robinetin (Weinges, 1958). In all these instances the suggested conversion of the flavan-3:4-diol into the corresponding 2:3-dihydroflavonol appears to predominate.

In some woods two flavan-3:4-diol precursors are present, for example 7:4'-dihydroxyflavan-3:4-diol and 7:3':4'-trihydroxyflavan-3:4-diol in certain *Guibourtia* spp. (Roux, 1959). In black-wattle-bark tannins similarly, two interrelated groups of components are present, namely (a) (-)-robinetinidol, robinetin (Kirby & White, 1955), leuco-robinetinidin (traces) and complex leuco-robinetinidin tannins and (b) fisetin, leuco-fisetinidin (traces) and complex leuco-fisetinidin tannins. These two groups of substances may arise from the monomeric flavan-3:4-diols by the same paths of interconversion and condensation as is evident for wattle-wood tannins.

SUMMARY

1. (-)-7:3':4'-Trihydroxyflavan-3-ol [(-)-fisetinidol], a new catechin, has been isolated from the heartwood of *Acacia mollissima*. The radial distribution of the substance in the wood has been estimated.

2. (-)-Fisetinidol has also been prepared synthetically by the hydrogenation of (+)-7:3':4'-trihydroxyflavan-3:4-diol.

3. The new catechin has the same configuration at C-2 and C-3 as (+)-7:3':4'-trihydroxyflavan-3:4-diol and as (+)-fustin, all of which occur in the wood.

4. The suggestion is made that the flavan-3:4-diol is the single precursor from which the corresponding catechin, 2:3-dihydroflavonol and flavonol

arise by interconversion, and the accompanying leuco-fisetinidin condensed tannins by condensation.

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The Effect of Riboflavin and Mepacrine on the Metabolism of 5-Hydroxytryptamine

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The monoamine-oxidase pathway has been identified as an important route of degradation of 5-hydroxytryptamine (serotonin) in many species. Because of the similarity of its action to that of D-amino acid oxidase, the coenzyme of which is flavinadenine dinucleotide, Richter (1937) suggested that monoamine oxidase may be a flavo-protein. Diamine oxidase has been shown to be a

yellow enzyme (Swedin, 1943; Kapeller-Adler, 1949; Goryachenkova, 1956). Hawkins (1952b) considered that the decreased activity of monoamine oxidase in the liver of rats, which is a concomitant of riboflavin deficiency, is due to impaired protein utilization and postulated that the vitamin is involved in synthesis of the apoenzyme of monoamine oxidase. Her findings have been

confirmed (Sourkes, 1958). Further evidence of the influence of riboflavin upon monoamine-oxidase activity is the observation that mepacrine, an inhibitor of yellow enzymes, blocks the metabolism of adrenaline *in vivo* and *in vitro* (Allegretti & Vukadinovic, 1950). Mepacrine also inhibits the tetrazolium salt-reducing system of liver suspensions incubated with amine substrates (Lagnado & Sourkes, 1956). These results have led to the tentative classification of monoamine oxidase as a yellow enzyme (Mahler, 1956), but the role of riboflavin in the metabolism of amines requires further clarification. With this aim, the effect of riboflavin deficiency and of mepacrine on the metabolism of 5-hydroxytryptamine has been examined.

MATERIALS AND METHODS

The method of McFarlane *et al.* (1956) was adapted for measurement of 5-hydroxyindolylacetic acid and that of Udenfriend, Weissbach & Clark (1955) for 5-hydroxytryptamine. The experimental animals used were male Sprague-Dawley rats (100–200 g.) and male albino guinea pigs (200 g.). Pooled urine collections were obtained from groups of three animals in plastic metabolism cages (Alpha Plastics Co., Montreal, Canada) for each treatment studied. At the end of the collection period the funnelled base of each cage was washed down with a small volume of water; washings were combined with the urine. Investigations were carried out *in vivo* by administering 2 mg. of 5-hydroxytryptamine/100 g. body wt. to rats by gastric tube; 24 hr.-urine specimens were then collected for determination of 5-hydroxyindolylacetic acid. The urinary output of this acid (above endogenous excretion) was considered a measure of monoamine-oxidase activity. The effect of mepacrine on monoamine-oxidase activity *in vivo* was studied by similarly administering 2 mg. of the drug/100 g. body wt. daily before 5-hydroxytryptamine (California Corporation for Biochemical Research, Los Angeles, Calif., U.S.A.) was given. Riboflavin deficiency was induced by omitting the vitamin from an otherwise fully supplemented artificial diet (Vitamin Test Diet, Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.). To 1 kg. of the basal diet were added (mg.): thiamine hydrochloride (30); biotin (1.2); calcium pantothenate (80); pyridoxine hydrochloride (30); menadione (4); folic acid (6); 0.1% of

