The Biochemistry of Aromatic Amines

3. ENZYMIC HYDROXYLATION BY RAT-LIVER MICROSOMES*

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Several hydroxylating systems showing different intracellular distributions but requiring a reduced phosphopyridine nucleotide, or the nucleotide and an enzyme system capable of reducing it, have been described. Mueller & Miller (1948) reported that a rat-liver homogenate, fortified with diphosphopyridine nucleotide (DPN^+), nicotinamide, magnesium ions and hexose diphosphate, demethylates and hydroxylates 4-dimethylaminoazobenzene to give 4'-hydroxy derivatives. Kensler, Miller & Hyatt (1956) showed that the hydroxylation could be carried out by rat-liver microsomes in the presence of the soluble liver fraction. The system responsible for the 11 β -hydroxylation of 11-deoxycorticosterone by suspensions of ox-adrenocortical mitochondria requires reduced triphosphopyridine nucleotide (TPNH) (Hayano & Dorfman, 1954; Grant & Brownie, 1955); the 21-hydroxylation of 17 α -hydroxyprogesterone requires the same co-enzyme, but for this reaction to take place both the microsomes and the soluble fraction are needed (Ryan & Engel, 1956). Aniline and acetanilide are hydroxylated, chiefly in the *p*-position, by rabbit-liver microsomes and this system requires TPNH and oxygen (Brodie *et al.* 1955; Mitoma & Udenfriend, 1955; Mitoma, Posner, Reitz & Udenfriend, 1956), whereas the conversion of 2-acetamidofluorene into the 7-hydroxy derivative by rat-liver homogenate takes place in the presence of DPN^+ , nicotinamide and succinate (Peters & Gutman, 1956).

Two examples of naturally occurring compounds which are hydroxylated by similar systems have been reported, but in these the microsomes are not involved. A soluble enzyme system from rat liver which catalyses the conversion of phenylalanine into tyrosine requires reduced diphosphopyridine nucleotide (DPNH) and oxygen (Udenfriend & Cooper, 1952; Mitoma, 1956), and the conversion of kynurenine into 3-hydroxykynurenine by liver mitochondria of cats and rats requires TPNH (de Castro, Price & Brown, 1956).

The isolation of derivatives of 2-amino-1-naphthol and 2-amino-6-naphthol from the urine of animals dosed with 2-naphthylamine and 2-acet-

amidonaphthalene has demonstrated that hydroxylation of the amines occurs *in vivo* (Wiley, 1938; Dobriner, Hofmann & Rhoads, 1941; Manson & Young, 1950; Boyland & Manson, 1955) and *in vitro* experiments have shown that the reaction also occurs in the presence of rat-liver slices (Booth, Boyland & Manson, 1955). The present paper reports an investigation on the enzyme system concerned with this reaction.

EXPERIMENTAL

Materials. Triphosphopyridine nucleotide (TPN^+) as the dihydrate of the sodium salt, TPNH as the sodium salt, glucose 6-phosphate (G 6-P) as the sodium salt or the heptahydrate of the barium salt and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co. Potassium 2-naphthyl sulphamate and potassium 6-hydroxy-2-naphthyl sulphamate were prepared by the action of chlorosulphonic acid on 2-naphthylamine and 2-amino-6-naphthol respectively [Boyland *et al.* 1957; Boyland & Manson (in preparation)]. Other derivatives of 2-naphthylamine were obtained as described in a previous paper (Booth *et al.* 1955). Pyrophosphate buffers were prepared from solutions of $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ and $\text{K}_4\text{P}_2\text{O}_7$.

Liver preparations. In experiments to determine the intracellular location of the enzyme, male rat-liver homogenates in 0.25 M-sucrose were fractionated by the method of Schneider (1948) and the sedimented material was resuspended in 0.2 M-pyrophosphate buffer, pH 7.2.

