- Junqueira, L. C. U., Hirsch, G. C. & Rothschild, H. A. (1955). *Biochem. J.* **61**, 275.
- Kaloušek, J., Liebster, J. & Babicky, A. (1957). *Nature, Lond.*, **179**, 521.
- Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.
- Loftfield, R. B. (1957). *Fed. Proc.* **16**, 82.
- Mandelstam, J. (1957). *Nature, Lond.*, **179**, 1179.
- Monod, J., Pappenheimer, A. M., jun. & Cohen-Bazire, G. (1952). *Biochim. biophys. Acta*, **9**, 648.
- Nomura, M., Hosoda, J., Yoshikawa, H. & Nishimura, S. (1958). *Proc. Int. Symp. Enzyme Chem., Japan*, 1957. (In the Press.)
- Peters, T. (1953). *J. biol. Chem.* **200**, 461.
- Peters, T. (1957). *J. biol. Chem.* **229**, 659.
- Pollock, M. R. (1952). *Brit. J. exp. Path.* **33**, 587.
- Pollock, M. R. (1953). In *Adaptation in Micro-organisms*, p. 150. 3rd Symp. Soc. gen. Microbiol. Ed. by Gale, E. F. & Davies, R. Cambridge University Press.
- Pollock, M. R. (1956a). *J. gen. Microbiol.* **14**, 90.
- Pollock, M. R. (1956b). *J. gen. Microbiol.* **15**, 154.
- Pollock, M. R. & Torriani, A. M. (1953). *C.R. Acad. Sci., Paris*, **237**, 276.
- Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. T. & Britten, R. J. (1955). *Studies of Biosynthesis in Escherichia coli*, pp. 318–319. Washington, D.C.: Carnegie Institution of Washington Publication 607.
- Rotman, B. & Spiegelman, S. (1954). *J. Bact.* **68**, 419.
- Straub, F. B. & Ullmann, A. (1957). *Biochim. biophys. Acta*, **23**, 665.
- Urbá, R. (1959). *Biochem. J.* (In the Press.)

Metabolism of Polycyclic Compounds

13. ENZYMIC HYDROXYLATION OF NAPHTHALENE BY RAT-LIVER MICROSOMES*

BY J. BOOTH AND E. BOYLAND

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W. 3

(Received 30 April 1958)

An enzyme system found by Mitoma & Udenfriend (1955) in microsomes of rabbit liver hydroxylates aniline, acetanilide, quinoline, naphthalene and diphenyl in the presence of oxygen, nicotinamide, adenosine triphosphate, inorganic phosphate and reduced triphosphopyridine nucleotide. Other substances are hydroxylated by microsomes, e.g. dimethylaminoazobenzene by rat-liver microsomes (Kensler, Miller & Hyatt, 1956), 17 α -hydroxyprogesterone to the 21-hydroxy derivative in the presence of reduced triphosphopyridine nucleotide by ox-adrenal microsomes (Ryan & Engel, 1956, 1957), [16-¹⁴C]oestradiol to 6-hydroxyoestradiol by mouse-liver microsomes in the presence of reduced triphosphopyridine nucleotide (Mueller & Rumney, 1957) and 3:4-benzopyrene to 8-hydroxy-3:4-benzopyrene and 10-hydroxy-3:4-benzopyrene by rat-liver microsomes in the presence of reduced triphosphopyridine nucleotide, reduced diphosphopyridine nucleotide and oxygen (Conney & Miller, 1957; Conney, Miller & Miller, 1957b). Other compounds hydroxylated in the presence of reduced triphosphopyridine nucleotide and oxygen are nitrobenzene and salicylic acid (Mitoma, Posner, Reitz & Udenfriend, 1956) and 2-naphthylamine, 2-acetamidonaphthalene, 2-aminofluorene and 2-acetamidofluorene (Booth & Boyland, 1957).

Naphthalene is a particularly interesting substrate for this system because it is converted into both 1-naphthol and 1:2-dihydro-1:2-dihydroxy-

naphthalene by liver microsomes in the presence of reduced triphosphopyridine nucleotide and oxygen (Mitoma *et al.* 1956). This paper presents evidence that two different enzymes are involved in the formation of these two hydroxylated products.

EXPERIMENTAL

Materials. Triphosphopyridine nucleotide (TPN), as the dihydrate of the sodium salt, reduced TPN (TPNH) as the sodium salt, glucose 6-phosphate (G6-P) as the disodium salt and flavine mononucleotide (FMN) as the monosodium salt dihydrate were obtained from Sigma Chemical Co. Ribonucleic acid (RNA) from rat liver was a gift from Professor J. A. V. Butler. Pyrophosphate buffers were prepared from K₄P₂O₇ and Na₂H₂P₂O₇ and tris buffers from 2-amino-2-hydroxymethylpropane-1:3-diol (tris) and HCl.