vitamin B₁₂ triturated in mannitol (10); inositol (2000); *p*-aminobenzoic acid (400); choline chloride (800); powdered sucrose to make 40 g. of mixture. For control animals 30 mg. of riboflavin was included in the vitamin mixture.

Experiments *in vitro* were carried out on livers of riboflavin-deficient rats. These tissues were stored in the frozen state until used, when they were allowed to thaw just before homogenizing in 0.2M-potassium phosphate buffer, pH 7.4. These homogenates, containing 20% (w/v) of liver, were centrifuged at 20 000 *g* for 30 min. at 2°; the supernatants were removed and respun, and the particulate fractions were washed with buffer, respun and made up to the original volume of the homogenate with buffer. Supernatants were also prepared from fresh guinea-pig livers by the method of Hogeboom (1955) by using 0.25M-sucrose. Tissue preparations equivalent to 0.5 or 1.0 g. (original wet wt.) of liver were incubated with 2 mg. of 5-hydroxytryptamine in 0.2M-potassium phosphate buffer, pH 7.4, for 1 hr. at 37° under a flow of O₂ + CO₂ (95:5). Final volume was 7.5 ml. After incubation, protein was precipitated with 2.5 ml. of 20% zinc sulphate and the clear supernatant obtained after centrifuging was used for the estimation of 5-hydroxytryptamine and 5-hydroxyindolylacetic acid.

RESULTS

Studies in vivo

The endogenous excretion of 5-hydroxyindolylacetic acid in urine of rats was 0.031 mg./100 g./24 hr. (s.e. 0.007). In many experiments with rats given 5-hydroxytryptamine, amine oxidation, estimated as the increase of 5-hydroxyindolylacetic acid excretion above endogenous, accounted for 3–30% of the administered 5-hydroxytryptamine.

If monoamine oxidase is in some way dependent upon adequate riboflavin nutrition of the animal, it would be expected that riboflavin-deficient rats would metabolize a smaller proportion of exogenous 5-hydroxytryptamine along the monoamine-oxidase pathway than would rats supplemented with the vitamin. The results in Table 1 show that this occurs only occasionally. In fact, the deficient rats often excreted more 5-hydroxyindolylacetic acid than did the control animals. These results cannot be considered definitive

Table 1. *Effect of mepacrine on the excretion of 5-hydroxyindolylacetic acid by riboflavin-deficient rats*

Results are given as mg. of 5-hydroxyindolylacetic acid/100 g. body wt./24 hr., after dosing with 5-hydroxytryptamine (2 mg./100 g.). Male Sprague-Dawley rats on fully supplemented diets were given only enough food to maintain the same body weight as their deficient pairs. Three rats were used for each treatment. Mepacrine, 2 mg./100 g., was administered by gastric tube 24 hr. and 1 hr. before the dose of 5-hydroxytryptamine.

Days on diet	Set I				Set II			Mean
	10	15	20	25	21	25	30	
Treatment with respect to riboflavin:								
Supplemented	0.24	0.20	0.38	0.07	0.25	0.49	0.18	0.26 (100%)
Deficient	0.15	0.23	0.37	0.08	0.40	0.54	0.36	0.30 (115%)
Supplemented + mepacrine	0.33	0.32	0.47	0.44	0.45	0.59	0.30	0.41 (158%)
Deficient + mepacrine	0.34	0.31	0.42	0.19	0.34	0.58	0.38	0.36 (138%)

because of the wide daily variation in output of 5-hydroxyindolylacetic acid by the control animals dosed with 5-hydroxytryptamine, an observation which has been made by others (Erspamer & Ciceri, 1957; Chak & De, 1958). Nevertheless, the results show that riboflavin deficiency does not result in the same change in the oxidation of 5-hydroxytryptamine *in vivo* as observed in extracts of liver, within the same time period, by Hawkins (1952*b*).

