Biochemical Studies of Toxic Agents

8. 1:2-DIHYDRONAPHTHALENE-1:2-DIOL AND ITS ROLE IN THE METABOLISM OF NAPHTHALENE*

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Studies of the metabolism of naphthalene have shown that conversion of this hydrocarbon into *trans*-1:2-dihydronaphthalene-1:2-diol takes place in the rat (Young, 1947; Booth & Boyland, 1949), the rabbit (Booth & Boyland, 1949), the guinea pig and the mouse (Corner & Young, 1954). It has been found, however, that different proportions of the dextro- and laevo-rotatory forms of the dihydrodiol are excreted by different species. 1:2-Dihydronaphthalene-1:2-diol can be broken down in an acid medium to yield 1- and 2-naphthol, and as both these naphthols, together with the sulphuric acid and glucuronic acid conjugates of 1-naphthol, are known to be metabolites of naphthalene (cf. Corner & Young, 1954), the question arises whether 1:2-dihydronaphthalene-1:2-diol is an intermediate in the metabolic conversion of naphthalene into naphthol. One approach to this problem lies in a study of the metabolism of the dihydrodiol itself, and one such investigation has been made by Boyland & Wiltshire (1953). These workers made a quantitative study of the metabolism of synthetic ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol in the rat and from the results of the colorimetric procedures they used they concluded that the dosed animals excreted unchanged 1:2-dihydronaphthalene-1:2-diol, free 1-naphthol, naphthylsulphuric acid and a glucuronide liberating 1-naphthol on hydrolysis.

In the present investigation the metabolism of ($\pm$)-, (+)- and (-)-*trans*-1:2-dihydronaphthalene-1:2-diol has been studied in the hooded rat. It has been found that administration of the dihydrodiol leads to a marked increase in the glucuronic acid content of the urine and also to a significant increase in the ethereal sulphate fraction. No increase in the neutral sulphur excretion was detected. Paper chromatography has been used to gain information about the nature of the hydroxy derivatives of naphthalene excreted in the free form and in conjugation with sulphuric acid and glucuronic acid. Experiments have also been carried out in which 1:2-dihydronaphthalene-1:2-diol and ^{35}S -labelled sodium sulphate have been

administered simultaneously to rats and these have thrown additional light on the character of the sulphuric acid conjugates in the urine.

Our experiments have revealed no qualitative differences in the metabolism of ($\pm$)-, (+)- and (-)-*trans*-1:2-dihydronaphthalene-1:2-diol in the rat, and every metabolite of this compound so far identified has also been shown to be a metabolite of naphthalene.

EXPERIMENTAL

Materials

($\pm$)-*trans*-1:2-Dihydronaphthalene-1:2-diol. Naphthalene was administered to male hooded rats by stomach tube and 1:2-dihydronaphthalene-1:2-diol was isolated from the urine by the method described by Young (1947). The compound showed no measurable optical rotation and it melted at 100°. (Found: C, 74.1; H, 6.1. Calc. for $\text{C}_{10}\text{H}_{10}\text{O}_2$: C, 74.0; H, 6.2%). The compound was acetylated by acetic anhydride in pyridine and the product so obtained showed no optical rotation and melted at 74°. (Found: C, 68.7; H, 5.5. Calc. for $\text{C}_{14}\text{H}_{14}\text{O}_4$: C, 68.3; H, 5.7%.)

(+)-*trans*-1:2-Dihydronaphthalene-1:2-diol. Naphthalene was administered to rabbits by stomach tube and a glucosiduronic acid fraction was separated from the urine by the procedure described by Corner, Billett & Young (1954). Incubation of the fraction at 37° with β -glucuronidase led to the isolation of 1:2-dihydronaphthalene-1:2-diol, m.p. 127–128°, $[\alpha]_D^{20} + 157^\circ$ in ethanol (c, 1). (Found: C, 73.5; H, 6.0. Calc. for $\text{C}_{10}\text{H}_{10}\text{O}_2$: C, 74.0; H, 6.2%). The diacetate of the compound was prepared, m.p. 77°, $[\alpha]_D^{25} + 421^\circ$ in ethanol (c, 0.5). (Found: C, 68.8; H, 6.1. Calc. for $\text{C}_{14}\text{H}_{14}\text{O}_4$: C, 68.3; H, 5.7%.)

(-)-*trans*-1:2-Dihydronaphthalene-1:2-diol. Naphthalene was administered to guinea pigs by intraperitoneal injection, and 1:2-dihydronaphthalene-1:2-diol was isolated from the urine by the method described by Corner & Young (1954). The compound melted at 126–127° and showed $[\alpha]_D^{20} - 157^\circ$ in ethanol (c, 1). (Found: C, 74.0; H, 6.1. Calc. for $\text{C}_{10}\text{H}_{10}\text{O}_2$: C, 74.0; H, 6.2%). The diacetate of the compound was prepared, m.p. 77–78°, $[\alpha]_D^{25} - 434^\circ$ in ethanol (c, 1). (Found: C, 68.8; H, 6.2. Calc. for $\text{C}_{14}\text{H}_{14}\text{O}_4$: C, 68.3; H, 5.7%.)

Quantitative studies

Dosing of animals and collection of urine. Pairs of male hooded rats (average body wt. 270 g.) were used. The animals were housed in metabolism cages designed to allow the collection of urine separate from faeces, and they

* Paper 7: Corner & Young (1954).

were fed on a bread and milk diet with vitamin supplements. Before an experiment was started the animals were kept in the metabolism cages for 3 days in order that they might become accustomed to the new environment.