Microsome suspensions. Livers from male rats were homogenized in 4 vol. of 0.25M-sucrose with an Ato-Mix Blender (Measuring and Scientific Equipment Ltd.) for 0.5 min. or with a Potter & Elvehjem (1936) type of homogenizer with a Teflon (polytetrafluoroethylene) pestle, and centrifuged at approx. 600 g for 1 hr. The supernatant was then centrifuged for 10 min. at 20 000 g in a Spinco Model L preparative ultracentrifuge with rotor no. 40. (Centrifugal forces refer to the centre of the tube.) This supernatant, containing the microsomes, was then centrifuged at 90 000 g for 30 min. and the microsomal pellet stored at -2° in 0.25M-sucrose. The activity remained reasonably constant for 3–4 days. For enzyme-activity estimations, the sucrose soln. was poured off and the microsomal pellet homogenized in water in a glass homogenizer with a Teflon pestle, so that 1 ml. of suspension corresponded to 750 mg. of original liver. All operations were carried out between 0

* Part 12: Booth & Sims (1957).

and 8° and glass-distilled water was used throughout. A typical microsome suspension contained 6.7 mg. of protein/ml., as estimated by a modification of the biuret method (Fincham, 1954), and 0.81 mg. of RNA/ml. as estimated by the orcinol method of Mejbaum (1939) as applied to microsome fractions by Simkin & Work (1957). This gave an RNA/protein ratio of 0.12, which is in agreement with the value found by other workers for microsomal material (Simkin & Work, 1957; Keller, Zamecnik & Loftfield, 1954). RNA prepared from rat liver by the method of Kay & Dounce (1953) was used as the standard.

Identification of metabolites. The metabolites 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene were identified by paper chromatography as previously described (Booth & Boyland, 1957). To determine if 2-naphthol was present in the reaction mixture, ascending-paper chromatograms were developed in aq. 0.1N-NH₃ soln. (Boyland & Sims, 1953).

Estimation of enzyme activity. A solution of TPNH was freshly prepared by the enzymic reduction of TPN by G6-P dehydrogenase present in the soluble liver fraction with added G6-P (Glock & McLean, 1953). TPN (2.4 mM), G6-P (12 mM), nicotinamide (24 mM) and soluble liver fraction (20%) were allowed to stand at room temperature for 30 min., by which time reduction of TPN as measured by the increase in optical density at 340 m μ was complete. The hydroxylation was started by the addition of 0.5 ml. of this solution to the reaction mixture, which had a final volume of 6 ml. Thus except for experiments involving inhibitors the reactions were carried out in the presence of G6-P and G6-P dehydrogenase, which enabled the initial reaction velocity to remain constant for longer periods of time than could be obtained by the addition of TPNH alone. The naphthalene was added as a solution in ethylene glycol monomethyl ether (0.1 ml.). A typical reaction mixture contained 1 ml. of microsome suspension, buffer solution (0.1M), naphthalene (0.4 mM) and 0.5 ml. of the TPNH solution described in a total volume of 6 ml. and was shaken in air at 37° for 15 min. In experiments testing the effect of various inhibitors on the reaction rate, TPNH was used in the absence of G6-P and G6-P dehydrogenase and the reaction mixture was incubated in 0.1M-tris buffer, pH 7.6, for 8 min. The reaction was stopped by the addition of 0.6 ml. of conc. HCl, two 3 ml. portions were removed into separate tubes and one of these was placed in a boiling-water bath for 8 min. to convert the 1:2-dihydro-1:2-dihydroxynaphthalene into 1-naphthol. Thus the difference in the amount of 1-naphthol before and after acid hydrolysis was proportional to the amount of 1:2-dihydro-1:2-dihydroxynaphthalene present in the reaction mixture. To estimate 1-naphthol, the acidified reaction mixture was shaken with ethyl acetate (3 ml.) and after centrifuging ethyl acetate (2.5 ml.) was removed from each tube. Freshly prepared 0.1% 2:6-dichloroquinonechloroimide in ethanol (0.2 ml.) was added, followed by 10% Na₂CO₃ soln. (1 ml.). After shaking well and centrifuging, the optical density of the blue colour in the ethyl acetate phase was read at 605 m μ in a Unicam SP. 500 spectrophotometer. In experiments with reaction mixtures containing G6-P and G6-P dehydrogenase, the addition of naphthalene caused increases of both 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene which were linear with time for 15 min. in either of the buffer systems used. When 1-naphthol was incubated with the complete reaction mixture (except for

