of *cis*-acenaphthene-1,2-diol, m.p. 206–207°, and 30 mg. of a mixture of (+)- and (±)-*trans*-acenaphthene-1,2-diol, m.p. 140–142°, $[\alpha]_{\text{D}}^{24}$ +15.2° (c 0.329 in CHCl_3) and –19.4° (c 0.310 in nitrobenzene).

In a similar experiment to that just described, except that the commercial β -glucuronidase preparation was used instead of a rat-liver suspension, 4 mg. of *cis*-acenaphthene-1,2-diol, m.p. 205–206° and mixed m.p. 206–207°, was obtained together with 36 mg. of a mixture of (+)- and (±)-*trans*-acenaphthene-1,2-diol, m.p. 142–145°, $[\alpha]_{\text{D}}^{24}$ +24° (c 0.251 in CHCl_3) and +50° (c 0.040 in benzene). In this experiment 12 rats received a total of 4.8 g. of acenaphthylene by subcutaneous injection.

The compound isolated in largest amount from the urine of rats dosed with acenaphthylene was naphthalic anhydride. From the urine of 35 rats which had received a total of 8.75 g. of acenaphthylene by stomach tube, 221 mg. of naphthalic anhydride was obtained as pale-yellow needles, m.p. 269–270°. This material when recrystallized from hot conc. HNO_3 yielded 106 mg. of colourless needles, m.p. 270–271°, not depressed by admixture

with authentic naphthalic anhydride (Found: C, 72.9; H, 3.1. Calc. for $\text{C}_{12}\text{H}_6\text{O}_3$: C, 72.7; H, 3.1%). The identity of the compound was confirmed by conversion into its derivative with phenylhydrazine, m.p. and mixed m.p. 215–216° (Found: C, 74.8; H, 4.1; N, 10.0. Calc. for $\text{C}_{18}\text{H}_{12}\text{N}_2\text{O}_2$: C, 75.0; H, 4.2; N, 9.7%). In an experiment in which 12 rats received a total of 4.8 g. of acenaphthylene by subcutaneous injection, 86 mg. of naphthalic anhydride, m.p. and mixed m.p. 272–273°, was isolated (Found: C, 73.2; H, 3.2%).

Not all the acenaphthylene administered to rats by stomach tube was absorbed, for by application of the method of Chang (1943) to the faeces of 18 rats which had received a total of 4.5 g. of the hydrocarbon it was possible to isolate 32 mg. of acenaphthylene, m.p. 91–93°, unchanged by admixture with authentic acenaphthylene.

Interconversion of cis- and trans-acenaphthene-1,2-diol

As *cis*- and *trans*-acenaphthene-1,2-diol had been shown to be present in the urine of rabbits and rats dosed with acenaphthylene, it was necessary to determine whether both diols were present in the urine as a result of the partial isomerization of one of them. In their study of the metabolism of indene, Brooks & Young (1956) showed that interconversion of *cis*- and *trans*-indane-1,2-diol occurred in hot acid solution, but that no detectable isomerization occurred in urine.

Synthetic *cis*-acenaphthene-1,2-diol (100 mg.) was heated with 0.2N- H_2SO_4 (80 ml.) under reflux in a boiling-water bath. After a measured period of time the solution was cooled and extracted with ether. The ether extract was evaporated to dryness and the amount of *cis*-acenaphthene-1,2-diol in the residue was determined by reaction with periodic acid. The acenaphthen-1-one present was determined by converting it into its 2,4-dinitrophenylhydrazone, which was weighed. The amount of *trans*-acenaphthene-1,2-diol was calculated by difference. Similar experiments were carried out with 100 mg. of *trans*-acenaphthene-1,2-diol as the starting material. The results summarized in Table 4 show

Table 4. *Interconversion of cis- and trans-acenaphthene-1,2-diol in acid solution*

Determinations were made of the percentages of the acenaphthene-1,2-diols and of acenaphthen-1-one present after reaction with 0.2N- H_2SO_4 at the temperature of the boiling-water bath. For details of methods see text.