Analytical methods. Glucuronic acid was determined by the naphthoresorcinol method of Hanson, Mills & Williams (1944) with the modification introduced by Bisset, Brooksbank & Haslewood (1948). It was found that when glucuronic acid determinations were carried out using rat urine, the blue colour normally obtained was modified considerably by urinary constituents which gave rise to a reddish purple colour. To test the significance of this effect a comparison was made of the results obtained when samples of 1-naphthyl glucosiduronic acid added to water and to normal rat urine were hydrolysed at various dilutions corresponding to those used in the metabolism experiments. Almost identical calibration graphs were obtained under the two sets of conditions. Complete hydrolysis of 1-naphthyl glucosiduronic acid occurs when the compound is heated in a boiling-water bath for 2 hr. in the presence of 3N-HCl and these conditions were used for the analysis of rat urine.

The excretion of total and inorganic sulphate in the urine was determined by means of the benzidine-precipitation procedure (cf. Corner *et al.* 1954) and the ethereal sulphate excretion was calculated from the difference between these values. The neutral sulphur content of the urine was calculated from the difference between the total sulphate sulphur and total sulphur excretions. The total sulphur content of the urine was determined by the Carius oxidation procedure described by Young, Edson & McCarter (1949).

During the metabolism experiments the urine of each pair of animals was collected daily, filtered and made up to 100 ml. Three 5 ml. samples of this diluted urine were used for estimations of inorganic sulphate, three 6 ml. samples for estimations of total sulphate. Two 6 ml. samples were concentrated and used for total sulphur estimations. The glucuronic acid content of the urine was determined by using two 1 ml. samples suitably diluted.

Results. Two pairs of animals were used in the first metabolism experiment and the urine of each pair was analysed separately. Daily determinations of inorganic and total sulphate, total sulphur and glucuronic acid were made. After a 4-day pre-dosing period each animal received an intraperitoneal injection of ($\pm$)-*trans*-1:2-dihydronaphthalene-

thalene-1:2-diol as a 2% (w/v) solution in water. Exactly 24 hr. after the first dose the animals received a second dose of the same size. Each pair of rats received a total of 212 mg./kg. body wt. of ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol. The urine was analysed daily for 4 days after the administration of the second dose of the compound. The mean results of this experiment are shown in Table 1.

Similar experiments were carried out in which (+)- and (-)-*trans*-1:2-dihydronaphthalene-1:2-diol were administered to rats. A summary of the main findings is given in Table 2, where the total effect of the administration of 1:2-dihydronaphthalene-1:2-diol on ethereal sulphate and glucuronic acid excretion is shown. A marked rise in the glucuronic acid content of rat urine occurred. There was also a significant increase in the ethereal sulphate content of the urine. There was no detectable rise in the neutral sulphur fraction (see Table 1).

Table 1. *Excretion of inorganic and ethereal sulphate S (IS and ES), neutral sulphur (NS) and glucuronic acid in the urine of four rats before and after the intraperitoneal injection of ($\pm$)-trans-1:2-dihydronaphthalene-1:2-diol*

Animals dosed twice. Total dose 212 mg./kg. body wt. as a 2% (w/v) solution in water.

Day	Sulphur (mg./kg. body wt.)			Glucuronic acid (mg./kg. body wt.)
	IS	ES	NS	
1	22.9	0.0	19.6	54
2	25.3	0.1	21.9	57
3	28.4	0.5	18.6	66
4	23.9	0.1	17.3	78
($\pm$)- <i>trans</i> -1:2-Dihydronaphthalene-1:2-diol administered				
5	20.7	3.8	20.0	127
($\pm$)- <i>trans</i> -1:2-Dihydronaphthalene-1:2-diol administered				
6	15.9	6.8	18.2	136
7	29.0	0.1	19.9	45
8	30.1	0.3	24.9	47
9	29.4	0.4	23.0	38

Table 2. *Conjugation of ($\pm$)-, (+)- and (-)-trans-1:2-dihydronaphthalene-1:2-diol and their metabolites with sulphuric acid and glucuronic acid in the rat*

The values given are percentages of the dose of 1:2-dihydronaphthalene-1:2-diol corresponding to the increases in ethereal sulphate and glucuronic acid excretions when calculated on the basis of one molecule of the compound per molecule of sulphuric acid or glucuronic acid. For experimental details see text and Table 1.

Form of <i>trans</i> -1:2-dihydronaphthalene-1:2-diol administered	Dose level (mg./kg. body wt.)	Percentage of dose conjugated		
		With sulphuric acid	With glucuronic acid	Total
($\pm$)	212	24	52	76
(+)	194	39	29	68
(-)	200	20	57	77

One possible explanation of these findings is that 1:2-dihydronaphthalene-1:2-diol was excreted in conjugation with glucuronic acid and sulphuric acid. Another possibility is that some 1:2-dihydronaphthalene-1:2-diol was converted into 1- and 2-naphthol which were excreted in the form of conjugates. It is known that some of the 1:2-dihydronaphthalene-1:2-diol formed from naphthalene in the rat and in the rabbit undergoes conjugation with glucuronic acid and is excreted as 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid (Corner *et al.* 1954; Corner & Young, 1954), and it appears likely, therefore, that this compound is present in the urine of rats dosed with 1:2-dihydronaphthalene-1:2-diol. In order to gain information concerning the nature of the free and conjugated hydroxy derivatives of naphthalene excreted by rats dosed with 1:2-dihydronaphthalene-1:2-diol the urine of the dosed animals was examined by the chromatographic procedures described in the following section.