To obtain an active 'microsome preparation', chilled livers of male rats were homogenized with an MSE Ato-Mix Blender (Measuring and Scientific Equipment Co. Ltd.) for 0.5 min. or with a Potter & Elvehjem (1936) homogenizer in 4 vol. of 0.05 M-pyrophosphate buffer, pH 7.2. The homogenates were centrifuged for 10 min. at 600 g to remove cell debris and nuclei and the supernatant was centrifuged for 10 min. at 22 000 g in a Spinco Model L preparative ultracentrifuge with rotor no. 40, since this force gave maximum activity in the supernatant under these conditions. The supernatant (100 ml.) was brought to pH 5.4 by the addition of 10% (v/v) acetic acid (approximately 6 ml.), allowed to stand for 0.5 hr. and centrifuged at 10 500 g for 30 min. The supernatant was discarded, and the precipitate, consisting of microsomes and some liver dehydrogenases, was suspended in 100 ml. of pyrophosphate buffer, pH 7.2, and dialysed against 5 l. of the same buffer solution for approximately 18 hr. All operations were carried out between 0° and 8° and the centrifugal forces refer to the centre of the tube employed. The preparations correspond to 200 mg. of

* Part 2: Boyland, Manson & Orr (1957).

liver/ml. and the protein content, determined by a modification of the biuret method (Fincham, 1954), was 9 mg. of protein/ml.

Estimation of hydroxylating activity with 2-acetamidonaphthalene as substrate. The activity was determined by estimating the rate of formation of 2-acetamido-6-naphthol from 2-acetamidonaphthalene. Incubations were carried out in air in flasks which were shaken for 30 min. at 38°. The standard reaction mixture consisted of 3 mM-G 6-P, 0.2 mM-TPN, 35 mM-nicotinamide, 2 mM-2-acetamidonaphthalene and 1 ml. of microsome preparation in a total volume of 3 ml. of pyrophosphate buffer (0.2M, pH 7.2). The 2-acetamidonaphthalene was added in ethylene glycol monomethyl ether (0.1 ml.), and the reaction was stopped by the addition of 10% (w/v) Na_2CO_3 (1 ml.). The reaction mixture was poured into a glass-stoppered tube, the flask washed out with *n*-butanol (3 ml.) and the washings were added to the tube. The addition of diazotized sulphanilic acid (0.5 ml.), freshly prepared by the addition of 0.5% NaNO_2 (1.6 ml.) to 0.2% sulphanilic acid in 0.1N-HCl (10 ml.), followed by shaking and centrifuging to separate the layers, produced an orange colour in the *n*-butanol phase; this phase (2.5 ml.) was removed to a 5 ml. graduated tube containing 2N-NaOH (2 ml.). After shaking and allowing the layers to separate, the *n*-butanol layer was yellow owing to the presence of 2-naphthylamine which had been formed by deacetylation of the substrate, and the red colour produced by the 2-acetamido-6-naphthol was in the alkaline layer. This was removed (2 ml.) and added to another tube containing ethanol (0.5 ml.) to prevent turbidity, and the optical density was read at 520 m μ on a Unicam SP. 500 spectrophotometer. A standard curve was prepared by the estimation of known amounts of 2-acetamido-6-naphthol added to a tissue preparation under the same conditions and the relationship between optical-density readings and μ moles of compound added was linear up to 0.4 μ mole. Flasks in which the tissue preparation had been boiled were run as blanks in each series of experiments.

In experiments to determine the intracellular distribution of the enzyme it was necessary to precipitate the protein by heating in a water bath at 70° for 2 min. After centrifuging, the precipitated protein was shaken with *n*-butanol (3 ml.) which was then added to the supernatant and the colour developed as before.

Identification of hydroxylated metabolites of various substrates. The microsome preparation was incubated for 2 hr. in the reaction mixture described for the quantitative experiments, the various substrates being added in ethylene glycol monomethyl ether (0.1 ml.). The addition of the less-soluble substrates resulted in some of them being precipitated, and in these experiments they remained partly in suspension throughout the incubation. With 2-naphthylamine it was not possible to detect 2-amino-1-naphthol, but when a phosphate buffer fortified with adenosine triphosphate (ATP), MgCl_2 and K_2SO_4 to facilitate sulphate conjugation (Bernstein & McGilvery, 1952) was employed, 2-amino-1-naphthylsulphuric acid could be identified.

