- Stern, I. J., Wang, C. H. & Gilmour, C. M. (1960). *J. Bact.* **79**, 601.
- Stoppani, A. O. M., Conches, L., de Favelukes, S. L. & Sacerdote, F. L. (1958). *Biochem. J.* **70**, 438.
- Tomlinson, N. (1954). *J. biol. Chem.* **209**, 597.
- Utter, M. F. & Kurahashi, K. (1954). *J. biol. Chem.* **207**, 821.
- Van Slyke, D. D., MacFayden, D. A. & Hamilton, P. B. (1943). *J. biol. Chem.* **150**, 251.
- Wang, C. H. & Jones, D. E. (1959). *Biochem. biophys. Res. Commun.* **1**, 203.
- Wang, C. H., Stern, I. J., Gilmour, C. M., Klungsoyr, S., Reed, D. J., Bialy, J. J., Christensen, B. E. & Cheldelin, V. H. (1958). *J. Bact.* **76**, 207.
- White, C. G. & Helf, S. (1956). *Nucleonics*, **14**, no. 10, 46.
- Wong, D. T. O. & Ayl, S. J. (1956). *J. Amer. chem. Soc.* **78**, 3230.
- Wood, H. G., Lifson, N. & Lorber, V. (1945). *J. biol. Chem.* **159**, 475.
- Wood, H. G., Werkman, C. H., Hemingway, A. & Nier, A. O. (1941). *J. biol. Chem.* **139**, 377.
- Wood, W. A. (1955). *Bact. Rev.* **19**, 222.

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The Metabolism of Naphthalene and 1-Naphthol by Houseflies and Rats

By L. C. TERRIERE, R. B. BOOSE AND W. T. ROUBAL

Department of Entomology, Oregon State College, Corvallis, Oregon, U.S.A.

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This report describes a study in which houseflies, *Musca domestica*, were fed with radioactive naphthalene or radioactive 1-naphthol and the resulting excretory products compared with those produced by rats. Kikal & Smith (1958) reported the excretion of free phenols and their cysteine conjugates by locusts given chlorobenzene. Friedler & Smith (1954) reported that locusts conjugate aromatic acids with glycine and Smith (1955) found that ethereal sulphates and glucosides were metabolic products. The present paper shows that houseflies metabolize naphthalene in a manner similar to rats but that 1-naphthol follows slightly different pathways of metabolism in the two species.

MATERIALS AND METHODS

Experiments with houseflies. Flies (3–4-day old) of mixed sexes were used. The compounds were administered by incorporation into the feed, a 5% sucrose solution. One-gallon glass jars, equipped with tight lids and a dehydrating agent, were used as containers for the flies. The jars were kept sealed for 8–10 hr. after adding the test compounds in order to reduce losses due to volatility of the radioactive material. The radioactive naphthalene and 1-naphthol were fed at the rate of approximately 25 µg./day until from 50 to 150 µg. had been used. Approximately 150 flies were used in each test. The specific activities of the radioactive compounds used were 2.0 mc/m-mole for naphthalene and 2.3 mc/m-mole for 1-naphthol. Neither of the compounds was toxic at the amounts fed and the mortality in the control and treated insects was parallel, about 50% after 4 days. No attempt was made to eliminate dead flies during the isolation of radioactivity. At the end of a feeding period, 4–8 days, both dead and living flies were ground with anhydrous sodium sulphate in a mortar and pestle and

extracted in a Soxhlet apparatus with ethanol. The excreted material in the feeding jars was extracted with 80% ethanol.

Experiments with rats. Each of three 50 g. weanling rats, one male and two females, was given approximately 70 µg. of radioactive naphthalene or 1-naphthol intraperitoneally in peanut oil. Urine was collected after 48 hr., extracted with ether and then with butan-1-ol and the two extracts were chromatographed.