Chromatographic studies

The reference compounds used were 1- and 2-naphthol, 1:2- and 1:4-dihydroxynaphthalene, 1:2-dihydronaphthalene-1:2-diol, 1- and 2-naphthylsulphuric acid, and 1- and

2-naphthyl glucosiduronic acid. A glucosiduronic acid fraction known to contain 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid (cf. Corner *et al.* 1954) was also used for reference purposes. The methods used for obtaining the reference compounds and details of the procedures for paper chromatography have been described previously (Corner & Young, 1954).

Animal experiments. Each of six male hooded rats of average body weight 260 g. received a single dose of 30 mg. of 1:2-dihydronaphthalene-1:2-diol by intraperitoneal injection. The first pair of animals was given the optically inactive compound, the second pair received the dextro-rotatory compound and the third pair was dosed with the laevorotatory compound. The urine from each pair of animals (pH 6.5–7.0) was collected for 24 hr. after dosing, filtered and extracted continuously with peroxide-free ether for 24 hr., and the ether extracts were combined and evaporated to dryness. The residue so obtained was dissolved in 1–2 ml. of ethanol, and samples of the solution were examined by paper chromatography using solvent systems 1–3 (see Table 3). After each urine had been extracted with ether, it was evaporated under reduced pressure to one-tenth of its volume and then shaken with an equal volume of *n*-butanol. The emulsion which formed was broken by centrifuging and the butanol layer was separated and examined by paper chromatography using solvent systems 4 and 5 (see Table 3) for the presence of conjugated hydroxy derivatives of naphthalene. The identity of each metabolite reported in Table 3 was deduced

Table 3. *The chromatographic examination of ether and butanol extracts of the urines of rats dosed with (±)-, (+)- and (-)-trans-1:2-dihydronaphthalene-1:2-diol*

R_F values, except those in heavy type, which refer to papers treated with HCl fumes before spraying, refer to papers sprayed immediately. Colours of spots: DR, dark red; O, orange; P, purple; Y, yellow. Solvent systems (the saturated organic phase obtained after mixing the volumes shown was used): 1, cyclohexane–water–ammonia (sp.gr. 0.88) (5:4:1); 2, benzene–water–ammonia (sp.gr. 0.88) (5:4:1); 3, cyclohexane–benzene–water–ammonia (sp.gr. 0.88) (5:5:8:2); 4, *n*-butanol–3*N*·(NH₄)₂CO₃–3*N*·NH₄OH (4:3:3); 5, isobutanol–3*N*·(NH₄)₂CO₃–3*N*·NH₄OH (4:3:3).

Solvent system	R_F values Form of <i>trans</i> -1:2-dihydronaphthalene-1:2-diol administered			Colours		Compounds
	(±)	(+)	(-)	Diazotized sulphanilic acid	NNCD reagent	
	Ether extracts					
1	0.14	0.16	0.16	O	Y	2-Naphthol
	0.01	0.01	0.01	DR	Y	1:2-Dihydronaphthalene-1:2-diol
2	0.76	0.76	0.76	O	Y	2-Naphthol
	0.26	0.27	0.27	DR	Y	1:2-Dihydronaphthalene-1:2-diol
3	0.50	0.51	0.51	O	Y	2-Naphthol
	0.10	0.11	0.11	DR	Y	1:2-Dihydronaphthalene-1:2-diol
Butanol extracts						
4	0.90	0.89	0.90	DR	Y	1:2-Dihydronaphthalene-1:2-diol
	0.81	0.81	0.80	DR	Y	1-Naphthylsulphuric acid
	0.74	0.73	0.74	P	P	Unknown*
	0.22	0.24	0.22	DR	Y	1-Naphthyl glucosiduronic acid
	0.09	0.10	0.09	DR	Y	1:2-Dihydro-2-hydroxy-1-naphthyl glucosiduronic acid
5	0.83	0.84	0.83	DR	Y	1:2-Dihydronaphthalene-1:2-diol
	0.76	0.77	0.77	DR	Y	1-Naphthylsulphuric acid
	0.67	0.66	0.67	P	P	Unknown*
	0.11	0.12	0.11	DR	Y	1-Naphthyl glucosiduronic acid
	0.08	0.09	0.09	DR	Y	1:2-Dihydro-2-hydroxy-1-naphthyl glucosiduronic acid

* Probably a conjugate of 1:2-dihydroxynaphthalene (see text).

by comparing its R_F values with those of reference compounds which were run simultaneously in the same solvent systems, and from the colours which the metabolites gave with diazotized sulphanilic acid and ammonia, and with 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate (NNCD reagent), either before or after the chromatograms were treated with HCl fumes.

Results. Ether extracts of the urines of rats dosed with ($\pm$), (+)- and (-)-*trans*-1:2-dihydronaphthalene-1:2-diol were found to contain 2-naphthol and 1:2-dihydronaphthalene-1:2-diol. No evidence for the presence in these extracts of 1-naphthol, 1:2-dihydroxynaphthalene or 1:4-dihydroxynaphthalene was obtained. 1-Naphthylsulphuric acid, 1-naphthyl glucosiduronic acid, and 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid were detected in butanol extracts of these urines, but no evidence for the presence of 2-naphthylsulphuric acid or 2-naphthyl glucosiduronic acid was obtained. As the R_F values of 1-naphthyl glucosiduronic acid are close to those of 2-naphthyl glucosiduronic acid in the two solvent systems used, it seemed possible that this may have accounted for failure to detect the latter compound. This possibility was tested in experiments in which an amount of 2-naphthyl glucosiduronic acid corresponding to 1% of the ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol administered to rats was dissolved in the urine of the dosed animals. Butanol extracts of this urine were prepared by means of the procedure described earlier and when these extracts were examined by paper chromatography, 2-naphthyl glucosiduronic acid was easily detectable on the paper chromatograms and could be differentiated from the corresponding derivative of 1-naphthol.