Identification of metabolites by paper chromatography. At the end of the incubation period the reaction mixture was poured into a glass-stoppered tube and shaken with ethyl acetate (1 ml.). After centrifuging to separate the layers the ethyl acetate phase was applied to Whatman no. 1 chromatography paper and descending chromatograms were run for approximately 18 hr. in the solvent system *n*-butanol-

n-propanol-water (2:1:1, by vol.). Authentic specimens of the hydroxy compounds were run alongside the metabolites in each case. The following sprays were employed for identification by colour reactions: (a) hexylresorcinol-N-HCl followed by NaNO_2 (0.5%) and then by hexylresorcinol (0.5% in 2N-NaOH); (b) 2,6-dichloroquinonechloroimide (0.5% in ethanol) followed by Na_2CO_3 (10%); (c) 1-naphthol-N-HCl followed by NaNO_2 (0.5%) and then by 1-naphthol (0.5% in 2N-NaOH); (d) diazotized sulphanilic acid [1.6 ml. of NaNO_2 (0.5%) added to 10 ml. of sulphanilic acid (0.2% in N-HCl)] followed by Na_2CO_3 (10%); (e) NNCD reagent (saturated 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate in 0.1N-HCl) followed by Na_2CO_3 (10%); (f) *p*-dimethylaminobenzaldehyde (0.5% in ethanol containing 1 ml. of conc. HCl/100 ml.); (g) diazotized *p*-nitroaniline [1.6 ml. of NaNO_2 (0.5%) added to 10 ml. of *p*-nitroaniline (0.2% in N-HCl)] followed by Na_2CO_3 (10%).

Identification of metabolites by colour tests on the reaction mixture. (a) 2-Acetamido-7-hydroxyfluorene and 2-amino-7-hydroxyfluorene were identified by the 'nitrite test' before and after hydrolysis with N-HCl for 1 hr. in a boiling-water bath (Damron & Dyer, 1953). (b) Phenol was identified by adding a saturated solution of NNCD reagent in 0.1N-HCl (1 ml.), 5N-NaOH (0.5 ml.) and *n*-butanol (3 ml.) to the reaction mixture and shaking. The red colour produced, both by the reaction mixture and by an authentic specimen of phenol, was extracted into the *n*-butanol phase and had a maximum absorption at 530 m μ . The formation of phenol from benzene was confirmed with 2,6-dichloroquinonechloroimide. The reaction mixture was shaken with *n*-butanol (3 ml.) and centrifuged. After breaking the emulsion with a glass rod and re-centrifuging, the *n*-butanol phase was removed. After the addition of 0.5% 2,6-dichloroquinonechloroimide in ethanol (0.1 ml.) and 0.1M-borate (2 ml.) the tube was allowed to stand for 0.5 hr. with occasional shaking. The blue colour in the *n*-butanol produced by the reaction mixture and by an authentic specimen of phenol had an absorption maximum at 640 m μ . A blank in which the tissue preparation has been boiled was run at the same time in both cases. (c) *p*-Acetamidophenol was detected by the indophenol reaction essentially as described by Brodie & Axelrod (1948).

RESULTS

Distribution and requirements of the enzyme system

When the rat-liver homogenate was fractionated according to the method of Schneider (1948) it was found that the hydroxylating activity was present in the microsome fraction (Table 1). Equal activity was obtained in the presence of either chemically prepared TPNH, TPN^+ and the soluble liver fraction or TPN, G 6-P and G 6-P dehydrogenase. In the microsome preparations used in the quantitative experiments both the microsomes and the dehydrogenases of the liver are present, only the material soluble at pH 5.4 having been discarded. Therefore with this preparation no activity was obtained unless a substrate for one of the dehydrogenases such as G 6-P, glucose or to a less extent malate, was also added. The replacement of TPNH

Table 1. Intracellular distribution and requirements of enzyme system responsible for the conversion of 2-acetamidonaphthalene into 2-acetamido-6-naphthol

Flasks contain 2 mm-2-acetamidonaphthalene in a total volume of 3 ml. of pyrophosphate buffer (0.2M), pH 7.2.