Paper chromatography. The solvent systems used were: (1) butan-1-ol-ethanol-water (17:3:20, by vol.) (Boyland & Solomon, 1956); (2) butan-1-ol-3N-(NH₄)₂CO₃-aq. 3N-NH₃ soln. (4:3:3, by vol.) (Corner & Young, 1955). Both ascending and descending techniques on Whatman no. 1 paper were used with no significant differences in *R_F* values. The developed chromatograms were examined for their radioactive spots with a gas-flow windowless scanning device and the measured radioactivity was recorded on charts synchronized with the scanning head. Occasional chromatograms were confirmed by radioautography on X-ray film.

Reference compounds. 1:2-Dihydro-2-hydroxy-1-naphthyl glucosiduronic acid, *N*-acetyl-*S*-(1:2-dihydro-2-hydroxynaphthyl)cysteine (1-naphthylpremercapturic acid), 2-hydroxy-1-naphthyl sulphate and *N*-acetyl-*S*-(1-naphthyl)cysteine (1-naphthylmercapturic acid) were kindly supplied by Professor E. Boyland. 1:2-Dihydro-1:2-dihydroxynaphthalene was isolated from rat urine by the method of Corner & Young (1955) and 1-naphthyl glucosiduronic acid by the method of Berenbom & Young (1951). 1-Naphthyl sulphate was synthesized by the method of Feigenbaum & Neuberg (1941).

RESULTS

When the excreta of [1-¹⁴C]naphthalene-fed houseflies were examined by paper chromatography with solvent system (1), seven to nine and occasion-

ally ten radioactive substances appeared to be present. With solvent (2), up to nine radioactive spots could be detected. Similar results were obtained when the flies were fed with 1-[1-¹⁴C]-naphthol. The urine of naphthalene-treated rats contained the same metabolites except for 1-naphthyl glucosiduronic acid, which was absent, with the conjugates appearing in the butanol extract and 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene appearing in the ether extract. The urine of 1-[1-¹⁴C]naphthol-treated rats contained six metabolites. Table 1 gives the R_F values found.

When houseflies were fed with sodium [³⁵S]-sulphate and non-radioactive naphthalene, radioactive excretory products with R_F 0.55 and 0.69 (solvent 1) and R_F 0.53 and 0.81 (solvent 2) were obtained. These were not produced by flies receiving only radioactive sodium sulphate. The compounds were probably *N*-acetyl-*S*-(1-naphthyl)cysteine and 1-naphthyl sulphate respectively.

In typical experiments with naphthalene-treated houseflies, zones corresponding to R_F (solvent 1) 0.07, 0.13 and 0.24 were excised from chromatograms and eluted with water. One portion of each eluate was incubated with β -glucuronidase (Sigma Chemical Co., St Louis, Mo., U.S.A.) for 24 hr. at 37° in 0.075M-phosphate buffer, pH 6.8. A portion without enzyme served as control. The activity of the enzyme was con-

firmed by the hydrolysis of phenolphthalein glucosiduronide. The incubated mixtures were extracted with ether and chromatograms of the extract were developed in solvent (1). The chromatogram of the product R_F 0.07, thought to be 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid, exhibited two radioactive spots with the same R_F values as 1:2-dihydro-1:2-dihydroxynaphthalene and 1-naphthol. The zone R_F 0.13, assumed to be 1-naphthyl glucosiduronic acid, yielded 1-naphthol. The zone R_F 0.24, thought to be 1:2-dihydro-1-naphthyl glucosiduronic acid, yielded radioactivity at the origin after spotting but none after development. An explanation might be that hydrolysis of this glucosiduronide would result in the production of naphthalene, which would disappear from the paper upon drying (Boyland & Solomon, 1955).

The metabolite R_F 0.38, thought to be 1-naphthylmercapturic acid, was eluted from its chromatogram, spotted again on fresh paper, exposed to HCl fumes for 1 hr. at room temperature and developed in solvent (1). Under these conditions radioactive products occurred at R_F 0.94 (1-naphthol). When the R_F 0.38 spot was treated with 1N-HCl for $\frac{1}{2}$ hr., the developed chromatogram exhibited radioactivity at R_F 0.55 (1-naphthylmercapturic acid) and R_F 0.94 (1-naphthol). In a similar test with HCl fumes, the material found in the region R_F 0.52–0.56 was hydrolysed to 1-naphthol.