When a similar experiment was carried out to detect the presence of 2-naphthylsulphuric acid on paper chromatograms the results were difficult to interpret because of the presence of an unknown metabolite of 1:2-dihydronaphthalene-1:2-diol with an R_F value slightly smaller than those of the sulphuric acid conjugates of 1- and 2-naphthol. Further evidence in favour of the view that 2-naphthylsulphuric acid was not present, however, was obtained as follows. A butanol extract of the urine of rats dosed with ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol was prepared by means of the procedure described earlier. A sample of the extract was spotted on a paper strip which was developed in solvent system 4 (see Table 3). After the chromatogram had been developed, the section of the paper strip which carried 1-naphthylsulphuric acid was removed and immersed in hot water (10 ml.) for 15 min. The aqueous extract was adjusted to pH 1 with conc. HCl and heated in a boiling-water bath for 30 min. The mixture was then cooled to room temperature, extracted with an

equal volume of ether and the evaporated ether extract, when examined by paper chromatography, was found to contain 1-naphthol but not 2-naphthol. The latter compound was easily detected, however, when the procedure just described was applied to rat urine containing 2-naphthylsulphuric acid in amounts corresponding to 1% of the dose of 1:2-dihydronaphthalene-1:2-diol which had been administered to these animals.

The results of the present investigation resemble in a number of respects those obtained from a chromatographic examination of the urine of rats dosed with naphthalene (Corner & Young, 1954). Thus, rats dosed either with naphthalene or with 1:2-dihydronaphthalene-1:2-diol excrete 2-naphthol but not 2-naphthylsulphuric acid or 2-naphthyl glucosiduronic acid. Whereas rats to which naphthalene has been administered excrete 1-naphthol together with 1-naphthyl glucosiduronic acid and 1-naphthylsulphuric acid, those dosed with 1:2-dihydronaphthalene-1:2-diol do not appear to excrete 1-naphthol in the free state, even though the sulphuric acid and glucuronic acid conjugates of this compound are present in the urine.

Isolation of unchanged 1:2-dihydronaphthalene-1:2-diol

The chromatographic examination of the urines of rats dosed with ($\pm$), (+)- and (-)-*trans*-1:2-dihydronaphthalene-1:2-diol showed that they contained 1:2-dihydronaphthalene-1:2-diol in the free state. This finding was confirmed by the isolation of the compound from the urine.

($\pm$)-*trans*-1:2-Dihydronaphthalene-1:2-diol. A dose of ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol as a 2% (w/v) solution in water was administered by intraperitoneal injection to each of four male hooded rats (average body wt. 180 g.) on each of 2 successive days. The total dose given to each animal was 40 mg. The urine was collected for 3 days after the first dose was administered, filtered and extracted continuously for 20 hr. with peroxide-free ether. The ether extracts were evaporated to dryness under reduced pressure and the brown tarry residue so obtained was dried thoroughly over P_2O_5 . *cyclo*Hexane was added to the residue and the mixture was boiled under reflux for 20 min. The extract was then concentrated at room temperature and the crystalline material which separated was dried thoroughly by suction on the filter. By this means 14 mg. of material in the form of pink silky needles were separated from the urine of the dosed animals. When an aqueous solution of the material was tested with diazotized sulphanilic acid in the presence of excess of 2N-NaOH, a very pale pink colour was obtained. When this test was repeated using an aqueous solution of the material which had first been heated in a boiling-water bath for 1 min. in the presence of acid, however, an intense cherry-red colour was formed. 1- and 2-Naphthol were identified by means of paper chromatography as the products formed when the compound was decomposed with acid. The compound melted at 101–102° and the m.p. was not depressed

by admixture with ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol, m.p. 100–101°.

(+)-*trans*-1:2-Dihydronaphthalene-1:2-diol. After a portion had been used for the chromatographic studies described in the previous section, the ethanolic solution of the residue obtained from evaporated ether extracts of the urine of rats dosed with (+)-*trans*-1:2-dihydronaphthalene-1:2-diol was treated as follows. The ethanolic solution was evaporated to dryness under reduced pressure and the brown tarry residue so obtained was dried thoroughly over P_2O_5 . By means of the procedure described for the isolation of ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol 3 mg. of pink crystalline material were isolated. When treated with diazotized sulphanilic acid in the presence of excess of 2N-NaOH an aqueous solution of the material gave a positive test for naphthol after, but not before, acid decomposition. The compound melted at 123–125° and after admixture with an authentic sample of (+)-*trans*-1:2-dihydronaphthalene-1:2-diol (m.p. 126–127°) at 124–126°. When the material was mixed with an equal quantity of (–)-*trans*-1:2-dihydronaphthalene-1:2-diol the m.p. was 99–101°.

(–)-*trans*-1:2-Dihydronaphthalene-1:2-diol. When the ethanolic solution of the residue obtained from the evaporated extract of the urine of rats dosed with (–)-*trans*-1:2-dihydronaphthalene-1:2-diol was subjected to the procedure just described it yielded 4 mg. of pink crystalline material, m.p. 125–126°, unchanged by admixture with (–)-*trans*-1:2-dihydronaphthalene-1:2-diol, m.p. 127°. When the material was mixed with an equal quantity of (+)-*trans*-1:2-dihydronaphthalene-1:2-diol the mixture melted at 102–105°.

