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Metabolism of Polycyclic Compounds

9. METABOLISM OF 2-NAPHTHYLAMINE IN RAT TISSUE SLICES*

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The increased excretion of ethereal sulphate and glucosiduronic acid found in the urine of dogs treated with 2-naphthylamine was considered by Engel (1920, 1924) to be due to conjugation products of 2-amino-1-naphthol, since this compound was identified in the acid-hydrolysed urine by a colour test described by Liebermann & Jacobson (1882). Quantitative studies showed that more ethereal sulphate and glucosiduronic acid was produced than was expected from the 2-naphthylamine administered. Wiley (1938a) isolated 2-amino-1-naphthyl sulphuric acid from the urine of dogs which had been dosed with 2-naphthylamine and confirmed the structure of this product by synthesis. Dobriner, Hofmann & Rhoads (1941) isolated 2-naphthylamine, 2-acetamidonaphthalene and 2-acetamido-6-naphthol from the urine of rats, rabbits and monkeys and obtained evidence for the presence of conjugated derivatives and of 2-amino-6-naphthol.

Manson & Young (1950) administered 2-naphthylamine to rats and isolated 2-amino-1-naphthyl sulphuric acid from the urine. They also confirmed the presence of 2-naphthylamine and 2-acetamido-6-naphthol, but concluded that 2-acetamidonaphthalene was either absent or present only in very small amounts. When the rats were dosed with 2-acetamidonaphthalene by stomach tube, 2-acetamido-6-naphthol and small amounts of the unchanged compound and of 2-naphthylamine were present in the urine. When 2-acetamidonaphthalene was administered by subcutaneous injection 2-acetamido-6-naphthol only was found in the urine.

Wiley (1938b) found normal Q_{O_2} values for rat liver and kidney slices in Ringer solution saturated with 2-naphthylamine, and for liver and kidney slices from animals which had been injected daily with 2-naphthylamine for 40 days. Rat liver homogenates are able to synthesize *m*-aminophenyl sulphuric acid from *m*-aminophenol (Bernstein & McGilvery, 1952). The hydroxylation of aromatic compounds by isolated liver tissue has been demonstrated by the conversion of benzene into phenol (Tschernikow, Gadaskin & Gurewitsch, 1930), and the conversion of naphthalene into dihydrodi-hydroxy derivatives (Boyland & Wiltshire, 1953).

MATERIALS

2-Naphthylamine was purified by the method described by Case (1951). 2-Acetamidonaphthalene was prepared by the acetylation of 2-naphthylamine in pyridine with acetic anhydride. 2-Amino-1-naphthol hydrochloride was prepared by the reduction of 2-nitroso-1-naphthol with SnCl_4 in HCl (Grandmougin & Michel, 1892). 2-Acetamido-1-naphthol was prepared by the alkaline hydrolysis of 2-acetamido-1-acetoxynaphthalene (Grandmougin, 1906). 2-Amino-1-naphthyl sulphuric acid was prepared by oxidation of 2-naphthylamine with $\text{K}_2\text{S}_2\text{O}_8$ as described by Boyland, Manson & Sims (1953). 2-Acetamido-1-naphthyl sulphuric acid: acetylation with acetic anhydride in pyridine caused hydrolysis of the sulphate group of 2-amino-1-naphthyl sulphuric acid but acetylation with thioacetic acid was effective. 2-Amino-1-naphthyl sulphuric acid (1 g.) was dissolved in pyridine (10 ml.) and thioacetic acid (0.3 g.) was added. After standing overnight a slight excess of 40% KOH was added, the solution evaporated to dryness in a desiccator over H_2SO_4 and the residue crystallized twice from ethanol to give pink prisms of the potassium salt of 2-acetamido-1-naphthyl sulphuric acid. (Found: N, 4.4. $\text{C}_{15}\text{H}_{10}\text{O}_4\text{NSK}$ requires N, 4.4%.)

* No. 8 of this series: Boyland & Solomon (1955).

2-Amino-6-naphthol hydrochloride was obtained from Imperial Chemical Industries Ltd. and recrystallized when necessary by solution in hot dil. HCl followed by addition of conc. HCl. 2-Acetamido-6-naphthol was prepared by hydrolysis of 2-acetamido-6-acetoxynaphthalene with warm 2N-NaOH. The product was precipitated with 2N-HCl and crystallized from ethanol-benzene (1:10) in plates, m.p. 222–223°.

2-Amino-6-naphthyl sulphuric acid: 6-nitro-2-naphthylamine was prepared from 2-naphthylamine (Saunders & Hamilton, 1932) and converted into 6-nitro-2-naphthol (Vesely & Jakes, 1923) of which 4.0 g. was added to a solution of chlorosulphonic acid (3.6 g.) in dry pyridine (30 ml.). After standing overnight the mixture was poured into water (750 ml.) containing KOH (7 g.) and the solution concentrated to small volume, when the *potassium salt* of 6-nitro-2-naphthyl sulphuric acid crystallized out as orange plates on cooling. (Found: K, 12.9; N, 4.1. $C_{10}H_6O_6NSK$ requires K, 12.7; N, 4.3%.) It was readily recrystallized from water and was soluble in ethanol. Reduction to 2-amino-6-naphthyl sulphuric acid was carried out equally well by ferrous hydroxide (Burkhardt & Wood, 1929) or by catalytic reduction over Adams's catalyst with H_2 in 30% ethanol. In either case the product was recrystallized by solution in dil. alkali and precipitation with conc. HCl to yield plates, m.p. 292–294°. (Found: S, 13.7. $C_{10}H_6O_4NS$ requires S, 13.4%.) The material tended to hydrolyse even in the dry state but recrystallization was sufficient to remove the aminonaphthol produced. 2-Acetamido-6-naphthyl sulphuric acid was prepared by acetylation of 2-amino-6-naphthyl sulphuric acid with thioacetic acid. The *potassium salt* crystallized from ethanol in plates. (Found: N, 4.3. $C_{12}H_{10}O_6NSK$ requires N, 4.4%.)