Table 1. R_F values of metabolites produced by houseflies and rats after treatment with [1-¹⁴C]naphthalene and 1-[1-¹⁴C]naphthol

Solvent systems: (1) butan-1-ol-ethanol-water (17:3:20) (15 hr.); (2) butan-1-ol-3N-(NH₄)₂CO₃-aq. 3N-NH₃ soln. (4:3:3) (15 hr.). Solvents are indicated by numbers at heads of columns.

Metabolites	R_F		R_F		Urine of rats given		Excreta of houseflies given			
					[1- ¹⁴ C]-Naphthalene	1-[1- ¹⁴ C]-Naphthol	[1- ¹⁴ C]-Naphthalene		1-[1- ¹⁴ C]-Naphthol	
	Standards	Literature	Standards	Literature			(1)	(2)	(1)	(2)
	(1)	(2)	(1)	(2)	(1)	(1)	(1)	(2)	(1)	(2)
1:2-Dihydro-2-hydroxy-1-naphthyl glucosiduronic acid	0.07	0.09	0.08*	0.09†	0.06	0.07	0.07	0.09	0.06	0.05
1-Naphthyl glucosiduronic acid	0.12	0.22	0.15*	0.22†	—	0.17	0.13	0.22	0.17	0.26
1:2-Dihydro-1-naphthyl glucosiduronic acid	—	—	0.22*	—	0.26	—	0.24	—	0.24	—
<i>N</i> -Acetyl- <i>S</i> -(1:2-dihydro-2-hydroxynaphthyl)cysteine	0.39	0.43	0.38†	—	0.36	—	0.38	—	0.35	—
2-Hydroxy-1-naphthyl sulphate	0.56	0.82	0.45§	—	0.43	0.44	0.45	—	0.43	—
<i>N</i> -Acetyl- <i>S</i> -(1-naphthyl)cysteine	0.56	0.51	0.55*	—	0.52	0.53	0.54	0.53	0.54	0.51
1-Naphthyl sulphate	0.68	0.78	0.69*	0.81†	0.65	0.66	0.66	0.80	0.65	0.80
1:2-Dihydro-1:2-dihydroxy-naphthalene	0.86	0.88	0.86*	0.90†	0.85	—	0.86	0.91	0.84	0.90
1-Naphthol	0.93	0.95	0.96*	—	0.94	0.94	0.93	0.95	0.93	0.95
Unidentified	—	—	—	—	0.75	—	0.77	—	0.73	—
Unidentified	—	—	—	—	—	—	—	0.13, 0.32, 0.65	—	0.13, 0.37, 0.66

* Boyland & Solomon (1956).

† Boyland & Sims (1958).

‡ Corner & Young (1955).

§ Boyland & Sims (1957).

The metabolite R_f 0.45 hydrolysed by exposure to HCl fumes as described above produced spots of R_f 0.01, 0.82 and 0.94, the last two regions corresponding to 1:2-naphthaquinone and 1-naphthol respectively. The spot at the origin is unidentified but has been observed whenever 1:2-dihydroxynaphthalene or 1:2-naphthaquinone is chromatographed in solvent (1). The results suggest that the metabolite was 2-hydroxy-1-naphthyl sulphate. The reference compound provided by Professor E. Boyland, however, had R_f 0.56.

In solvents (1) and (2), 1- and 2-naphthol have the same R_f but in aq. 0.1N-NH₃ soln. they are separated (Boyland & Sims, 1958). With this solvent it was shown that the radioactive material in the R_f 0.93-0.96 region of systems (1) and (2) was 1-naphthol and that 2-naphthol was not present in excreta of either houseflies or rats treated with naphthalene.