naphthalene) it was destroyed linearly so that 20% disappeared in 15 min. Therefore in all experiments with incubations for 15 min., standard curves were constructed by adding known amounts of 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene to the complete (except for naphthalene) reaction mixture and incubating for 15 min., so that the values quoted represent the amount of metabolites which were actually formed. Added 1:2-dihydro-1:2-dihydroxynaphthalene was not destroyed by incubation with the reaction mixture. Similar standard curves were prepared for inhibition experiments carried out in the absence of G6-P and G6-P dehydrogenase and with incubations for 8 min. The addition of caffeine reduced the destruction of 1-naphthol but had no effect on added 1:2-dihydro-1:2-dihydroxynaphthalene. A different standard curve for 1-naphthol in the presence of caffeine was therefore employed. Added cysteine had no effect on the final concentration of either metabolite when the metabolites were incubated with the reaction mixture. In experiments on the effect of cysteine on the course of the reactions (Fig. 4; Table 2), the results represent the actual amount of the metabolites present at the various times, and for 1-naphthol are the overall results of both formation and destruction. The metabolism of 2-naphthol has not been investigated. The relationship between optical density and concentration of metabolites added was linear over the range used (0–0.03 μ mole/ml.). In most experiments the blank contained boiled-microsome suspension and all the other components, but fresh microsomes without either naphthalene or TPNH also gave colourless blanks.

RESULTS

The only metabolites of naphthalene identified by paper chromatography were 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene. Although aq. 0.1N-NH₃ soln. gave good separation of 1-naphthol and 2-naphthol (Boyland & Sims, 1953), no 2-naphthol could be detected in the reaction mixture. When 1-naphthol, 2-naphthol or 1:2-dihydro-1:2-dihydroxynaphthalene was incubated with microsomes and TPNH, only the added compound could be identified, indicating that none of these substances is an intermediate in the formation of one of the others under these conditions.

Effect of pH. The reaction rates in 0.1M-pyrophosphate buffer were determined at various pH values in the range 6.4–8.9, and in 0.1M-tris buffer between 6.8 and 9.0, the pH-activity curves being shown in Fig. 1. The optimum pH for 1:2-dihydro-1:2-dihydroxynaphthalene formation was 7.5 in pyrophosphate buffer and 8.2 in tris buffer, and the optimum conditions for 1-naphthol formation were 7.2 in pyrophosphate buffer and 7.6 in tris buffer. Thus in each buffer the higher pH value favoured 1:2-dihydro-1:2-dihydroxynaphthalene formation. The proportional amounts of 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene formed by the microsomes varied slightly with different preparations, but at the optimum pH values relatively more 1:2-dihydro-1:2-dihydroxynaphthalene than

1-naphthol was formed in tris buffer than in pyrophosphate buffer. The initial rates remained constant for 15 min. and this incubation time was used to determine the pH-activity curves. Pre-incubation of the microsomes without TPNH for these times at the highest and lowest pH values used did not alter the activity, showing that the enzymes were stable under these conditions over the pH range tested.

Effect of caffeine. The addition of caffeine in 0.1M-pyrophosphate buffer, pH 7.4, had no effect on the rate of formation of 1:2-dihydro-1:2-dihydroxynaphthalene but caused an increase in the formation of 1-naphthol, a maximum effect being obtained with 8 mM-caffeine (Fig. 2). Although caffeine increases the solubility of hydrocarbons (Brock, Druckrey & Hamperl, 1938), the activation of 1-naphthol formation is unlikely to be due to an increase in the solubility of the substrate because the effect of caffeine was greater at lower substrate concentrations. With the substrate concentration (0.4 mM), which is near the maximum solubility of naphthalene in water at 37° (Bohon & Claussen, 1951), the reaction is increased by 120 % in 8 mM-

caffeine, but by 165 % if the naphthalene concentration is 0.1 mM. Furthermore, the addition of 8 mM-tetramethyluric acid, which is a more effective solubilizing agent than caffeine (Weil-Malherbe, 1946), has an inhibitory effect.

Since liver microsomes in the presence of TPNH and O_2 are known to *N*-demethylate narcotic drugs such as morphine and codeine (Axelrod, 1956), it is possible that caffeine may undergo a similar reaction and thus influence the rate of hydroxylation. However, both of these narcotics at a concentration of 8 mM caused inhibition.

At higher pH values the rate of 1:2-dihydro-1:2-dihydroxynaphthalene formation was also increased on addition of caffeine and the pH of optimum activity shifted from 7.5 to 8.1 (Fig. 3). The addition of caffeine with or without ethylenediaminetetra-acetic acid (EDTA) in 0.1M-tris buffer, pH 7.6, did not increase the rate of formation of 1-naphthol but caused inhibition of 1:2-dihydro-1:2-dihydroxynaphthalene formation. These results are summarized in Table 1.