2-Amino-1-naphthyl phosphate was prepared during a study of the oxidation of aromatic amines by perphosphoric acids (Boyland & Manson, in preparation). 2-Amino-1-naphthyl glucosiduronic acid, 2-acetamido-1-naphthyl glucosiduronic acid, 2-amino-6-naphthyl glucosiduronic acid and 2-acetamido-6-naphthyl glucosiduronic acid were all crude products from rat or rabbit urines identified by colour reactions and paper chromatography. The properties and isolation of these compounds are under investigation.

METHODS

Moist tissue slices, dried on filter paper, were weighed on a torsion balance and transferred to Warburg flasks containing Krebs-Ringer phosphate solution (1.5 ml.) in the flasks, and a solution of substrate dissolved in Ringer solution (1.5 ml.) in the side arms. As 2-acetamidonaphthalene had a lower solubility, it was dissolved in Ringer solution (3 ml.) and shaken at 38° for 10 min. The weighed slices were then added, the flasks equilibrated with O_2 and readings commenced. The centre wells contained 20% (w/v) KOH solution (0.2 ml.). The ratio wet wt./dry wt. was found to be 5.9:1 for liver and 6.4:1 for kidney; Q_{O_2} values were calculated on the basis of these ratios. After equilibrating with O_2 at 38° the contents of the side arms were tipped into the flasks. At the end of the incubation period, the KOH was removed with filter paper and the contents of the flask were transferred to a centrifuge tube, heated in a boiling-water bath for 5 min. to precipitate the proteins, and then centrifuged. The supernatant liquid was poured off and the residue homogenized in 2 ml. acetone. After addition of

1 ml. of water, the acetone was removed by evaporation and the contents of the tube centrifuged again. The two supernatant layers were then combined. This acetone treatment was necessary for extraction of metabolites.

Identification of the metabolites by paper chromatography

About 250 mg. (wet wt.) of tissue were shaken in approximately $2 \times 10^{-4}M$ solutions of the required substrate in Ringer solution for 2 hr. and the supernatant solutions were applied to Whatman no. 1 filter paper (ten applications each of approximately 0.01 ml.) along a line 10 cm. from the edge of the paper and descending chromatograms developed (about 17 hr.). Markers of synthetic preparations of possible metabolites were applied to the same papers for comparison of R_f values and colour reactions. The reaction mixtures from the incubation of tissue slices in Ringer solution and boiled tissue slices in substrate were run as controls. The solvent systems used are given in Table 1.

Detection of metabolites. After development, the chromatograms were dried at room temperature and examined under an ultraviolet 'Chromatolite'. Compounds containing free amino groups showed a bright blue fluorescence and could be marked on the papers; the acetylated derivatives were not fluorescent. Colours were developed by spraying the papers with the following reagents: (a) *p*-dimethylaminobenzaldehyde (0.5% in ethanol containing 1 ml. conc. HCl/100 ml.); (b) *N*-HCl followed by $NaNO_2$ (0.5%) then by hexylresorcinol (0.5% in 2N-NaOH); (c) Na_2CO_3 (10%, w/v) followed by diazotized sulphanilic acid (1.6 ml. $NaNO$ (0.5%) added to 10 ml. sulphanilic acid (0.2% in *N*-HCl)). Sprays (a) and (b) gave colour reactions with the compounds possessing free amino groups and (c) with phenolic compounds. Acetamido compounds and conjugated phenols (except glucosiduronic acids) were hydrolysed by spraying the paper lightly with *N*-HCl and placing it in an oven at 70° for 0.5 hr. between two sheets of glass. The paper was then removed and the colours developed as before (Table 1).

Formation of glucosides. In the first experiments the Ringer solution contained 0.2% glucose, and it was noticed that each of the compounds containing free amino groups produced two spots on the chromatograms. The additional spot gave colour reactions identical with those of the amine itself but had R_f values approximately 0.1 unit lower in each case.

Saturated solutions of 2-naphthylamine, 2-amino-1-naphthyl sulphuric acid, 2-amino-6-naphthyl sulphuric acid and 2-amino-6-naphthol were shaken for 2 hr. at 38° in water and in 0.2% glucose solution, and chromatograms of the solutions run in the usual way. The addition of glucose to the solutions produced a second spot which gave the same colour reactions as the amine itself but had a lower R_f value in each case. These additional spots were probably due to amine *N*-glucosides since the formation of 2-naphthylamine-*N*-D-ribopyranoside (Berger & Lee, 1946) and aniline-*N*-glucoside (Honeyman & Tatchell, 1950) had been reported under similar conditions. 2-Naphthylamine-*N*-glucoside was prepared by a modification of the method used for aniline-*N*-glucoside by Honeyman & Tatchell (1950). The reaction mixture, prepared by allowing 60% (v/v) ethanol saturated with 2-naphthylamine and glucose to stand for 2 weeks, was applied to an alumina column and the unreacted 2-naphthylamine eluted with ether; the column was washed with ethanol and then the glucoside was eluted

Table 1. R_F values and colour reactions of 2-naphthylamine and derivatives

All compounds with free amino groups gave a yellow or orange colour with *p*-dimethylaminobenzaldehyde. Hydrolysis refers to treatment with N-HCl at 70° as described in the text. Colours observed were: B, blue; Brk, brick red; M, mauve; O, orange; P, pink; R, red; Y, yellow.