The metabolite of R_f 0.84 from 1-naphthol-treated houseflies was eluted, chromatographed again, eluted a second time, spotted, exposed to HCl fumes and developed in aq. 0.1N-NH₃ soln. The product had the R_f of 1-naphthol, suggesting that the original radioactivity was probably present as 1:2-dihydro-1:2-dihydroxynaphthalene (Young, 1947). In agreement with Berenbom & Young (1951) and Boyland & Wiltshire (1953), the diol could not be detected in the urine of rats treated with 1-naphthol.

The unidentified material found in the region R_f 0.73-0.77 (solvent 1) yielded on acid hydrolysis radioactive spots at R_f 0.04, 0.77 and 0.93 (1-naphthol). This material occurred in both rat and fly experiments with naphthalene and in fly experiments with 1-naphthol.

Houseflies were given a single injected dose of [1-¹⁴C]naphthalene or 1-[1-¹⁴C]naphthol followed by a determination of radioactivity in the tissues and excreta of survivors by the Van Slyke wet-combustion method (Van Slyke, Steele & Plazin, 1951). After 24 hr. 7 % of the radioactivity from the naphthalene injection remained in the tissues of the survivors and 34 % was found in the excreta. With 1-naphthol the values were 10 and 43 % respectively. Expired CO₂ was not collected in these tests.

DISCUSSION

On the basis of results from 15 species of insects, Smith (1955) concluded that the conversion of phenolic substances into glucosides rather than glucosiduronides was a characteristic of insects. This conclusion is not supported by the present study of species of flies which were not included in Smith's survey.

There seems little doubt that houseflies produce all of the metabolites of naphthalene which are

known to be excreted by rats. It also appears that 1-naphthol and naphthalene are converted by this insect into the same excretory products. There does seem to be a difference between houseflies and rats in the metabolism of 1-naphthol. Only six metabolites were excreted by the naphthol-treated rats, whereas nine and possibly ten were produced by flies given this compound.

Berenbom & Young (1951) and Boyland & Wiltshire (1953) have concluded that rats and rabbits receiving 1-naphthol produce 1-naphthol, 1-naphthyl sulphate and 1-naphthyl glucosiduronic acid. Two of the additional metabolites which we have found in rats, 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid and 1-naphthylmercapturic acid, may be the result of conjugations involving 1:2-dihydro-1:2-dihydroxynaphthalene, which has not been identified as a product of 1-naphthol metabolism in rats. The failure to detect 1-naphthylpremercapturic acid in these experiments may be due to its instability since no precautions were taken to prevent its conversion into 1-naphthylmercapturic acid.

SUMMARY

1. Houseflies and rats have been treated with [1-¹⁴C]naphthalene and 1-[1-¹⁴C]naphthol and the products of their metabolism examined.

2. By paper chromatography, these products have been identified as: glucosiduronides of 1-naphthol, 1:2-dihydro-1:2-dihydroxynaphthalene and 1:2-dihydro-1-hydroxynaphthalene; 1-naphthylsulphate; mercapturic acid conjugates of 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene; free 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene. 2-Hydroxy-1-naphthyl sulphate may also be present.

3. All of the metabolites produced by naphthalene- and 1-naphthol-treated flies are found in the urine of rats treated with naphthalene, but only six are produced by rats given 1-naphthol.

4. Metabolites not previously detected in the urine of 1-naphthol-treated rats are 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid and 1-naphthylmercapturic acid.

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REFERENCES

- Berenbom, M. & Young, L. (1951). *Biochem. J.* **49**, 165.
- Boyland, E. & Sims, P. (1957). *Biochem. J.* **66**, 38.
- Boyland, E. & Sims, P. (1958). *Biochem. J.* **68**, 440.
- Boyland, E. & Solomon, J. B. (1955). *Biochem. J.* **59**, 518.