Liver preparation (1 ml.)	Phosphopyridine nucleotide (0.2 mm)	Substrate for dehydrogenase (3 mm)	G 6-P dehydrogenase (0.1 %)	Gas	2-Acetamido- 6-naphthol (μ moles/g. of liver/hr.)
Whole homogenate	TPN ⁺	—	—	Air	1.0
Nuclei	TPN ⁺	—	—	Air	0
Nuclei	TPNH	—	—	Air	0
Mitochondria	TPN ⁺	—	—	Air	0
Mitochondria	TPNH	—	—	Air	0
Microsomes	TPN ⁺	—	—	Air	0
Soluble fraction	TPN ⁺	—	—	Air	0
Microsomes + soluble fraction	TPN ⁺	—	—	Air	1.1
Microsomes + soluble fraction	TPN ⁺	—	—	N ₂	0
Microsomes	TPNH	—	—	Air	1.2
Microsomes	TPN ⁺	G 6-P	+	Air	1.3
Microsomes	DPNH	—	—	Air	0.6
Microsome preparation	TPN ⁺	—	—	Air	0
Microsome preparation	TPN ⁺	G 6-P	—	Air	1.2
Microsome preparation	TPN ⁺	G 6-P	+	Air	1.2
Microsome preparation	TPN ⁺	Glucose	—	Air	1.0
Microsome preparation	TPN ⁺	Malate	—	Air	0.3
Microsome preparation	TPNH	—	—	Air	1.1
Microsome preparation	DPNH	—	—	Air	0.5

Fig. 1. Effect of pH on the rate of hydroxylation of 2-acetamidonaphthalene. Reaction mixtures (3 ml.) contained 1 ml. of microsome preparation, 3 mm-G 6-P, 0.2 mm-TPN, 35 mm-nicotinamide, 2 mm-2-acetamidonaphthalene and 0.2M-pyrophosphate buffer, and were incubated for 30 min. at 38°.

by DPNH resulted in a loss of approximately half of the activity and complete loss of activity resulted when air was replaced by nitrogen.

Formation of 2-acetamido-6-naphthol from 2-acetamidonaphthalene

Rate of reaction and effect of pH. The rate of reaction was constant over 30 min. and was directly proportional to the volume of microsome preparation added between 0.2 and 1 ml. The estimation of activity at various pH values in the range 5.8–8.2 and with 1 ml. of microsome preparation incubated for 30 min., showed optimum activity at

pH 7.2, the rate of formation of 2-acetamido-6-naphthol being 1 μ mole/g. of liver/hr. (Fig. 1). The activity of the microsome preparation was dependent on either TPNH or a TPNH-generating system such as TPN⁺, G 6-P and G 6-P dehydrogenase. Sufficient liver-G 6-P dehydrogenase was precipitated by the treatment with acetic acid, since the microsome preparation showed the same activity as a suspension of microsomes in the soluble liver fraction, and addition of G 6-P or G 6-P dehydrogenase did not increase the activity or affect the pH-activity curve.

Effect of concentrations of the reactants. The rate of hydroxylation was determined in a series of experiments in which the concentration of one of the reactants was varied while the others were present in concentrations giving maximum activity. The effect of variations in TPN concentration is shown in Fig. 2, a half-maximal rate being obtained with a concentration of 0.018 mm-TPN.

The results of experiments in which the activity was measured in the presence of various concentrations of G 6-P (Fig. 3) show a half-maximal rate with 0.25 mm-G 6-P.

When the substrate concentration was varied, maximal activity occurred at a concentration of 2 mm; at higher substrate concentrations the reaction rate was diminished (Fig. 3). Examination by paper chromatography showed that some of the substrate was deacetylated to 2-naphthylamine and this compound may have inhibited the oxidation. However, estimations of 2-naphthylamine at the end of the incubations showed that the concentration was 0.07 mm, whereas a concentration of

0.25 mM-2-naphthylamine caused only 32% inhibition, suggesting that it was the substrate itself which caused a decrease in activity at concentrations above 2 mM.

Hydroxylation of other aromatic compounds

The hydroxylated metabolites identified after treating other aromatic compounds with the microsome preparation in the presence of TPN⁺, nicotinamide and G 6-P are listed in Table 2. These experiments were purely qualitative and the conditions which produce maximum hydroxylation of 2-acetamidonaphthalene were employed.

When the three acetylated amines acetanilide, 2-acetamidonaphthalene and 2-acetamidofluorene were used as substrates, deacetylation occurred in each case, the free amine being identified on the paper chromatograms. No hydroxy derivatives of the deacetylated products, however, were identified except 2-amino-7-hydroxyfluorene from 2-acetamidofluorene, but it is uncertain whether the deacetylation or the hydroxylation occurs first. No acetylated hydroxy compounds were found in experiments in which the free amines were used as substrates.