Compound	R_F in solvent system						Colours with spray reagents					
	n -Butanol-acetic acid (2:1:1)		n -Butanol-water (10:1:1)		n -Butanol- n -propanol-0.1N-NH ₃ (2:1:1)		Diazotization followed by hexyl resorcinol		Diazotized sulphamic acid			
	water (2:1:1)		water (10:1:1)		n -propanol-0.1N-NH ₃ (2:1:1)		Before hydrolysis		Before hydrolysis			
	0.90	0.95	0.93	0.79	0.92	0.94	Y	Y	O	O	After hydrolysis	After hydrolysis
2-Naphthylamine	—	—	—	0.79	0.75	—	—	—	—	—	—	—
2-Naphthylamine- <i>N</i> -glucoside	0.92	0.97	0.94	0.95	0.96	—	—	Y	—	—	O	—
2-Acetamidonaphthalene	0.69	0.56	0.56	0.43	0.40	—	M	—	—	—	—	—
2-Amino-1-naphthyl sulphuric acid	—	—	—	0.32	0.25	—	—	—	—	—	—	—
2-Amino-1-naphthyl sulphuric acid <i>N</i> -glucoside	0.71	0.59	0.65	0.55	0.50	—	—	B	—	—	P	—
2-Acetamido-1-naphthyl sulphuric acid	0.64	0.42	0.24	0.18	0.16	—	Brk	Brk	—	—	—	—
2-Amino-1-naphthyl glucosiduronic acid	0.71	0.47	0.29	0.22	0.18	—	—	Brk	—	—	—	—
2-Amino-1-naphthyl phosphate	0.70	0.49	0.17	0.07	0.06	—	Brk	O	—	—	—	—
2-Acetamido-1-naphthol	0.93	0.97	0.97	0.95	0.98	—	*	B	P	—	—	—
2-Amino-6-naphthol	0.81	0.80	0.85	0.83	0.86	—	R	R	M	M	M	—
2-Amino-6-naphthol- <i>N</i> -glucoside	—	—	0.71	0.70	—	—	—	—	—	—	—	—
2-Acetamido-6-naphthol	0.85	0.94	0.89	0.87	0.91	—	—	R	R	R	M	—
2-Amino-6-naphthyl sulphuric acid	0.54	0.30	0.39	0.27	0.23	—	O	R	—	—	M	—
2-Amino-6-naphthyl sulphuric acid <i>N</i> -glucoside	—	—	0.20	0.14	—	—	—	—	—	—	—	—
2-Acetamido-6-naphthyl sulphuric acid	0.58	0.40	0.47	0.39	0.36	—	—	R	—	—	M	—
2-Amino-6-naphthyl glucosiduronic acid	0.45	0.20	0.12	0.13	0.10	—	P	P	—	—	—	—
2-Acetamido-6-naphthyl glucosiduronic acid	0.52	0.26	0.16	0.15	0.14	—	—	P	—	—	—	—

* Mauve with NaNO_2 .

with 60% ethanol. On evaporation of this eluate, buff crystals were obtained and recrystallized from 60% ethanol. The compound was characterized by the formation of the tetraacetate (Honeyman & Tatchell, 1950). The 2-naphthylamine-*N*-glucoside tetraacetate had m.p. 168–170° (decomp.). (Found: C, 61.0; H, 5.6; N, 2.8. $C_{24}H_{27}O_9N$ requires C, 60.9; H, 5.7; N, 3.0%.)

2-Naphthylamine-*N*-glucoside had an R_F value identical with that of the substance produced by the incubation of 2-naphthylamine in 0.2% glucose solution at 38°. These glucosides are very easily decomposed by dilute mineral acids, and as both colour reactions used for amines are carried out under acid conditions it is probable that the glucosides are decomposed on the paper and hence the colour is due to the free amine. Glucose was omitted from the Ringer solution in subsequent experiments in order to avoid this complication.

as solvent. Since this compound was not fluorescent it was located by applying solutions of approximately the concentration to be measured to each end of the starting line of the chromatogram as 'markers'. The papers were dried, the strips of paper containing the 'markers' were cut off, hydrolysed, sprayed with *N*-HCl and heated; the colour was developed with the hexylresorcinol spray, which then gave the red colour of 2-amino-6-naphthol. The bands of 2-acetamido-6-naphthyl sulphuric acid were then cut out and shaken for 10 min. in 0.01*N*-NaOH (3 ml.) in 25 ml. conical flasks. The contents of the flask were transferred to a sintered glass funnel and as much liquid as possible removed by suction. The paper was washed with 0.01*N*-NaOH (1 ml.) and the liquid again removed by suction. A sample of the filtrate (3 ml.) was added to 0.2 ml. conc. HCl in a test-tube, and placed in a boiling-water bath for 0.5 hr. The tubes were cooled and 0.1 ml. of 0.005% $NaNO_2$ solution

Table 2. *Spectrophotometric properties of the coloured derivatives used in the estimation of 2-naphthylamine and some metabolites*

The coloured derivatives were formed as described in the text.