From these experiments it is apparent that excretion of unchanged ($\pm$)-, (+)- and (–)-*trans*-1:2-dihydronaphthalene-1:2-diol follows the administration of these compounds to rats.

Tracer isotope studies

Information concerning the nature of the sulphuric acid derivatives excreted by rats dosed with naphthalene (Corner & Young, 1954) and 1:2-dihydronaphthalene-1:2-diol (this paper) has been obtained by the use of paper chromatography. Laidlaw & Young (1948, 1953) have shown that administered sodium sulphate labelled with ^{35}S can participate in the formation in the rat of 2-amino-1-naphthylsulphuric acid from 2-naphthylamine and 2-naphthylsulphuric acid from 2-naphthol. This suggested that the administration of ^{35}S -labelled sodium sulphate to the rat, together with 1:2-dihydronaphthalene-1:2-diol, followed by a radio-chromatographic examination of the urine, might lead to the detection of sulphuric acid derivatives for which no reference compounds were available. Experiments of this type were carried out and, for the purposes of comparison, the same method of investigation was applied to the study of the sulphuric acid derivatives in the urines of rats dosed with 1- and 2-naphthol and with naphthalene.

Methods. Chromatograms of the urines of the dosed animals were developed in the solvent system 5 (see Table 3). Paper strips 2.54 cm. wide were used, and after the chromatograms had been developed each strip was dried and marked along its length into 1 cm. sections. The paper strip was drawn underneath a metal plate in which there was a slot measuring 2.5 $\times$ 1.0 cm. Centred over the slot and immediately above it was a Geiger-Müller tube (type EHM2S, General Electric Co. Ltd., London) connected to a counting apparatus, and the radioactivity of each 1 cm. section of the strip was measured in turn. Further details of this method of measuring radioactivity on paper chromatograms are reported elsewhere (Lowenstein, 1953).

Animal experiments. Five male hooded rats, each weighing about 200 g., were housed in separate metabolism cages fitted with glass systems for the collection of urine separate from faeces. The animals were fed on the diet described earlier and were dosed subcutaneously as follows: rat 1, 32 mg. of ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol in 2 ml. of water; rat 2, 1 ml. of arachis oil; rat 3, 200 mg. of naphthalene in 1 ml. of arachis oil; rat 4, 40 mg. of 1-naphthol in 2 ml. of arachis oil; rat 5, 40 mg. of 2-naphthol in 2 ml. of arachis oil. Immediately afterwards each animal was given an intraperitoneal injection of 0.5 ml. of an aqueous solution of ^{35}S -labelled sodium sulphate containing 1 mg. of sulphate-S and 0.1 mc of ^{35}S . The urine of each animal was collected for 24 hr. after dosing and filtered. A chromatogram was developed from a 40 μ l. sample of each urine and the paper strip was examined for radioactivity by the procedures already described.

Results. By plotting radioactivity as counts/min. against R_f values graphs were obtained which indicated the positions on the chromatograms of radioactive compounds present in the urine. Diagrammatic representations of these radio-chromatograms are shown in Fig. 1. After the chromatograms had been examined for radioactivity they were suspended in fumes of HCl for 30 min. at room temperature and then sprayed with diazotized sulphanilic acid followed by ammonia or with 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate (cf. Corner & Young, 1954). Except for a radioactive area with R_f 0.49–0.53, this treatment produced coloured spots at positions on the chromatograms which corresponded to regions with peaks of radioactivity (see Table 4). The radioactive area which gave no colour reaction was one of low activity, and it was present on the chromatograms of all the urines including that obtained in the control experiment. Inorganic sulphate remained in the vicinity of the starting-line of the chromatograms.

The information summarized in Table 4 shows that the urine of rats receiving either ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol or naphthalene, together with ^{35}S -labelled sodium sulphate, contained two radioactive metabolites. The R_f value of one of these compounds and the colours which it gave with the diazo reagents after its decomposition with

acid showed that it was 1-naphthylsulphuric acid. The second radioactive metabolite could not be identified with certainty because no reference compound was available. There was no evidence for the presence of this latter compound in the urines of rats dosed with ^{35}S -labelled sodium sulphate and either 1- or 2-naphthol. These urines were found to contain only one radioactive metabolite, namely the sulphuric acid conjugate of the naphthol administered. The relative sizes of the peaks of radioactivity observed on chromatograms of the urines of rats dosed with either ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol or naphthalene show that these urines contained more of this unknown metabolite than of 1-naphthylsulphuric acid.

Information concerning the rate of hydrolysis of the unknown ^{35}S -labelled metabolite was obtained from the following experiments. The urines of rats dosed with 1:2-dihydronaphthalene-1:2-diol and 1- and 2-naphthol were extracted with ether. A sample (1 ml.) of each urine was then adjusted to pH 1 with conc. HCl and heated in a boiling-water bath for 1 min. After being cooled the acidified urine was neutralized with NaOH, and 40 μl . of the solution so obtained were placed on a paper chromatogram which was developed in solvent system 5 (see Table 3). Similar experiments were performed in which 1 ml. samples of each urine were hydrolysed for 3 and 30 min. The three chromatograms for each urine were developed in the presence of a fourth spotted with 40 μl . of urine which had been adjusted to pH 1 with conc. HCl and neutralized immediately afterwards with NaOH. This fourth solution, therefore, contained approximately the same amount of inorganic material as each of the other three. All the chromatograms were examined for radioactivity by the procedures already described. Comparison of the patterns of the radiochromatograms showed that in each case the

Fig. 1. Radiochromatograms of the urines of rats dosed with ^{35}S -labelled sodium sulphate together with ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol, arachis oil (control experiment), naphthalene, 1-naphthol, 2-naphthol. For details see text.