- Boyland, E. & Solomon, J. B. (1956). *Biochem. J.* **63**, 679.
- Boyland, E. & Wiltshire, G. H. (1953). *Biochem. J.* **53**, 636.
- Corner, E. D. S. & Young, L. (1955). *Biochem. J.* **61**, 132.
- Feigenbaum, J. & Neuberg, C. A. (1941). *J. Amer. chem. Soc.* **63**, 3529.
- Friedler, L. & Smith, J. N. (1954). *Biochem. J.* **57**, 396.
- Kikal, T. & Smith, J. N. (1958). *Biochem. J.* **69**, 52f.
- Smith, J. N. (1955). *Biochem. J.* **60**, 436.
- Van Slyke, D. D., Steele, R. & Plazin, J. (1951). *J. biol. Chem.* **192**, 769.
- Young, L. (1947). *Biochem. J.* **41**, 417.

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Further Purification and Properties of the Amine Oxidase of Pea Seedlings

By P. J. G. MANN

Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts.

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Although amine oxidases have been studied intensively for more than 20 years, the nature of their prosthetic groups has not been established. The amine oxidase of pea seedlings belongs to the group of amine oxidases that is inhibited by carbonyl reagents such as hydrazine and semicarbazide; the group also includes the spermine, benzylamine and diamine oxidases of animal tissues. The sensitivity of animal diamine oxidase to carbonyl reagents led to the suggestion that the enzyme contains a functional aldehyde or ketone group (Zeller, 1938). Much evidence has since been reported that the prosthetic groups both of animal diamine oxidase (Davison, 1956; Goryachenkova, 1956) and of the related plant enzyme (Werle & Pechmann, 1949; Goryachenkova, 1956) contain pyridoxal phosphate. It has also been suggested that the prosthetic group of spermine oxidase contains pyridoxal phosphate (Tabor, Tabor & Rosenthal, 1954).

Hydrogen peroxide is a product of the reactions catalysed by amine oxidases, so it has been suggested that some of these enzymes are flavoproteins. Kapeller-Adler (1949) and Goryachenkova (1956) confirmed and extended the work of Zeller, Stern & Wenk (1940) and Swedin (1943, 1944) on the flavoprotein nature of animal diamine oxidase and concluded that the prosthetic group of the enzyme contains flavinadenine dinucleotide. Goryachenkova (1956) also reported evidence that flavinadenine dinucleotide forms a part of the prosthetic group of the amine oxidase of pea and clover seedlings.

The finding that animal diamine oxidase is inhibited by cyanide suggested that the enzyme is a metalloprotein. Zeller (1940) found that other

reagents that react with heavy metals, such as sodium azide, thiourea and sodium sulphide, caused only small inhibitions, which were attributed to effects on secondary oxidations. It was concluded that animal diamine oxidase was inhibited by cyanide because this compound acts as a carbonyl reagent. The inhibition of the related plant enzyme by cyanide was attributed to the same cause by Kenten & Mann (1952). Mann (1955), working with 300-fold purified preparations of pea-seedling amine oxidase, found that the enzyme was inhibited by sodium diethyldithiocarbamate, salicylaldoxime and potassium ethyl xanthate and suggested that it might be a metalloprotein. Preliminary investigations of the heavy-metal content of the preparations showed that copper and manganese were present in possibly significant amounts. Werle & Hartung (1956) found that both the plant enzyme and animal diamine oxidase are inhibited by sodium diethyldithiocarbamate, salicylaldoxime, 8-hydroxyquinoline and dithizone. In the experiments on animal diamine oxidase, with 1:5-diaminopentane as substrate, the ammonia formation was completely inhibited by 8-hydroxyquinoline and sodium diethyldithiocarbamate. Such inhibitions cannot be attributed to effects on secondary oxidations. Suzuki (1959) reported that the plant enzyme is also inhibited by 2:2'-dipyridyl and 1:10-phenanthroline and, on the basis of the inhibition by metal-chelating agents, suggested the presence of iron, molybdenum or zinc in the prosthetic group of the enzyme.

The evidence so far obtained suggests the possibility that the enzymes contain pyridoxal phosphate, flavinadenine dinucleotide and a heavy metal in the prosthetic groups. Direct evidence for