Compound	Min. concn. required for estimation (μ mole/ml.)	λ of E_{max} ($m\mu$.)	Optical density at E_{max} for 0.08 μ mole/ml.
2-Naphthylamine	0.01	450	0.42
2-Amino-1-naphthyl sulphuric acid	0.006	450	0.66
2-Amino-6-naphthyl sulphuric acid	0.01	450	0.43
2-Acetamido-6-naphthyl sulphuric acid	0.01	520	0.46
2-Acetamido-6-naphthol	0.005	510	0.78

Quantitative methods

When tissue slices were incubated in 2-naphthylamine or 2-acetamidonaphthalene solutions, the initial concentration of substrate was usually $1.1 \times 10^{-3}M$. The concentration of metabolites after incubation for 2 hr. was approximately $1.7 \times 10^{-4}M$ and this was the initial concentration used in studying the further reactions of the primary metabolites. The supernatant layer from each flask (1 ml. in four 0.25 ml. portions) was applied to Whatman no. 3MM filter paper along a 5 cm. line 10 cm. from the edge of the paper and descending chromatograms developed. The supernatant layer from a flask containing tissue slices without substrate was run in each experiment and a band in the same position as the compound to be estimated cut out and used as the blank. A sample of the solution of the substrate incubated with boiled tissue was also examined.

2-Amino-1-naphthyl sulphuric acid and 2-amino-6-naphthyl sulphuric acid. Chromatograms were run in *n*-butanol-*n*-propanol-water (2:1:1) and the position of the sulphates marked by the fluorescence shown under ultra-violet light. The bands were cut out and shaken in 0.5% KOH in methanol (3 ml.) for 20 min. A sample (2 ml.) was removed and added to 2% *p*-dimethylaminobenzaldehyde in acetic acid (1 ml.). The intensity of the yellow colour produced was measured in a Unicam SP. 500 spectrophotometer at $\lambda 450 m\mu$.

2-Naphthylamine. This was estimated by the same method as that used for the aminonaphthyl sulphuric acids except that the bands were eluted with ethanol.

2-Acetamido-6-naphthyl sulphuric acid. Chromatograms were developed with *n*-butanol-acetic acid-water (10:1:1)

added. After shaking and allowing to stand for 2 min., 0.5 ml. of 2% α -naphthol in 5*N*-NaOH was added and the intensity of the red colour read on the spectrophotometer at $\lambda 520 m\mu$.

A standard curve of each of these four substances was prepared using 1 ml. portions of known concentrations of each compound in Ringer solution (cf. Table 2).

2-Acetamido-6-naphthol. At the end of the incubation period the KOH was removed from the centre well and the flask contents transferred to a centrifuge tube. This was placed in a boiling-water bath for 5 min. and centrifuged. The supernatant was poured off and the solid material homogenized with 3 ml. *n*-butanol which was then added to the supernatant liquid, and shaken thoroughly. After centrifuging to separate the layers, the *n*-butanol layer was removed into another centrifuge tube and the colour was developed by the addition of 10% Na_2CO_3 (1 ml.) and diazotized sulphanilic acid (0.5 ml.), shaking and again centrifuging to separate the layers. The *n*-butanol layer was then pipetted off, one drop of ethanol added to prevent turbidity and the colour read at $\lambda 510 m\mu$ on the spectrophotometer. Solutions of known concentrations of 2-acetamido-6-naphthol in Ringer solution were treated in the same way to obtain a standard curve (cf. Table 2).

Detection of 2-amino-1-naphthol

When 2-amino-1-naphthol hydrochloride was added to Ringer solution, it dissolved easily but the solution rapidly turned mauve and in 0.5 hr. a fine dark purple precipitate formed. Attempts to develop a satisfactory colour reaction for the detection of this decomposition product on paper were unsuccessful. On shaking the suspension with benzene

the purple pigment was extracted and the intensity of the colour in the benzene was proportional to the amount of 2-amino-1-naphthol hydrochloride originally present (Clayson, 1950). To determine whether 2-amino-1-naphthyl sulphuric acid was hydrolysed by kidney the sensitivity of this test was increased by incubating 3 g. of kidney slices in 30 ml. 1.7×10^{-4} M 2-amino-1-naphthyl sulphuric acid in Ringer solution for 2 hr. At the end of the incubation the supernatant liquid was poured off, the slices were homogenized in 2 ml. acetone, 3 ml. benzene were added and this mixture was added to the original supernatant liquid. This was then shaken and centrifuged to separate the layers. The addition of known amounts of 2-amino-1-naphthol to similar experiments before incubation showed that a concentration of $0.034 \mu\text{mole/ml.}$ or the formation of $0.2 \mu\text{mole/g.}$ dry wt. tissue could easily be detected in this way by the purple colour in the benzene.

RESULTS

The chemical oxidation of 2-naphthylamine and 2-acetamidonaphthalene

Udenfriend, Clark, Axelrod & Brodie (1952, 1954) and Brodie, Axelrod, Shore & Udenfriend (1954) have examined the oxidation of aromatic compounds in an ascorbic acid-ferrous sulphate system containing ethylenediaminetetraacetic acid in the presence of oxygen. A reagent was prepared containing $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.435 g.), ethylenediaminetetraacetic acid (disodium salt) (2.62 g.), ascorbic acid (2.96 g.) dissolved in 60 ml. $0.1 \text{ M-KH}_2\text{PO}_4$ and $0.1 \text{ M-Na}_2\text{HPO}_4$ solution added until the pH was 6.7, about 330 ml. of the latter solution being required. The solution was divided into three portions and oxygenated for 2 hr. at 38° . One flask was used as a control, the others contained 2-naphthylamine (0.34 g.) and 2-acetamidonaphthalene (0.44 g.) respectively.