Table 4. The radiochromatographic examination of the urines of rats dosed with ^{35}S -labelled sodium sulphate simultaneously with ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol, naphthalene and 1- and 2-naphthol

For details of dosing see text. Solvent system 5 of Table 3 was used. The R_f values refer to those obtained from the peaks of radioactivity. The colours refer to those given by the radioactive spots when the papers were treated with HCl fumes and then sprayed with diazo reagents. Colours of spots: DR, dark red; O, orange; P, purple; Y, Yellow.

Material administered	<i>R_F</i> values	Radioactive compounds present in the urine			Metabolites
		Colours			
		Diazotized sulphanilic acid	NNCD reagent		
Arachis oil	0.50	—	—		—
Naphthalene	0.76	DR	Y		1-Naphthylsulphuric acid
	0.68	P	P		Unknown*
	0.53	—	—		—
1:2-Dihydronaphthalene-1:2-diol	0.77	DR	Y		1-Naphthylsulphuric acid
	0.66	P	P		Unknown*
	0.51	—	—		—
1-Naphthol	0.77	DR	Y		1-Naphthylsulphuric acid
	0.50	—	—		—
2-Naphthol	0.76	O	Y		2-Naphthylsulphuric acid
	0.49	—	—		—

* Probably a conjugate of 1:2-dihydroxynaphthalene (see text).

^{35}S -labelled compound was completely hydrolysed in 30 min. at pH 1 in a boiling-water bath, and that about 80% of the compound was hydrolysed in 3 min. under these conditions. In the case of 1- and 2-naphthylsulphuric acid this is in accord with other observations on the rates of hydrolysis of these compounds (e.g. Boyland & Wiltshire, 1953). The radiochromatograms obtained in the hydrolysis experiments with the urine of rats dosed with ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol are shown in Fig. 2. They show that the unknown ^{35}S -labelled metabolite was hydrolysed at about the same rate as the 1-naphthylsulphuric acid present together with it in the urine, and that, in this respect, the unknown compound showed the characteristics of an arylsulphuric acid.

After exposure to HCl fumes the region of the chromatogram on which the unknown ^{35}S -labelled metabolite was located gave a purple colour with diazotized sulphanilic acid and also with the NNCD reagent. These colour reactions are shown by 1:2-dihydroxynaphthalene. Tests for the presence of this compound in free and conjugated forms were made on the urines of rats dosed with naphthalene, 1:2-dihydronaphthalene-1:2-diol, 1- and 2-

Fig. 2. Radiochromatograms showing the effect of time of heating on the acid hydrolysis of ^{35}S -labelled compounds in the urine of rats dosed with ^{35}S -labelled sodium sulphate and ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol. For details see text.

naphthol. The urines were adjusted to pH 6-7, extracted with ether, and the ether extracts were examined by paper chromatography. A sample of each urine was then adjusted to pH 1 with conc. HCl and the acidified urine was heated in a boiling-water bath for 30 min. The urine was then adjusted to pH 6-7 with NaOH, extracted with ether, and the ether extract of each urine was examined by paper chromatography for the presence of 1:2-dihydroxynaphthalene and other hydroxy derivatives of naphthalene. Synthetic 1:2-dihydroxynaphthalene was run on the chromatograms as a reference compound.

The results obtained are given in Table 5. They show that 1:2-dihydroxynaphthalene was present in the form of an acid-decomposable precursor in the urine of rats dosed with naphthalene and with 1:2-dihydronaphthalene-1:2-diol, but not in the urine of those dosed with 1- and 2-naphthol. In addition, it has been shown: (1) that after exposure to HCl fumes the radiochromatograms of the urines of rats dosed with naphthalene and with 1:2-dihydronaphthalene-1:2-diol gave colour reactions for 1:2-dihydroxynaphthalene in the areas corresponding to the location of the unknown ^{35}S -labelled metabolite; (2) that the unknown radioactive metabolite had an R_f value close to that of 1- and 2-naphthylsulphuric acid; (3) that it underwent hydrolysis at a similar rate to 1- and 2-naphthylsulphuric acid.

These various observations suggest that 1:2-dihydroxynaphthalene may have been present as a sulphuric acid conjugate in the urines of the rats dosed with naphthalene and with 1:2-dihydronaphthalene-1:2-diol. No evidence was obtained for the presence in these urines of a sulphuric acid conjugate of 1:2-dihydronaphthalene-1:2-diol. If such a compound was present in smaller amounts than other sulphuric acid conjugates, and if its R_f value was similar to the values shown by these compounds, it is unlikely that it would have been detected.

Table 5. *The chromatographic examination of ether extracts of neutralized and acid-hydrolysed urines of rats dosed with naphthalene, ($\pm$)-*trans*-1:2-dihydronaphthalene-1:2-diol, and 1- and 2-naphthol*

R_f values, except those in heavy type, which refer to papers treated with HCl fumes before spraying, refer to papers sprayed immediately. Colours of spots: DR, dark red; O, orange; P, purple; Y, yellow. Chromatogram *a*: prepared from an ether extract of the neutralized urine. Chromatogram *b*: prepared from an ether extract of the acid-hydrolysed urine. Solvent system: benzene-glacial acetic acid-water (volumes mixed, 5:1:4).