The hydroxy compounds formed from the acetylated amines acetanilide, 2-acetamidonaphthalene and 2-acetamidofluorene were *p*-acetamidophenol, 2-acetamido-6-naphthol and 2-acetamido-7-hydroxyfluorene respectively, the hydroxylation having taken place at the carbon atom which is furthest from the amino group. On the other hand, the free amines aniline and 2-naphthylamine were oxidized to the *o*-hydroxy derivatives as well. 2-Naphthyl sulphamate appeared to behave in the same way as 2-acetamidonaphthalene in that hydroxylation occurred in the 6-position.

The metabolites identified from naphthalene were 1-naphthol and 1:2-dihydronaphthalene-1:2-diol, but 2-naphthol was not seen, and phenol was the only product identified from benzene.

Fig. 2. Effect of TPN⁺ concentration on the rate of hydroxylation of 2-acetamidonaphthalene. Reaction mixtures (3 ml.) contained 1 ml. of microsome preparation, 3 mM-G 6-P, 35 mM-nicotinamide, 2 mM-2-acetamidonaphthalene and 0.2 M-pyrophosphate buffer, pH 7.2, and were incubated for 30 min. at 38°.

Fig. 3. Effect of G 6-P and substrate concentration on the rate of hydroxylation of 2-acetamidonaphthalene. Reaction mixture (3 ml.) contained 1 ml. of microsome preparation, 0.2 mM-TPN, 35 mM-nicotinamide and 0.2 M-pyrophosphate buffer, pH 7.2, and was incubated for 30 min. at 38°. ○, Hydroxylation with variations in G 6-P concentrations in the presence of 2 mM-2-acetamidonaphthalene; ●, hydroxylation with variations in 2-acetamidonaphthalene concentration in the presence of 3 mM-G 6-P.

DISCUSSION

Since Mueller & Miller (1948) found that a rat-liver homogenate required DPN and an oxidizable substrate for the hydroxylation of 4-dimethylaminoazobenzene in the 4'-position, the enzymic hydroxylation of several compounds has been reported, all the reactions requiring a reduced phosphopyridine nucleotide and oxygen.

The enzyme system responsible for the conversion of 2-acetamidonaphthalene into 2-acetamido-6-naphthol is present in the microsomes of rat liver and requires TPNH and oxygen. Apart from the buffer system employed the conditions are similar to those described by Mitoma *et al.* (1956) for the hydroxylation of acetanilide by rabbit-liver microsomes. Although these workers reported a maximum activity at pH 8.2 for the hydroxylation of acetanilide, whereas the pH optimum for 2-acetamidonaphthalene is 7.2, it would seem that the same enzyme system is involved, and qualitative experiments with some of the substrates used by Mitoma *et al.* (1956) such as aniline, acetanilide and naphthalene yielded essentially the same metabolites.

The mechanism of the hydroxylation reaction is still not understood, since it is difficult to explain the requirement for TPNH in an oxidative reaction. As aromatic compounds can be hydroxylated non-enzymically in the presence of H_2O_2 and Fe^{2+} ions (Loebl, Stein & Weiss, 1949) the possibility that H_2O_2 was involved has been investigated. Thus Mitoma (1956) found that H_2O_2 -generating systems such as D-amino acid oxidase, uricase or xanthine oxidase and their respective substrates could not

replace the DPN^+ or either of the liver fractions needed for the hydroxylation of phenylalanine; similarly the TPNH required for the hydroxylation of acetanilide could not be replaced by H_2O_2 generated by glucose oxidase or D-amino acid oxidase (Mitoma *et al.* 1956). Furthermore, neither of these reactions was inhibited by the addition of catalase, indicating that H_2O_2 was not involved. With $^{18}O_2$ and $H_2^{18}O$ it has been demonstrated that molecular oxygen, but not water, is utilized by the

Table 2. *Compounds hydroxylated by the rat-liver microsome preparation in the presence of TPN, nicotinamide and G 6-P*

Colours were developed by spraying paper chromatograms with the reagents described in Methods unless stated otherwise. Substrates were added in ethylene glycol monomethyl ether (0.1 ml.) to give the final concentration stated, but some of the less soluble compounds were present partly in suspension.