2-Naphthylamine. Addition of ammonia to the oxidation mixture produced the green colour due to 2-amino-1-naphthol extractable as a purple colour by benzene. Extraction of the reaction mixture with ether followed by paper chromatography of the extract indicated the presence of 2-naphthylamine and 2-amino-6-naphthol.

2-Acetamidonaphthalene. Boiling a portion of the reaction mixture with HCl followed by the addition of ammonia gave the colour reaction for 2-amino-1-naphthol, not seen before acid treatment. After extraction of the reaction mixture with ether and washing the ether extract with dilute NaOH solution, paper chromatography showed the presence of 2-acetamidonaphthalene in the ether extract and of 2-acetamido-1-naphthol and 2-acetamido-6-naphthol in the NaOH extract. Treatment of the control solution by the same methods elicited no colour reactions.

2-Acetamido-1-naphthol has not been detected as a metabolite in the urine of rats and rabbits injected

with 2-naphthylamine or from rat tissue slices incubated with 2-naphthylamine or 2-acetamidonaphthalene but the chemical oxidation experiments carried out seem to indicate that the 2-acetamido group does not prevent oxidation in the 1 position of the naphthalene nucleus. Udenfriend *et al.* (1952, 1954) and Brodie *et al.* (1954) have shown that acetanilide is oxidized to *p*-acetamidophenol and *o*-acetamidophenol by this system.

Metabolites formed by rat liver and kidney slices

The different compounds detected as metabolic products from 2-naphthylamine and 2-acetamidonaphthalene and from the primary metabolites of these two compounds are listed in Table 3. Although 2-naphthylamine is oxidized to 1-hydroxy and 6-hydroxy derivatives by liver slices it is not attacked by kidney slices. All the phenolic compounds are converted into sulphuric esters or glucosiduronic ethers by liver but not by kidney tissue. Whereas kidney but not liver can hydrolyse 2-amino-6-naphthyl sulphuric acid (or the corresponding 2-acetamido compound) neither tissue can liberate the aminonaphthol from 2-amino-1-naphthyl sulphuric acid. Both liver and kidney tissue acetylate 2-amino-6-naphthyl sulphuric acid but not the isomeric 2-amino-1-naphthyl sulphuric acid. On the other hand, both tissues deacetylate 2-acetamidonaphthalene and 2-acetamido-1-naphthyl sulphuric acid, but not 2-acetamido-6-naphthol or 2-acetamido-6-naphthyl sulphuric acid.

The conversion of 2-amino-1-naphthyl phosphate into the corresponding sulphuric ester probably involves hydrolysis of the phosphate ester and esterification of the liberated aminonaphthol with sulphate.

Quantitative experiments

None of the substances used in estimating the rates of the different reactions studied had any effect on the respiration of the tissues (Table 3).

The rate of formation of metabolites was proportional to the amount of tissue present between 25 and 75 mg. dry wt. (Table 4), and the maximum rate of formation was produced by an initial concentration of 2-naphthylamine of $1.1 \times 10^{-3} \text{ M}$ (Table 5). The recovery of 2-naphthylamine after incubation with liver slices over a period of 2 hr. is shown in Table 6.

After incubation for 1 hr. the recovery of 2-amino-1-naphthyl sulphuric acid from liver and kidney slices respectively was $90\% \pm 3$ (79–100) and $95\% \pm 4$ (82–113) (mean of ten experiments $\pm$ S.E., range in brackets).

The quantitative estimation of the three sulphuric esters formed by the incubation of liver slices with 2-naphthylamine at intervals over a period of 2 hr. shows a steady increase in each

Table 3. *Compounds identified after incubation of tissue slices with 2-naphthylamine and some derivatives and their rate of formation*

Results are given as the mean of ten experiments $\pm$ s.e. Ranges are given in parentheses. Initial concentrations: 2-naphthylamine 1.1×10^{-3} M or 45 μ moles/g. dry wt. tissue; other substrates 1.7×10^{-4} M or 8–20 μ moles/g. dry wt. tissue. No metabolites were identified from liver slices with 2-amino-1-naphthyl and 2-acetamido-6-naphthyl sulphuric acids and from kidney slices with 2-naphthylamine, 2-acetamido-6-naphthol, 2-acetamido-1-naphthol and 2-amino-1-naphthyl sulphuric acid. Control $-Q_{O_2}$ values for liver and kidney slices were 8.8 ± 0.5 (6.8–10.7) and 17.9 ± 0.6 (13.7–22.1) μ l./mg. dry wt. tissue/hr. respectively.