Effect of cysteine in tris buffer. The addition of 2.5 mM-cysteine or EDTA to the reaction mixture had no effect on the initial rates of formations of the two metabolites but the rate of 1:2-dihydro-1:2-dihydroxynaphthalene formation was maintained for longer periods of time (Fig. 4). In tris

Fig. 1. pH-Activity curve for the hydroxylation of naphthalene. Reaction mixtures (6 ml.) contain 1 ml. of microsome suspension, 2 mM-nicotinamide, 0.2 mM-TPNH, 2 mM-G 6-P, 0.4 mM-naphthalene and 0.1 ml. of soluble liver fraction and were incubated in air for 15 min. at 37°. Rate of formation of ●, 1-naphthol in 0.1M-pyrophosphate buffer; ■, 1-naphthol in 0.1M-tris buffer; ○, 1:2-dihydro-1:2-dihydroxynaphthalene in 0.1M-pyrophosphate buffer; □, 1:2-dihydro-1:2-dihydroxynaphthalene in 0.1M-tris buffer.

Fig. 2. Effect of concentration of caffeine on the rate of hydroxylation of naphthalene in 0.1M-pyrophosphate buffer, pH 7.4. Reaction mixtures and incubations were the same as those used in Fig. 1. Rate of formation of ○, 1-naphthol; ●, 1:2-dihydro-1:2-dihydroxynaphthalene.

buffer, pH 8.2, the rate of formation of 1:2-dihydro-1:2-dihydroxynaphthalene remained constant for only 15 min., whereas in the presence of cysteine or cysteine ethyl ester the initial rate was maintained for 50 min., but the same concentration

of mercaptoethylamine caused slight inhibition. On the other hand, the increase produced in the overall 1-naphthol formation, which is an equi-

Fig. 3. Effect of caffeine on the pH-activity curve in 0.1M-pyrophosphate buffer. Reaction mixtures and incubations were the same as those used in Fig. 1. Rate of formation of ●, 1-naphthol; ■, 1-naphthol in the presence of 8 mM-caffeine; ○, 1:2-dihydro-1:2-dihydroxynaphthalene; □, 1:2-dihydro-1:2-dihydroxynaphthalene in the presence of 8 mM-caffeine.

Fig. 4. Effect of cysteine and EDTA on the course of the reactions in 0.1M-tris buffer, pH 8.2. Reaction mixtures and incubations were the same as those used in Fig. 1. Formation of 1-naphthol: ●, with no additions; ■, with 2.5 mM-EDTA; ▲, with 2.5 mM-cysteine. Formation of 1:2-dihydro-1:2-dihydroxynaphthalene: ○, with no additions; □, with 2.5 mM-EDTA; △, with 2.5 mM-cysteine.

Table 1. *Effect of various compounds on the rate of hydroxylation of naphthalene*

Reaction mixtures (6 ml.) contained 1 ml. of microsome suspension, 2 mM-nicotinamide, 0.2 mM-TPNH, 2 mM-G-6-P, 0.4 mM-naphthalene and 0.1 ml. of soluble liver fraction and were incubated in air at 37°.

Buffer	pH	Addition (8 mm)	1-Naphthol			1:2-Dihydro-1:2-dihydroxynaphthalene		
			(μmole/g. of liver/hr.)		Change in activity due to addition (%)	(μmole/g. of liver/hr.)		Change in activity due to addition (%)
			Without addition	With addition		Without addition	With addition	
Pyrophosphate	7.2	Caffeine	0.43	0.88	+105	0.43	0.40	-7
Pyrophosphate	7.5	Caffeine	0.40	0.93	+132	0.51	0.49	-4
Pyrophosphate	8.1	Caffeine	0.14	0.75	+435	0.37	0.65	+76
Pyrophosphate	7.5	Tetramethyluric acid	0.40	0.25	-37	0.51	0.32	-37
Pyrophosphate	7.5	Codeine	0.40	0.22	-45	0.51	0.20	-61
Pyrophosphate	7.5	Morphine	0.40	0.18	-55	0.51	0.21	-60
Tris	7.6	Caffeine	0.34	0.32	-6	0.51	0.20	-61
Tris	8.2	Caffeine	0.26	0.25	-4	0.68	0.25	-63
Tris	7.6	Caffeine + 2.5 mM-EDTA	0.34	0.34	0	0.51	0.36	-30

librium between formation and destruction, after incubation for 50 min. was comparatively small and the rate of the reaction, even in the presence of cysteine, began to fall off considerably after 20 min. This decrease in the rate of formation of 1-naphthol with or without cysteine also occurred at lower pH values, which are more favourable to 1-naphthol production.

Experiments in which some of the components of the system were pre-incubated are summarized in Table 2. Expts. 1 and 2 showed the increase in the amounts of metabolites formed in 50 min. due to the maintenance of the reaction rate by the addition of cysteine, and Expts. 3 and 4 demonstrated that pre-incubation of microsomes in tris buffer for 30 min. had no effect on the activity. Expts. 5 and 6 showed that after incubation of microsomes with TPNH for 30 min. the system was no longer able to form either 1-naphthol or 1:2-dihydro-1:2-dihydroxynaphthalene, although measurements of

the optical density at 340 m μ showed that about 90 % of the TPNH remained after this time. The addition of cysteine did not restore the activity. Addition of cysteine at the beginning of the incubation affords partial protection (Expt. 7). Furthermore, when all the components are present from the start (Expt. 8) the reaction had ceased after 30 min. (Fig. 4) and addition of cysteine at this stage caused no increase in the amount of metabolites. The addition of 2.5 mM-EDTA also caused a large increase in the amount of 1:2-dihydro-1:2-dihydroxynaphthalene with long incubation times but the initial rate was slightly lower in the presence of this compound.