Substrate	Hydroxylated metabolite	Method of identification	R_F	Colour
Aniline (20 mM)	<i>o</i> -Aminophenol	Hexylresorcinol	0.82	Mauve
		2:6-Dichloroquinonechloroimide	—	Grey-green
	<i>p</i> -Aminophenol	Hexylresorcinol	0.71	Red
		1-Naphthol	—	Violet
Acetanilide (2 mM)	<i>p</i> -Acetamidophenol	1-Naphthol after hydrolysis of reaction mixture	—	Violet
		Indophenol test on hydrolysed reaction mixture	—	Blue
2-Naphthylamine (2 mM)	2-Amino-6-naphthol	Hexylresorcinol	0.85	Red
		Diazotized sulphanilic acid	—	Mauve
		NNCD reagent (acid)	—	Mauve
		NNCD reagent (alkaline)	—	Blue
	2-Amino-1-naphthol (identified by formation of 2-amino-1-naphthyl sulphuric acid)	Hexylresorcinol <i>p</i> -Dimethylaminobenzaldehyde	0.56 —	Mauve Yellow
2-Acetamidonaphthalene (2 mM)	2-Acetamido-6-naphthol	Diazotized sulphanilic acid	0.89	Red
		2:6-Dichloroquinonechloroimide	—	Blue
2-Naphthylsulphamate (20 mM)	6-Hydroxy-2-naphthyl sulphamate	Hexylresorcinol after hydrolysis	0.21	Red
		<i>p</i> -Dimethylaminobenzaldehyde after hydrolysis	—	Orange
		1-Naphthol after hydrolysis	—	Puce
2-Aminofluorene (2 mM)	2-Amino-7-hydroxyfluorene	<i>p</i> -Dimethylaminobenzaldehyde	0.76	Orange
		Nitrite test on reaction mixture	—	Yellow
2-Acetamidofluorene (2 mM)	2-Amino-7-hydroxyfluorene	<i>p</i> -Dimethylaminobenzaldehyde	0.76	Orange
		Nitrite test on reaction mixture	—	Yellow
	2-Acetamido-7-hydroxyfluorene	Diazotized <i>p</i> -nitroaniline Nitrite test on hydrolysed reaction mixture	0.79 —	Blue Yellow
Benzene (20 mM)	Phenol	2:6-Dichloroquinonechloroimide test on the reaction mixture	—	Blue
		NNCD reagent test on the reaction mixture	—	Red
Naphthalene (20 mM)	1-Naphthol	Diazotized sulphanilic acid	0.97	Red
		Diazotized <i>p</i> -nitroaniline	—	Blue
		2:6-Dichloroquinonechloroimide	—	Blue
	1:2-Dihydronaphthalene-1:2-diol	Diazotized sulphanilic acid after hydrolysis	0.86	Red
		Diazotized <i>p</i> -nitroaniline after hydrolysis	—	Blue
		2:6-Dichloroquinonechloroimide after hydrolysis	—	Blue

adrenal gland in the 11- β -hydroxylation of steroids (Hayano, Lindberg, Dorfman, Hancock & Doering, 1955). Further evidence for the utilization of molecular oxygen was provided by the finding that the oxygenation of the hydroxyl group which is introduced into salicylate by the model hydroxylase ferrous ion, ethylene diaminetetra-acetate, oxygen and ascorbate (Brodie, Axelrod, Shore & Udenfriend, 1954) arose from molecular oxygen (Mason & Onoprienko, 1956), and Mason, Fowls & Paterson (1955) have shown that molecular oxygen contributes directly in phenolase-catalysed hydroxylations of some aromatic compounds. It has in fact been suggested (Velick, 1956) that phenolase may provide a model for the TPNH and oxygen-requiring systems, since if molecular oxygen and oxidizable metallo-enzymes were involved the TPNH would serve to regenerate the reduced and active enzyme. Both the hydroxylation of phenylalanine and acetanilide are inhibited by the ferrous ion-complexing agent, $\alpha\alpha'$ -dipyridyl (Mitoma, 1956; Mitoma *et al.* 1956), whereas the 11 β -hydroxylation of 11-deoxycorticosterone is strongly inhibited by diethyl dithiocarbamate (Sweat *et al.* 1956), suggesting that the enzymic activity in this case is associated with a copper complex.