Substrate	Tissue	Metabolite	Rate of formation (μ moles/g. dry wt. tissue/hr.)	$-Q_{O_2}$ (μ l./mg. dry wt. tissue/hr.)
2-Naphthylamine	Liver	2-Amino-1-naphthyl sulphuric acid 2-Amino-6-naphthyl sulphuric acid 2-Acetamido-6-naphthyl sulphuric acid	0.73 ± 0.04 (0.54–0.90) 0.32 ± 0.03 (0.23–0.52) 0.38 ± 0.03 (0.16–0.48)	8.5 ± 0.3 (7.0–10.0)
2-Acetamidonaphthalene	Liver	2-Naphthylamine 2-Amino-1-naphthyl sulphuric acid 2-Amino-6-naphthyl sulphuric acid 2-Acetamido-6-naphthyl sulphuric acid	1.48 ± 0.15 (0.91–2.30)	9.2 ± 0.4 (7.0–11.9)
2-Amino-6-naphthol	Kidney	2-Naphthylamine	1.18 ± 0.06 (0.77–1.47)	17.0 ± 0.9 (14.1–20.2)
	Liver	2-Amino-6-naphthyl sulphuric acid 2-Acetamido-6-naphthyl sulphuric acid 2-Amino-6-naphthyl glucosiduronic acid	5.91 ± 0.45 (3.36–7.98)	9.2 ± 0.4 (7.5–11.4)
2-Acetamido-6-naphthol	Kidney	2-Acetamido-6-naphthol		
	Liver	2-Acetamido-6-naphthyl sulphuric acid	8.43 ± 0.74 (5.60–12.60)	8.9 ± 0.3 (7.6–10.0)
2-Acetamido-1-naphthol	Liver	2-Amino-1-naphthyl sulphuric acid 2-Acetamido-1-naphthyl glucosiduronic acid		
2-Amino-6-naphthyl sulphuric acid	Liver	2-Acetamido-6-naphthyl sulphuric acid	1.19 ± 0.05 (0.96–1.42)	9.0 ± 0.3 (7.4–10.5)
	Kidney	2-Acetamido-6-naphthyl sulphuric acid 2-Acetamido-6-naphthol	1.08 ± 0.09 (0.78–1.60)	19.6 ± 0.4 (18.7–22.5)
2-Acetamido-1-naphthyl sulphuric acid	Liver	2-Amino-1-naphthyl sulphuric acid	5.00 ± 0.23 (3.52–5.94)	9.3 ± 0.4 (7.4–11.4)
	Kidney	2-Amino-1-naphthyl sulphuric acid	3.72 ± 0.37 (3.31–4.44)	17.7 ± 1.5 (12.3–25.4)
2-Acetamido-6-naphthyl sulphuric acid	Kidney	2-Acetamido-6-naphthol	0.76 ± 0.06 (0.50–1.14)	19.5 ± 0.6 (14.8–21.6)
2-Amino-1-naphthyl phosphate	Liver	2-Amino-1-naphthyl sulphuric acid		
	Kidney	2-Amino-1-naphthol		

Table 4. *Effect of concentration of liver slices on the rate of formation of metabolites*Slices incubated in 3 ml. 2-naphthylamine ($1.1 \times 10^{-3}M$) in Ringer solution for 2 hr.

Metabolite	Dry wt. of slices (mg.)			
	25	53	76	104
	Rate of formation ($\mu\text{mole/g. dry wt. tissue/hr.}$)			
2-Amino-1-naphthyl sulphuric acid	0.59	0.63	0.65	0.36
2-Amino-6-naphthyl sulphuric acid	0.23	0.21	0.27	0.21
2-Acetamido-6-naphthyl sulphuric acid	0.53	0.53	0.50	0.30

Table 5. *Effect of initial concentration of 2-naphthylamine on the rate of formation of metabolites by liver slices*

Tissue (50 mg. dry wt.) incubated for 2 hr. in 3 ml. 2-naphthylamine solution.

Metabolite	Initial concentration of 2-naphthylamine ($M \times 10^{-3}$)				
	0.14	0.28	0.55	1.10	2.20
	Rate of formation ($\mu\text{mole/g. dry wt. tissue/hr.}$)				
2-Amino-1-naphthyl sulphuric acid	0.69	0.73	0.78	0.84	0.82
2-Amino-6-naphthyl sulphuric acid	0.39	0.40	0.39	0.38	0.38
2-Acetamido-6-naphthyl sulphuric acid	0.28	0.30	0.39	0.44	0.43

Table 6. *Recovery of 2-naphthylamine*Liver slices (50 mg. dry wt.) incubated in $0.55 \times 10^{-3}M$ 2-naphthylamine in Ringer solution (3 ml.).

Compound recovered	Time of incubation (hr.)			
	0.5	1.0	1.5	2.0
	Percentage			
Excess 2-naphthylamine	83	76	73	70
2-Amino-1-naphthyl sulphuric acid	3	4	5	6
2-Amino-6-naphthyl sulphuric acid	1	2	2	3
2-Acetamido-6-naphthyl sulphuric acid	1	3	3	3
Total	88	85	83	82

Fig. 1. Formation of metabolites on incubation of rat-liver slices with 2-naphthylamine ($1.1 \times 10^{-3}M$). ○—○, 2-Amino-1-naphthyl sulphuric acid; △····△, 2-amino-6-naphthyl sulphuric acid; ×---×, 2-acetamido-6-naphthyl sulphuric acid.

metabolite. Hydroxylation in the 1 and the 6 positions occurred at approximately equal rates but about half the 2-amino-6-naphthyl sulphuric acid was acetylated (cf. Fig. 1 and Table 3).