Time of reaction (hr.)	Starting material	Components of mixture (%)		
		<i>cis</i> -Diol	<i>trans</i> -Diol	Acenaphthen-1-one
5	<i>cis</i> -Diol	54	39	7
10	<i>cis</i> -Diol	29	65	6
5	<i>trans</i> -Diol	13	82	5
10	<i>trans</i> -Diol	19	76	5

that even in hot acid solution interconversion of the two dihydrodiols occurs quite slowly.

The stability of the acenaphthene-1,2-diols in rabbit urine at pH 5, 6.5 and 8.5 was studied by the method used by Brooks & Young (1956). No conversion of one geometrical form of acenaphthene-1,2-diol into the other was detected.

DISCUSSION

The dihydrodiols isolated from the urine of experimental animals after the administration of naphthalene (Young, 1947; Booth & Boyland, 1949), anthracene (Boyland & Levi, 1935) and phenanthrene (Young, 1947; Boyland & Wolf, 1948, 1950) have all been assigned the *trans*-configuration (Boyland, 1950; Cook, 1950). Indene, which gives rise to *cis*- and *trans*-indane-1,2-diol in the animal body (Brooks & Young, 1956), was the first hydrocarbon shown to be metabolized to a *cis*- and a *trans*-dihydrodiol. The present work (see

Fig. 1) has revealed a second example of this type of metabolic change, namely the conversion of acenaphthylene (III) into *cis*- and *trans*-acenaphthene-1,2-diol (IV and VI). Consideration has to be given to the possibility that the appearance of both isomers in the urine can be explained by the metabolic production of one isomer and its partial isomerization to the other in the urine. Brooks & Young (1956) concluded that although interconversion of the indanediols could occur under very acid conditions it does not take place under the conditions prevailing in the urine. A similar conclusion has been reached in the present work with respect to the isomerization of *cis*- and *trans*-acenaphthene-1,2-diol.

Boyland (1950) has suggested that epoxide formation may occur during the metabolic oxidation of aromatic hydrocarbons. van Loon (1919) reported that in an aqueous medium indene epoxide is converted into *cis*- and *trans*-indane-1,2-diol and this has been confirmed by the observation

Fig. 1. Metabolism of acenaphthylene. Broken arrows indicate possible metabolic pathways.

(Hopkins, 1959) that this change takes place at 37° in phosphate buffer, pH 7.4. Epoxide formation, therefore, could explain the appearance of *cis*- and *trans*-indane-1,2-diol in the urine of animals dosed with indene. Whether a similar possibility can be entertained with respect to the metabolism of acenaphthylene is uncertain, for attempts to prepare the appropriate epoxide (V) have been unsuccessful.

cis-Acenaphthene-1,2-diol exists only in an optically inactive form, but the *trans*-isomer can occur in dextrorotatory and laevorotatory forms. The isolation of *trans*-acenaphthene-1,2-diol from rabbit urine in the racemic form as well as the dextrorotatory form presents a situation analogous to that encountered in the metabolism of naphthalene, for it is possible to isolate a racemic form of *trans*-1,2-dihydronaphthalene-1,2-diol from the urine of rats and rabbits dosed with naphthalene (Booth & Boyland, 1949) in addition to a laevorotatory form from rat urine (Young, 1947; Booth & Boyland, 1949) and a dextrorotatory form from rabbit urine (Booth & Boyland, 1949). The appearance of unequal amounts of the dextro- and laevo-isomers can be explained if the acenaphthylene is first converted into the epoxide, which then changes spontaneously into a mixture of *cis*- and ($\pm$)-*trans*-acenaphthene-1,2-diol. If the (+) and (−) forms of *trans*-acenaphthene-1,2-diol then undergo some further reaction such as formation of naphthalic acid (I) by ring fission (Hopkins & Young, 1961) and if this does not take place to the same extent for one optical form as for the other, then unequal amounts of the (+) and (−) forms of the free dihydrodiols will be excreted in the urine.