Although naphthalene is converted into both 1-naphthol and 1:2-dihydronaphthalene-1:2-diol by this system, it seems likely that two different enzyme systems having the same location and requiring the same coenzyme are responsible for these two different types of reactions. 1:2-Dihydronaphthalene-1:2-diol is readily dehydrated to 1-naphthol by mineral acid, but when it is incubated with the enzyme system no 1-naphthol can be detected, so that 1:2-dihydronaphthalene-1:2-diol is unlikely to be an intermediate between naphthalene and 1-naphthol. A further study of these two reactions may throw some light on the hydroxylation mechanism.

SUMMARY

1. The enzyme system responsible for the conversion of 2-acetamidonaphthalene into 2-acet-amido-6-naphthol is associated with the microsomes of rat liver and requires reduced triphosphopyridine nucleotide (TPNH) and oxygen.

2. The TPNH can be replaced by triphosphopyridine nucleotide, glucose 6-phosphate (G 6-P) and G 6-P dehydrogenase, and conditions affecting the rate of hydroxylation of 2-acetamidonaphthalene by this system have been studied.

3. The system is not specific for 2-acetamidonaphthalene but will hydroxylate other aromatic amines, *N*-substituted amines and also benzene and naphthalene.

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REFERENCES

- Bernstein, S. & McGilvery, R. W. (1952). *J. biol. Chem.* **198**, 195.
 Booth, J., Boyland, E. & Manson, D. (1955). *Biochem. J.* **60**, 62.
 Boyland, E. & Manson, D. (1955). *Biochem. J.* **60**, ii.
 Boyland, E., Manson, D. & Orr, S. F. D. (1957). *Biochem. J.* **65**, 417.
 Brodie, B. B. & Axelrod, J. (1948). *J. Pharmacol.* **94**, 22.
 Brodie, B. B., Axelrod, J., Cooper, J. R., Gaudette, L., La Du, B. N., Mitoma, C. & Udenfriend, S. (1955). *Science*, **121**, 603.
 Brodie, B. B., Axelrod, J., Shore, P. A. & Udenfriend, S. (1954). *J. biol. Chem.* **208**, 741.
 Damron, C. M. & Dyer, H. M. (1953). *J. nat. Cancer Inst.* **14**, 279.
 de Castro, F. T., Price, J. M. & Brown, R. R. (1956). *J. Amer. chem. Soc.* **78**, 2904.
 Dobriner, K., Hofmann, K. & Rhoads, C. P. (1941). *Science*, **93**, 600.
 Fincham, J. R. S. (1954). *J. gen. Microbiol.* **11**, 236.
 Grant, J. K. & Brownie, A. C. (1955). *Biochim. biophys. Acta*, **18**, 433.
 Hayano, M. & Dorfman, R. I. (1954). *J. biol. Chem.* **211**, 227.
 Hayano, M., Lindberg, M. C., Dorfman, R. I., Hancock, J. E. H. & Doering, W. von E. (1955). *Arch. Biochem. Biophys.* **59**, 529.
 Kensler, C. J., Miller, A. & Hyatt, M. (1956). *Proc. Amer. Ass. Cancer Res.* **2**, 124.
 Loeb, H., Stein, G. & Weiss, J. (1949). *J. chem. Soc.* p. 2074.
 Manson, L. A. & Young, L. (1950). *Biochem. J.* **47**, 170.
 Mason, H. S., Fowls, W. L. & Paterson, E. J. (1955). *J. Amer. chem. Soc.* **77**, 2914.
 Mason, H. S. & Onoprienko, I. (1956). *Fed. Proc.* **15**, 310.
 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. & Miller, J. C. (1948). *J. biol. Chem.* **176**, 535.
 Peters, J. H. & Gutman, H. R. (1956). *Arch. Biochem. Biophys.* **62**, 234.
 Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 4951.
 Ryan, K. J. & Engel, L. L. (1956). *J. Amer. chem. Soc.* **78**, 2654.
 Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.
 Sweat, M. L., Aldrich, R. A., de Bruin, C. H., Fowls, W. L., Heiselt, L. R. & Mason, H. S. (1956). *Fed. Proc.* **15**, 367.
 Udenfriend, S. & Cooper, J. R. (1952). *J. biol. Chem.* **194**, 503.
 Velick, S. F. (1956). *Annu. Rev. Biochem.* **25**, 277.
 Wiley, F. H. (1938). *J. biol. Chem.* **124**, 627.