R_f values								Colours		Compounds
Naphthalene urine		Dihydronaphthalene diol urine		1-Naphthol urine		2-Naphthol urine		Diazotized sulphanilic acid	NNCD reagent	
<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>			
0.95	0.96	—	0.96	0.95	0.95	—	—	DR	Y	1-Naphthol
0.90	0.90	0.90	0.89	—	—	0.89	0.90	O	Y	2-Naphthol
—	0.44	—	0.44	—	—	—	—	P	P	1:2-Dihydroxynaphthalene
0.35	—	0.34	—	—	—	—	—	DR	Y	1:2-Dihydronaphthalene-1:2-diol

DISCUSSION

The present work on the metabolism of 1:2-dihydronaphthalene-1:2-diol has provided evidence for the presence of the following compounds in the urine of rats dosed with this compound: free 1:2-dihydronaphthalene-1:2-diol, 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid (i.e. the glucuronide of the dihydrodiol), 1-naphthylsulphuric acid, 1-naphthyl glucosiduronic acid, 2-naphthol, and a compound which yields 1:2-dihydroxynaphthalene on acid decomposition. All these compounds, with the exception of the precursor of 1:2-dihydroxynaphthalene, have previously been shown to be excreted by rats dosed with naphthalene (cf. Corner & Young, 1954). Free 1:2-dihydroxynaphthalene has been reported previously as a constituent of the urine of guinea pigs dosed with naphthalene (Corner & Young, 1954), and in the present work evidence has been obtained for the excretion of an acid-decomposable precursor of this compound in the urine of rats dosed with naphthalene. The metabolic reactions which 1:2-dihydronaphthalene-1:2-diol has been shown to undergo in the present work, together with some of the changes which naphthalene is known to undergo in the rat are given in Fig. 3.

Various authors (e.g. Boyland, 1948; Smith, 1950; Young, 1950) have drawn attention to the possibility that the conversion of aromatic hydrocarbons into phenols *in vivo* may involve perhydroxylation of the hydrocarbon to form a dihydrodiol followed by conversion of this compound into a phenol by loss of the elements of water. Following the work in which they administered 1:2-dihydronaphthalene-1:2-diol to rats by injection, Boyland & Wiltshire (1953) concluded that their results, while they did not prove the point, were in agreement with the hypothesis that a dihydroxydihydro derivative is the normal precursor of naphthol.

In the present work it was found that whereas free 2-naphthol was present in the urine of rats dosed with 1:2-dihydronaphthalene-1:2-diol, no evidence could be obtained for the presence of conjugates of 2-naphthol. On the other hand, no free 1-naphthol could be detected in the urine of the dosed animals, whereas both 1-naphthylsulphuric acid and 1-naphthyl glucosiduronic acid were present. In attempting to interpret these findings it is necessary to recall that in the rat 1- and 2-naphthol follow metabolic pathways which are similar from both the qualitative and quantitative standpoint (Berenbom & Young, 1951). It seems likely, therefore, that if free 1- and 2-naphthol were formed during the metabolism of 1:2-dihydronaphthalene-1:2-diol they would give similar excretory products. A possible explanation of our

Fig. 3. The metabolism of 1:2-dihydronaphthalene-1:2-diol in the rat compared with that of naphthalene. Reactions undergone by 1- and 2-naphthol in the rat have been included and are indicated by broken arrows. Brackets enclosing 1:2-dihydroxynaphthalene imply that this compound is present in the urine as an unidentified acid-decomposable precursor (see text). Figures on arrows refer to: 1, this paper; 2, Corner & Young (1954); 3, Young (1947); Booth & Boyland (1949); 4, Berenbom & Young (1951).

observations following the administration of 1:2-dihydronaphthalene-1:2-diol to rats is that some of this compound is first conjugated with glucuronic acid or sulphuric acid in the 1-position, and that decomposition of the conjugated compound in the organism gives rise to the corresponding conjugate of 1-naphthol in addition to free 2-naphthol:

Some support for this hypothesis is provided by the finding that the urine of rats dosed with 1:2-dihydronaphthalene-1:2-diol contains 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid, a compound originally shown to be present in the urine of animals dosed with naphthalene (Corner *et al.* 1954; Corner & Young, 1954). As yet there is no evidence, however, to show that 1:2-dihydronaphthalene-1:2-diol undergoes conjugation with sulphuric acid in the animal body.

The present work has shown that when either naphthalene or its dihydrodiol is administered to rats, together with ^{35}S -labelled sodium sulphate, at least two radioactive metabolites are excreted in the urine. One of these is 1-naphthylsulphuric acid. The other ^{35}S -labelled metabolite has not been identified, but there are grounds for believing that it may be a sulphuric acid conjugate of 1:2-dihydroxynaphthalene. If this is so, it suggests that dehydrogenation of 1:2-dihydronaphthalene-1:2-diol or its conjugates takes place in the organism (cf. Young, 1950).

Our finding that 1:2-dihydronaphthalene-1:2-diol and all the compounds to which it has so far been shown to give rise in the organism are also metabolites of naphthalene is consistent with the view that dihydrodiol formation is a step in the metabolic conversion of hydrocarbons into phenolic compounds. It does not exclude the possibility, however, that there are other metabolic pathways which lead to phenol formation.