Effect of inhibitors. The inhibitory effect of various compounds in tris buffer, pH 7.6, is shown in Table 3. The most powerful inhibitor of both 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene formation was methylene blue, which caused about 50 % inhibition at a concentration of

Table 2. *Effect of pre-incubation of microsomes with 2.5 mM-cysteine and various components of the hydroxylating system*

Incubation was in tris buffer, pH 8.2, with additions as given in Table 1, so that when TPNH was added it was in the presence of G6-P and G6-P dehydrogenase.

Expt. no.	Components present at beginning of expt.	Components added after pre-incubation for 30 min.	Amount present after 50 min. (μ mole/g. of liver)	
			1-Naphthol	1:2-Dihydro-1:2-dihydroxynaphthalene
1	Nil	Microsomes + naphthalene + TPNH	0.09	0.18
2	Nil	Microsomes + naphthalene + TPNH and cysteine	0.16	0.51
3	Microsomes	Naphthalene + TPNH	0.09	0.18
4	Microsomes + cysteine	Naphthalene + TPNH	0.15	0.50
5	Microsomes + TPNH	Naphthalene	0	0
6	Microsomes + TPNH	Naphthalene + cysteine	0	0
7	Microsomes + TPNH + cysteine	Naphthalene	0.08	0.23
8	Microsomes + TPNH + naphthalene	Cysteine	0.09	0.18

Table 3. *Effect of inhibitors on the hydroxylation of naphthalene*

Reaction mixtures (6 ml.) contain 1 ml. of microsome suspension, 2 mM-nicotinamide, 0.2 mM-TPNH, 0.4 mM-naphthalene and 0.1 M-tris buffer, pH 7.6, and were incubated for 8 min. at 37°.

Inhibitor	Final concn. (mM)	Approximate inhibition (%)	
		1-Naphthol	1:2-Dihydro-1:2-dihydroxynaphthalene
Methylene blue	0.006	50	50
CuCl ₂ ·2H ₂ O	0.06	60	50
HgCl ₂	0.1	70	70
p-Chloromercuribenzoate	0.1	60	50
MnCl ₂	0.12	30	30
FMN	0.8	70	70
KCN	1.0	0	0
α -Dipyridyl	1.0	-15*	0
o-Phenanthroline	1.0	-35*	0
EDTA	2.5	0	0
Cysteine	2.5	0	0

* Activation.

6 μM , and another electron carrier FMN also inhibited both reactions, although a higher concentration was required. The metal-binding compounds $\alpha\alpha$ -dipyridyl, *o*-phenanthroline and KCN at mM and EDTA and cysteine at 2.5 mM did not inhibit the reactions; in fact the first two compounds caused a slight increase of 1-naphthol formation. Both reactions were very sensitive to Cu^{2+} ions, being inhibited about 50 % by 60 μM - CuCl_2 and 0.12 mM- Mn^{2+} ions caused about 30 % inhibition. This latter effect is, however, complicated by the fact that Mn^{2+} ion apparently reacts with tris buffer to form a complex (De Moss, 1954).

DISCUSSION

Both 1-naphthol and 2-naphthol are excreted in the urine of rats which have been dosed with naphthalene (Corner & Young, 1954), so that it is remarkable that the hydroxylating system of liver microsomes produces 1-naphthol only. Although the failure to identify 2-naphthol may be due to the fact that the tests applied are not sufficiently sensitive, this seems unlikely since Mitoma *et al.* (1956) obtained similar results with rabbit-liver microsomes and spectrofluorometrical methods. It has been suggested by Parke & Williams (1956) that an *ortho*-hydroxylase and a *para*-hydroxylase are involved in the hydroxylation of aniline since they have found considerable differences in the *para/ortho* formation ratio in different species. However, rat-liver microsomes hydroxylate aniline in the *ortho*- and *para*-positions and 2-naphthylamine in the 1- and 6- positions (Booth & Boyland, 1957), so that the system producing 2-naphthol from naphthalene *in vivo* probably involves a third enzyme system which may either be situated outside the microsomes as suggested by Mitoma *et al.* (1956) or have different coenzyme requirements.

The formation of 1-naphthol from naphthalene but not from 1:2-dihydro-1:2-dihydroxynaphthalene suggests that the latter is not an intermediate in the formation of 1-naphthol by liver microsomes. Experiments *in vivo* have shown that 1:2-dihydro-1:2-dihydroxynaphthalene is converted into 1-naphthyl hydrogen sulphate and 2-naphthol by the rat (Corner & Young, 1955), but no evidence has been obtained that this occurs in liver microsomes.