The free dihydrodiols isolated from rabbit urine in the present work corresponded to only a small proportion of the acenaphthylene administered, namely 0.2–0.5% for *cis*-acenaphthene-1,2-diol and 3–4% for the *trans*-isomer. There is little doubt, however, that substantial amounts of the dihydrodiols were excreted in conjugated form, as evidenced by the production of acenaphthen-1-one (VIII) on acid treatment of the urine after removal of the free dihydrodiols. Moreover, the liberation of the dihydrodiols when the urine was incubated with rat liver or with bacterial β -glucuronidase indicates that glucuronides (VII and IX) of the dihydrodiols were present. The administration of acenaphthylene to rabbits and rats was followed by an increase in glucuronic acid excretion in the following 48 hr., corresponding on an equimolar basis to 37% and 15% respectively of the hydrocarbon administered. Neither rabbits nor rats appear to excrete an acid-decomposable acenaphthylene precursor analogous to that formed from naphthalene and identified by Boyland & Solomon (1955) as 1,2-dihydro-1-naphthyl glucosiduronic acid.

Considerable interest attaches to the separation of naphthalic anhydride (II) from the urine of rabbits and rats dosed with acenaphthylene. In view of the insolubility of naphthalic anhydride in water and the ease with which naphthalic acid (I) is converted into its anhydride it is reasonable to suppose that naphthalic acid is the excretory product. Attention has been drawn to the possibility that dihydrodiols may undergo ring fission in the organism to give dicarboxylic acids (Boylund, 1950; Young, 1950), and evidence has recently been obtained that both *cis*- and *trans*-acenaphthene-1,2-diol may serve as intermediates in the metabolic conversion of acenaphthylene into naphthalic acid (Hopkins & Young, 1961).

SUMMARY

1. The metabolism of acenaphthylene in rabbits and rats has been studied after the administration of the hydrocarbon by stomach tube or by subcutaneous injection.

2. *cis*- and *trans*-Acenaphthene-1,2-diol have been shown to be metabolites of acenaphthylene by their isolation from the urine of dosed rabbits and by confirmation of their identity by synthesis. The presence of these compounds in the urine of dosed rats has been demonstrated by means of paper chromatography.

3. Liberation of *cis*- and *trans*-acenaphthene-1,2-diol has been shown to occur when the urine of rabbits and rats dosed with acenaphthylene is hydrolysed with β -glucuronidase.

4. The absence of any significant interconversion of *cis*- and *trans*-acenaphthene-1,2-diol in urine has been demonstrated.

5. Naphthalic acid has been isolated in the form of its anhydride from the acidified urine of animals dosed with acenaphthylene.

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Studies on Zinc in Blood

TRANSPORT OF ZINC AND INCORPORATION OF ZINC IN LEUCOCYTES

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It is widely recognized that zinc is essential for the normal growth of both animals and plants, but our present knowledge of zinc metabolism and transport in the animal body is still incomplete.

It has long been known that blood contains zinc and it is reasonable to expect that, since a significant fraction of the blood zinc is present in the plasma, it is the plasma form which is the main transport medium. Varying values for the plasma zinc have been recorded; thus Delezenne (1919) reported that about one-tenth of the blood zinc is contained in the plasma and a similar value of 12% for the serum zinc as a percentage of the whole-blood zinc (of 880 $\mu\text{g.}/100\text{ ml.}$) is given by Vallee (1959). Analyses on plasma and serum give identical results, and, whichever fluid is used, there is fairly general agreement that the plasma or serum zinc may be divided into two fractions, in one of which the metal is more firmly bound than it is in the other.

There appears to be no evidence of any plasma-saturation limit for zinc (Berfenstam, 1952) similar to that for iron. Indeed, the mode of transport of zinc in plasma is still not clear; studies have been made with several species of animals and it is

possible that species differences account for some of the recorded differences. Another factor is the possible exchange of zinc between the plasma proteins and the zinc compounds in erythrocytes and leucocytes, these cells accounting respectively for about 85 and 3% of the whole-blood zinc (Vallee, 1959). Part of the plasma zinc is probably associated with zinc-containing enzymes, but this amount is probably small and has not been completely identified, though Vessel & Bearn (1957) have found, in electrophoretic separation of isotopically labelled serum proteins, that part of the zinc is associated with the α_2 -globulins, coinciding with one of the peaks of lactic dehydrogenase.

In view of this uncertainty about the distribution of the zinc in normal plasma, we have investigated this problem in the rabbit, with ^{65}Zn as tracer, and have studied the labelled blood to determine which protein fractions bind zinc injected intravenously as a zinc-glycine complex.

A study has also been made of the extent to which the injected zinc is incorporated into erythrocytes and leucocytes, and the possibility of exchange of this zinc between the plasma and the cells in the blood.