SUMMARY

1. A study has been made of the metabolism of 1:2-dihydronaphthalene-1:2-diol in the rat.

2. By means of paper chromatography the urines of rats dosed with ($\pm$)-, (+)- and (-)-*trans*-1:2-dihydronaphthalene-1:2-diol have been shown to contain 1:2-dihydronaphthalene-1:2-diol, 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid, 1-naphthyl glucosiduronic acid, 1-naphthylsulphuric acid (1-naphthyl hydrogen sulphate) and 2-naphthol.

3. Isolation experiments have provided evidence that the free 1:2-dihydronaphthalene-1:2-diol excreted is of the same optical form as that administered.

4. The urines of rats dosed with naphthalene and with 1:2-dihydronaphthalene-1:2-diol have been found to contain a compound which yields 1:2-dihydroxynaphthalene on acid decomposition.

5. Additional evidence that 1-naphthylsulphuric acid is a metabolite of naphthalene and of 1:2-dihydronaphthalene-1:2-diol has been obtained from experiments in which these last two compounds were administered to rats dosed with ^{35}S -labelled sodium sulphate. The excretion of a second acid-decomposable metabolite containing ^{35}S occurred in these experiments, and the possibility that this compound was a sulphuric acid conjugate of 1:2-dihydroxynaphthalene is discussed.

6. All the metabolites of 1:2-dihydronaphthalene-1:2-diol identified in the present work are known to be formed from naphthalene in the rat. This and other aspects of the metabolism of the dihydrodiol are discussed.

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The Branched-Chain Fatty Acids of Ox Fat

2. THE ISOLATION OF 15-METHYLHEXADECANOIC ACID*

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Earlier investigations on the minor constituents of ox suet fat indicated the presence of trace quantities of the C₁₇ *anteiso*-acid 14-methylhexadecanoic acid (Hansen, Shorland & Cooke, 1952). Examination of a further sample of the fat surrounding the kidneys of oxen has shown that the C₁₇ *iso*-acid, 15-methylhexadecanoic acid, also is present.

EXPERIMENTAL

Approximately 14.5 kg. ox perinephric fat (sample J/21, comprising the fatty tissue surrounding the kidneys and extending about 6 in. along the body) were steam-rendered

to yield 11.6 kg. glycerides (acidity 0.83% expressed as oleic acid). After the free fatty acids had been neutralized with aqueous NaOH and the soaps removed by repeated washing with water, the glycerides (sap. equiv. 285.0, iodine value 33.3) were hydrogenated at 180° and at atmospheric pressure with the aid of a nickel catalyst supported on kieselguhr. Part of the hydrogenated fat (iodine value 0.3, m.p. 59.0–61.5°) was saponified, converted into fatty acids and steam-distilled. Of the fatty acids non-volatile in steam, 7150 g. were crystallized four times from 10 vol. acetone at approximately –20°, resulting in 734.5 g. 'liquids' and 6285 g. 'solids'. The 'liquid' acids were converted into methyl esters and 727.2 g. were fractionated in a Stedman column, yielding 22 fractions and a residue as shown in Table 1.

* Part 1: Hansen, Shorland & Cooke (1952).

Table 1. Fractional distillation of the 'liquid' methyl esters (727.2 g.) of ox perinephric fat

Fraction	Wt. of methyl esters (g.)	Sap. equiv. methyl esters	Iodine value of methyl esters	M.p. methyl esters (°)	Sap. equiv. acids†	M.p. acids† (°)
J/21, L1	5.01	237.2	4.5	–25.0 to –23.1	169.5	18.0–21.2
J/21, L2	6.27	252.3	3.4	–9.5 to –8.5	185.7	23.0–26.5
J/21, L3	9.10	245.1	2.0	–1.8 to –0.3	199.7	36.8–38.2
J/21, L4	14.70	252.5	0.9	11.5–12.7	222.9	44.8–47.0
J/21, L5	31.33	245.4	0.0	10.0–15.0	228.9	52.0–52.4
J/21, L6	30.01	254.6	0.0	17.1–17.6	229.2	52.8–53.2
J/21, L7	38.00	243.2	0.2	15.2–19.5	229.2	53.8–54.1
J/21, L8	37.97	245.0	0.1	16.3–17.0	230.5	52.2–52.8
J/21, L9	38.61	248.5	0.0	13.7–14.5	233.5	47.7–49.3
J/21, L10	40.48	258.3	0.6	–1.0–0.2	239.7	37.0–39.0
J/21, L11	35.36	266.6	0.0	5.0–6.5	244.0	40.0–40.7
J/21, L12	38.06	272.1	0.9	22.8–23.8	254.2	55.0–56.3
J/21, L13	41.59	272.3	0.0	23.9–25.9	256.8	57.2–59.3
J/21, L14	41.72	273.1	0.0	25.8–26.5	257.7	58.0–59.8
J/21, L15	40.78	275.2	0.0	17.2–19.2	260.8	57.3–58.2
J/21, L16	38.83	282.2	1.4	10.4–13.5	265.7	47.8–49.0
J/21, L17	40.42	288.6	1.8	4.8–8.2	271.1	37.8–39.5
J/21, L18	34.94	298.6	4.0	22.0–22.9	280.7	42.0–48.0
J/21, L19	36.95	302.6	4.8	28.9–30.6	288.0	60.0–61.1
J/21, L20	42.69	299.2	15.5	22.0–24.0	283.4	61.8–63.9
J/21, L21	32.61	299.4	32.3	26.8–29.0	275.8	46.3–48.5
J/21, L22	6.05	375.4	40.3	16.8–17.2	282.1	49.0–52.5
J/21, LR	31.90	723.5	58.6	—	283.3	—

† Freed from unsaponifiable matter.