The presence of caffeine has been shown to have a different effect on the rate of formation of the two metabolites; in pyrophosphate buffer, pH 7.4, 1:2-dihydro-1:2-dihydroxynaphthalene formation is unaffected and 1-naphthol formation is doubled. Little is known about the action of caffeine on enzymes except that it inhibits oxidases such as cytochrome oxidase (Torda & Wolff, 1950) and D-amino acid oxidase (Burton, 1951). The system

containing microsomes, TPNH and oxygen is known to demethylate many compounds, e.g. narcotic drugs (Axelrod, 1956), lanosterol (Olson, Lindberg & Bloch, 1957), ephedrine (Axelrod, 1955) and 3-methoxy-4-monomethylaminoazobenzene (Conney, Brown, Miller & Miller, 1957a). If caffeine were demethylated it would be expected to reduce hydroxylation because it would compete for TPNH, unless it is the demethylating reaction or one of its products which stimulates the rate of formation of 1-naphthol. This possibility was investigated by adding morphine or codeine, which are known to be *N*-demethylated by the system (Axelrod, 1956), to the reaction mixture, but both compounds caused inhibition of hydroxylation. The finding that the increase in 1-naphthol formation caused by the addition of caffeine was produced in pyrophosphate buffer but not in tris buffer suggested that a metal-binding compound may be necessary, but pyrophosphate buffer could not be replaced by EDTA in tris buffer.

In tris buffer, pH 8.2, the initial rates of formation of 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene are maintained only for 15 min. Gillette, Brodie & La Du (1957) have shown that although liver microsomes catalyse the disappearance of TPNH, the addition of a TPNH-generating system, such as G 6-P and G 6-P dehydrogenase, restores the characteristic peak at 340 $\text{m}\mu$ to its original value, showing that the TPNH had been oxidized to TPN, which was then completely reduced again. Since the experiments in tris buffer were carried out in the presence of G 6-P and G 6-P dehydrogenase it is unlikely that failure to maintain the rate of hydroxylation is due to loss of TPNH. In fact measurement of the optical density at 340 $\text{m}\mu$ showed that 90 % of the coenzyme was still in the reduced form after incubation for 30 min. with microsomes. The addition of cysteine to the reaction mixture enables the initial rate of formation of 1:2-dihydro-1:2-dihydroxynaphthalene, but not of 1-naphthol, to continue for at least three times as long, presumably due to the protection of either the 1:2-dihydro-1:2-dihydroxynaphthalene-forming enzyme or an intermediate essential to its production. Cysteine may protect the enzyme by acting as a metal-complexing agent, since EDTA is nearly as effective.

All the inhibitors tested decreased the formation of both 1:2-dihydro-1:2-dihydroxynaphthalene and 1-naphthol. Compounds which react with sulphhydryl groups such as *p*-chloromercuribenzoate, mercuric chloride and copper sulphate were powerful inhibitors of both reactions, which indicates that sulphhydryl groups are involved. Similar results were obtained in the hydroxylation of acetanilide (Mitoma *et al.* 1956) and benzopyrene (Conney *et al.* 1957b).

The fact that the enzyme systems are active in pyrophosphate buffer and are not inhibited by potassium cyanide, $\alpha\alpha$ -dipyridyl, *o*-phenanthroline, EDTA or cysteine suggests that either they have no metal-ion requirement or the metal is firmly bound to the enzyme or some microsomal material. In this respect they are similar to benzopyrene hydroxylase (Conney *et al.* 1957*b*) and kynurenine hydroxylase (de Castro, Brown & Price, 1957), and differ from phenylalanine hydroxylase (Mitoma, 1956), which requires Fe^{3+} ion for activity.

Methylene blue was the most effective inhibitor found for the hydroxylation of naphthalene, but the interpretation of the effect of inhibitors is complicated by the fact that the reaction involves an enzyme system rather than a single enzyme. A TPNH oxidase from human erythrocytes, which oxidizes TPNH, with oxygen, methaemoglobin or cytochrome *c* as the terminal electron acceptor, and requires methylene blue as an electron carrier, has been described by Huennekens, Liu, Myers & Gabrio (1957) and Huennekens, Caffrey, Basford & Gabrio (1957). Rabbit-liver microsomes contain a TPNH oxidase which catalyses the oxidation of TPNH to TPN and hydrogen peroxide in the absence of added substrate and this enzyme has been implicated in the dealkylation of foreign alkylamines such as monomethyl-4-aminoantipyrine. The rate of oxidation of TPNH is greatly increased by methylene blue, which is an inhibitor of dealkylation, probably acting by diverting the transfer of electrons (Gillette *et al.* 1957). If methylene blue had a similar effect on the hydroxylation of naphthalene, the inhibition by FMN could be explained in the same way. Since some of the liver TPNH-cytochrome reductase is present in the microsome fraction (Hogeboom & Schneider, 1950) it is possible that methylene blue could replace cytochrome as an electron acceptor and thus cause rapid oxidation of the TPNH, as pyridine nucleotide-cytochrome *c* reductases can also show diaphorase activity (Mahler, Sarkar & Vernon, 1957).