With 2-acetamidonaphthalene as substrate, deacetylation to 2-naphthylamine was the fastest reaction observed and 2-amino-1-naphthyl sulphuric acid was formed. Only traces of 2-amino-6-naphthyl sulphuric acid were present while 2-acetamido-6-naphthyl sulphuric acid occurred in comparatively large amounts suggesting that acetylation of the amino group favours hydroxylation in the 6 position (Fig. 2).

The amounts of 2-amino-6-naphthyl sulphuric acid and 2-acetamido-6-naphthyl sulphuric acid formed on incubation of 2-amino-6-naphthol and 2-acetamido-6-naphthol respectively with liver slices were much greater than the amounts formed from 2-naphthylamine.

The deacetylation of 2-acetamidonaphthalene and 2-acetamido-1-naphthyl sulphuric acid were fairly rapid reactions which could be effected by

both liver and kidney slices; the same tissues acetylated 2-amino-6-naphthyl sulphuric acid but at a slower rate.

The hydrolysis of 2-acetamido-6-naphthyl sulphuric acid to the free acetamidonaphthol by kidney slices was comparatively slow (approximately $0.8 \mu\text{mole/g./hr.}$), while 2-amino-1-naphthyl sulphuric acid was not hydrolysed.

Fig. 2. Formation of metabolites on incubation of rat-liver slices with 2-acetamidonaphthalene ($1.1 \times 10^{-3} \text{M}$). ●—●, 2-Naphthylamine; ○—○, 2-amino-1-naphthyl sulphuric acid; ×—×—×, 2-acetamido-6-naphthyl sulphuric acid.

DISCUSSION

The formation of 2-amino-1-naphthyl sulphuric acid and 2-amino-6-naphthyl sulphuric acid from 2-naphthylamine by the rat liver probably involves oxidation to 2-amino-1-naphthol and 2-amino-6-naphthol and subsequent conjugation with sulphuric acid. Neither of these aminonaphthols could be detected in experiments with rat liver or kidney slices, but this would be expected since rates of formation of 2-amino-6-naphthyl sulphuric acid and 2-acetamido-6-naphthyl sulphuric acid from the corresponding aminonaphthols were very much

greater than the oxidation of 2-naphthylamine. As the tissues were incubated in Ringer solution containing sulphate any aminonaphthol formed would be conjugated rapidly, so that the amounts of 2-amino-1-naphthyl sulphuric acid and 2-amino-6-naphthyl sulphuric acid and its acetyl derivative formed by the liver would be determined by the rate at which hydroxyl groups were introduced into the parent amines.

The fact that 2-acetamido-6-naphthyl sulphuric acid is the metabolite found in the greatest quantity when 2-acetamidonaphthalene is the substrate, suggests that the presence of the acetamido group has directed the hydroxylation to the 6 position. Similar results have been obtained with other amines such as the acetylation of the amino group in the metabolism of aniline and acetanilide which was studied by Smith & Williams (1948, 1949). They found that in the rabbit aniline was oxidized in both *ortho*- and *para*- positions, since *o*- and *p*-aminophenyl sulphuric acids, 4-aminoresorcinyl sulphuric acid, and *p*-acetamidophenyl and *p*-aminophenyl glucosiduronic acids were present in the urine of animals which had been dosed with aniline. Acetanilide, however, was oxidized almost entirely to *p*-acetamidophenol which was excreted in the urine as *p*-acetamidophenyl sulphuric acid. They pointed out that biological oxidation in the *ortho* position appeared to be hindered when the amino group was acetylated so that animals which could deacetylate the amine would be expected to produce *ortho*- and *para*-substituted metabolites in the urine.

Michel, Bernheim & Bernheim (1937) showed that acetanilide is hydrolysed by the liver of the dog, cat, rabbit and ox and by both rat liver and kidney. Bray, James, Raffan, Ryman & Thorpe (1949) obtained evidence to show that the enzyme responsible for the hydrolysis of acetanilide was different from that which hydrolysed acetyl glycine and later measured the deacetylase activity of the liver and kidney of various species (Bray, James, Thorpe & Wasdell, 1950). These workers also measured the deacetylase activity of rat-liver extracts on the *ortho*-, *meta*- and *para*- isomers of acetanilide substituted with $-\text{COOH}$, $-\text{CONH}_2$, $-\text{CH}_3$, $-\text{OH}$, $-\text{Cl}$ and $-\text{NO}_2$, and found that *ortho*-substituted acetanilides were deacetylated very much more rapidly than the *meta*- and *para*- isomers. 2-Naphthylamine derivatives behave similarly since 2-acetamido-1-naphthyl sulphuric acid (which corresponds to the *ortho*-substituted acetanilides) has not been identified as a metabolite of 2-naphthylamine, and it is interesting that 2-acetamido-1-naphthyl sulphuric acid is deacetylated by both liver and kidney slices to give 2-amino-1-naphthyl sulphuric acid, which does not appear to be further metabolized by these tissues. On the

other hand, 2-amino-6-naphthyl sulphuric acid (corresponding to the *p*-aminophenol) is acetylated by both liver and kidney slices and the acetyl derivative is not deacetylated.

The hydrolysis of 2-acetamido-6-naphthyl sulphuric acid to 2-acetamido-6-naphthol by rat-kidney slices which do not attack 2-amino-1-naphthyl sulphuric acid probably explains the fact that previous workers have isolated 2-amino-1-naphthyl sulphuric acid, and 2-acetamido-6-naphthol but not its sulphuric acid ester from the urine of animals dosed with 2-naphthylamine. Furthermore, Clayson (1950) found that in the dog

sulphuric acid, 2-amino-6-naphthyl sulphuric acid and 2-acetamido-6-naphthyl sulphuric acid have been determined.