Isotopic studies have shown that the oxygen utilized by 11β -hydroxylase of steroids (Hayano, Lindberg & Dorfman, 1955), acetanilide hydroxylase (Hayaishi, Rothberg & Mehler, 1956) and aniline hydroxylase (Udenfriend, Mitoma & Posner, 1956) is derived from oxygen. These enzymes fall into class II (or mixed-function) oxidases, in which one atom of oxygen is added to the substrate, according to the classification suggested by Mason (1956, 1957). The formation of 1-naphthol from naphthalene could also be included; the formation of 1:2-dihydro-1:2-dihydroxynaphthalene is different, because the elements of hydrogen peroxide are added to the substrate.

The different effects of pH, caffeine and cysteine

on the rate of formation of the two metabolites indicate that two different enzyme systems which are affected in a similar manner by several types of inhibitors are involved, but more information on the mechanisms of the reactions is required to decide whether the metabolites are derived from a common intermediate.

SUMMARY

1. The oxidation of naphthalene to 1-naphthol and 1:2-dihydro-1:2-dihydroxynaphthalene by rat-liver microsomes in the presence of reduced triphosphopyridine nucleotide and oxygen appears to involve two different enzyme systems. The system does not form 2-naphthol.

2. In pyrophosphate buffer, pH 7.4, the rate of formation of 1-naphthol is doubled by 5 mM-caffeine, and the rate of formation of 1:2-dihydro-1:2-dihydroxynaphthalene is not altered. At pH 8.1 the rate of formation of 1:2-dihydro-1:2-dihydroxynaphthalene is also increased.

3. In 2-amino-2-hydroxymethylpropane-1:3-diol buffer, pH 8.2, the rate of formation of both metabolites falls off after 15 min., but the initial rate of formation of 1:2-dihydro-1:2-dihydroxynaphthalene only is maintained if 2.5 mM-cysteine is present.

4. The formation of both metabolites is inhibited by methylene blue, flavine mononucleotide and mercaptide-forming compounds but not by metal-binding compounds.

This work has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

- Axelrod, J. (1955). *J. Pharmacol.* **114**, 430.
Axelrod, J. (1956). *J. Pharmacol.* **117**, 322.
Bohon, R. L. & Claussen, W. F. (1951). *J. Amer. chem. Soc.* **73**, 1571.
Booth, J. & Boyland, E. (1957). *Biochem. J.* **66**, 73.
Boyland, E. & Sims, P. (1953). *J. chem. Soc.* p. 2966.
Boyland, E. & Sims, P. (1957). *Biochem. J.* **68**, 440.
Brock, N., Druckrey, H. & Hamperl, H. (1938). *Arch. exp. Path. Pharmacol.* **189**, 709.
Burton, K. (1951). *Biochem. J.* **48**, 458.
Conney, A. H. & Miller, A. J. (1957). *Proc. Amer. Ass. Cancer Res.* **2**, 194.
Conney, A. H., Brown, R. R., Miller, A. J. & Miller, E. C. (1957*a*). *Cancer Res.* **17**, 628.
Conney, A. H., Miller, E. C. & Miller, A. J. (1957*b*). *J. biol. Chem.* **228**, 753.
Corner, E. D. S. & Young, L. (1954). *Biochem. J.* **58**, 647.
Corner, E. D. S. & Young, L. (1955). *Biochem. J.* **61**, 132.

- de Castro, F. T., Brown, R. R. & Price, J. M. (1957). *J. biol. Chem.* **228**, 777.
- De Moss, R. D. (1954). *J. Bact.* **68**, 252.
- Fincham, J. R. S. (1954). *J. gen. Microbiol.* **11**, 236.
- Gillette, J. R., Brodie, B. B. & La Du, B. N. (1957). *J. Pharmacol.* **119**, 532.
- Glock, G. E. & McLean, P. (1953). *Biochem. J.* **55**, 400.
- Hayaishi, O., Rothberg, S. & Mehler, A. H. (1956). *Abstr. 130th Meeting Amer. chem. Soc., Atlantic City*, p. C53.
- Hayano, M., Lindberg, M. C. & Dorfman, R. I. (1955). *Arch. Biochem. Biophys.* **59**, 529.
- Hogebloom, G. H. & Schneider, W. C. (1950). *J. biol. Chem.* **186**, 417.
- Huennekens, F. M., Caffrey, R. W., Basford, R. E. & Gabrio, B. W. (1957). *J. biol. Chem.* **227**, 261.
- Huennekens, F. M., Liu, L., Myers, H. A. P. & Gabrio, B. W. (1957). *J. biol. Chem.* **227**, 253.
- Kay, E. R. M. & Dounce, A. L. (1953). *J. Amer. chem. Soc.* **75**, 4041.
- Keller, E. B., Zamecnik, P. C. & Loftfield, R. B. (1954). *J. Histochem. Cytochem.* **2**, 378.
- Kensler, C. J., Miller, A. & Hyatt, M. (1956). *Proc. Amer. Ass. Cancer Res.* **2**, 124.
- Mahler, H. R., Sarkar, N. K. & Vernon, L. P. (1957). *J. biol. Chem.* **199**, 585.
- Mason, H. S. (1956). *Abstr. 130th Meeting Amer. chem. Soc., Atlantic City*, p. C55.
- Mason, H. S. (1957). *Advanc. Enzymol.* **19**, 79.
- Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
- Mitoma, C. (1956). *Arch. Biochem. Biophys.* **60**, 476.
- Mitoma, C., Posner, H. S., Reitz, H. C. & Udenfriend, S. (1956). *Arch. Biochem. Biophys.* **61**, 431.
- Mitoma, C. & Udenfriend, S. (1955). *J. Pharmacol.* **113**, 40.
- Mueller, G. C. & Rumney, G. (1957). *J. Amer. chem. Soc.* **79**, 1004.
- Olson, J. A., Lindberg, M. & Bloch, K. (1957). *J. biol. Chem.* **226**, 941.
- Parke, D. V. & Williams, R. T. (1956). *Biochem. J.* **63**, 12 p.
- Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
- Ryan, K. J. & Engel, L. L. (1956). *J. Amer. chem. Soc.* **78**, 2654.
- Ryan, K. J. & Engel, L. L. (1957). *J. biol. Chem.* **225**, 103.
- Simkin, J. L. & Work, T. S. (1957). *Biochem. J.* **65**, 307.
- Torda, C. & Wolff, H. G. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 744.
- Udenfriend, S., Mitoma, C. & Posner, H. S. (1956). *Abstr. 130th Meeting Amer. chem. Soc., Atlantic City*, p. C54.
- Weil-Malherbe, H. (1946). *Biochem. J.* **40**, 351.

Studies in Detoxication

77. THE METABOLISM OF PHENYLHYDRAZINE AND SOME PHENYLHYDRAZONES*

BY W. M. McISAAC, D. V. PARKE AND R. T. WILLIAMS

Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(Received 31 March 1958)

Phenylhydrazine was discovered by Fischer (1875). It is highly toxic and Fischer himself soon became sensitized to it, developing a severe chronic eczema. Although phenylhydrazine is a haemolytic poison, it was used at one time in the treatment of polycythaemia vera. *N*-Acetyl-*N'*-phenylhydrazine (Pyrocin or Hydraceticin), however, was later preferred to the free base in treatment, since it was less toxic and had a greater margin of safety (see Whitby & Britton, 1953).

The metabolism of phenylhydrazine in mammals has not been investigated in detail. Hoppe-Seyler (1885) and Lewin (1901) reported that, in warm-blooded animals, it was rapidly destroyed since its presence in the blood or urine could not be demonstrated after its administration.

In the present work it was possible to demonstrate by the isotope-dilution technique that [^{14}C]-phenylhydrazine was extensively transformed *in vivo*. Its urinary metabolites appeared to be mainly conjugated *p*-hydroxyphenylhydrazine and the phenylhydrazones of the natural keto acids.

Some of the radioactivity of the administered compound, however, remained in the body for longer than 10 days after dosing and this was largely found in the erythrocytes. Observations have also been made on the urinary excretion of metabolites after the administration of acetyl[^{14}C]-phenylhydrazine and of pyruvic acid and α -oxoglutaric acid [^{14}C]phenylhydrazones.

MATERIALS AND METHODS

All melting points are corrected.

Animals. Chinchilla doe rabbits kept on a diet of 50 g. of rat cubes (diet 41: Associated London Flour Millers) per day and water *ad lib.*, were used. The [^{14}C]phenylhydrazine hydrochloride, acetyl[^{14}C]phenylhydrazine, and the sodium salts of pyruvic acid [^{14}C]phenylhydrazone, and α -oxoglutaric acid [^{14}C]phenylhydrazone were administered by stomach tube or by subdermal injection as 10% (w/v) aqueous solutions. Urine was collected daily.

Materials. [^{14}C]Phenylhydrazine hydrochloride, m.p. 242–243° (decomp.), of specific activity 7.65 $\mu\text{C/g.}$, was prepared in 85% yield from randomly labelled [^{14}C]aniline by a standard method of diazotization and reduction (see Gilman & Blatt, 1948). This hydrochloride (1 g.) in water

* Part 76: Jondorf, Parke & Williams (1958).