2. Both liver and kidney slices deacetylate 2-acetamidonaphthalene and 2-acetamido-1-naphthyl sulphuric acid and acetylate 2-amino-6-naphthyl sulphuric acid.

3. Neither kidney nor liver slices hydrolyse 2-amino-1-naphthyl sulphuric acid but kidney slices hydrolyse 2-amino-6-naphthyl sulphuric acid and 2-acetamido-6-naphthyl sulphuric acid to 2-amino-6-naphthol and 2-acetamido-6-naphthol respectively. On the other hand, liver slices synthesize

and cat the amounts of 2-amino-1-naphthol derivatives produced when 2-naphthylamine was administered were much greater than after the administration of 2-acetamidonaphthalene; while the 2-amino-6-naphthol, estimated as diazotizable amine in the hydrolysed urine of the dog, was small when 2-naphthylamine was administered and relatively large after the administration of 2-acetamidonaphthalene.

The above reactions, which have been shown to take place in the presence of tissue slices, would account for the presence of 2-amino-1-naphthyl sulphuric acid and 2-acetamido-6-naphthol as two of the chief metabolites found in the urine of rats dosed with 2-naphthylamine. Whereas 2-amino-6-hydroxynaphthalene sulphuric acid was acetylated in both kidney and liver, it was hydrolysed by kidney and not by liver. On the other hand, the free 2-acetamido-6-naphthol is conjugated to the sulphuric ester by liver tissue but not by kidney. Thus, the synthesis and hydrolysis of the sulphuric ester occur in different tissues, which would suggest that the sulphatase is not concerned in the synthesis of sulphuric esters.

SUMMARY

1. The rates at which 2-naphthylamine is metabolized by rat-liver slices to 2-amino-1-naphthyl

sulphuric esters from 2-amino-6-naphthol, 2-acetamido-6-naphthol and 2-acetamido-1-naphthol.

4. The oxidation of 2-naphthylamine and 2-acetamidonaphthalene has been demonstrated in an ascorbic acid-ferrous sulphate-oxygen system.

5. All the aromatic amines examined form *N*-glucosides on incubation with glucose solution. 2-Naphthylamine-*N*-glucoside is stable on chromatograms but is decomposed by weak acid.

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The Oxidation of Glutathione by a Lipoxidase Enzyme from Pea Seeds

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Snow & Zilva (1938) were among the first to show the enzymic oxidation of glutathione (GSH) by both dialysed and undialysed cucumber juice. They also showed an appreciable oxygen uptake on adding GSH to these juices, and supplied evidence that the enzyme catalysing this reaction was different from ascorbic acid oxidase. Ames & Elvehjem (1945) showed the existence of an enzyme system oxidizing GSH in mouse-kidney homogenates, and found that this system was more active when cytochrome *c* was added. They suggested a respiratory system in which GSH and cytochrome *c* were involved. Many investigators have studied the indirect oxidation of GSH through the ascorbic acid-dehydroascorbic acid system (Szent-Gyorgyi, 1928; Hopkins & Morgan, 1936; Crook, 1941). This oxidation was found to be catalysed by the enzyme dehydroascorbic acid reductase (Crook & Hopkins, 1938; Crook, 1941).

During the course of work on the role of GSH in plant respiration, it was observed that GSH, under certain circumstances, was rapidly oxidized by dialysed extracts prepared from ungerminated pea seeds. Since the dialysed extract was devoid of ascorbic acid (AA) or dehydroascorbic acid (DHA) the oxidation could not be attributed to the action of DHA reductase. Furthermore, it was found that one of the enzyme systems concerned was cyanide-insensitive, which excludes the possibility that ascorbic acid oxidase, polyphenol oxidase or the cytochrome system could be involved.

In the present work an attempt has been made to elucidate the oxidizing systems concerned.

MATERIALS AND METHODS

Source of enzyme

Extracts were prepared from powdered dry ungerminated pea seeds, variety Laxton Superb, which had been rapidly extracted with cold acetone (Mapson & Goddard, 1951). The acetone-powder preparation was preferred to unextracted powder owing to the greater ease of subsequent extraction. Control experiments have shown that results essentially similar to those reported below were obtained from unextracted powdered pea seeds.

Dialysed enzyme

Acetone powder (30 g.) was mixed with 100 ml. of 0.01M potassium phosphate buffer, pH 6.8, and allowed to stand 15 min. at room temperature. The suspension was centrifuged and the supernatant was dialysed against the same phosphate buffer for 48 hr. This operation was carried out at +1° and air was bubbled through the external solution to facilitate dialysis.

Determination of lipoxidase activity

The lipoxidase activity was determined by measuring the O₂ uptake in the presence of either methyl linoleate or methyl linolenate, using Warburg manometers at 25°. The volume of the reaction mixture was 3 ml.; 0.2 ml. of 20% KOH in the central well was used to absorb CO₂.

Measurement of the enzymic oxidation of GSH

This was carried out in flasks fitted with delivery tubes for bubbling air or nitrogen, shaken at a speed of about 80 cyc./min. The reaction mixture, unless otherwise stated, consisted of 15 ml. of the dialysed pea extract and 15 ml. of 0.2M phosphate buffer, pH 6.8. At this pH the rate of the non-enzymic oxidation of GSH was low. GSH was added at