It was considered that treatment of the animals with mepacrine, an inhibitor of yellow enzymes (Haas, 1944), would result in a decrease in the

Table 2. *Effect of mepacrine on excretion of 5-hydroxyindolylacetic acid by rats dosed with 5-hydroxytryptamine*

All animals (three for each treatment) received a dose of 5-hydroxytryptamine (2 mg./100 g. body wt.) before collection of urine for 24 hr. Mepacrine, 2 mg./100 g. body wt., was administered daily by gastric tube.

Treatment	Days on treatment	5-Hydroxyindolylacetic acid in urine	
		(mg./100 g. body wt./ 24 hr.)	(% of control)
None	2	0.13	100
Mepacrine		0.18	138
None	4	0.17	100
Mepacrine		0.23	135
None	7	0.17	100
Mepacrine		0.22	129
None	11	0.11	100
Mepacrine		0.13	118

Table 3. *Effect of galactoflavin on excretion of 5-hydroxyindolylacetic acid by rats dosed with 5-hydroxytryptamine*

Galactoflavin, 2 mg./100 g. body wt., was administered intragastrically 1 hr. before the dose (2 mg./100 g. body wt.) of 5-hydroxytryptamine. Riboflavin-supplemented animals were pair-weighted to the deficient animals, as in Table 1.

Treatment	5-Hydroxyindolylacetic acid in urine	
	(mg./100 g. body wt./ 24 hr.)	(% of control)
None	0.43	100
Galactoflavin	0.49	114
None	0.26	100
Galactoflavin	0.35	135
Riboflavin-supplemented	0.07	100
Riboflavin-supplemented + galactoflavin	0.13	186
Riboflavin-deficient	0.08	114
Riboflavin-deficient + galactoflavin	0.05	71

amount of 5-hydroxyindolylacetic acid excreted, but it was found that the treatment with mepacrine led to the excretion of a greater proportion of administered 5-hydroxytryptamine as the acidic metabolite (Table 2) than in the controls. This occurred in both riboflavin-supplemented and -deficient animals (Table 1). However, the (weighted) average increase in the former was 60 %, compared with only 17 % for the deficient rats. This result was checked with the antivitamin, galactoflavin (Emerson, Wurtz & Johnson, 1945) (Merck, Sharpe and Dohme Inc., Rahway, N.J., U.S.A.). In rats receiving a complete diet (Table 3) a single dose of this compound caused an increased output of 5-hydroxyindolylacetic acid (14, 35 and 86 % in three experiments). In riboflavin-deficient rats, however, the administration of galactoflavin did not increase the metabolism of exogenous 5-hydroxytryptamine along the monoamine-oxidase pathway but, rather, decreased it.

To assess the value of the 5-hydroxytryptamine-load test for estimating monoamine-oxidase activity in the rat *in vivo* (Tabachnick & Rubin 1959), known inhibitors of monoamine oxidase were studied. Iproniazid (Hoffman-La-Roche Ltd., Montreal, Canada), harmaline and harmine (Dr I. I. A. Tabachnick, Schering Corp., Bloomfield, N.J., U.S.A.) and tranlycypromine (SKF *trans* 385) (Smith, Kline and French Labs., Phil., Pa., U.S.A.) all decreased the excretion of 5-hydroxyindolylacetic acid (Table 4).

Studies in vitro

Monoamine oxidase is mainly associated with the particulate fraction of rat livers (Hawkins, 1952*a*). Both particulate and supernatant fractions were allowed to act upon 5-hydroxytryptamine at pH 7.4 for 1 hr. at 37°, when the 5-hydroxytryptamine remaining was measured. Much pigment was formed in both fractions. From Table 5 it is seen that the riboflavin deficiency did not appreciably affect the rate of disappearance of the amine in the particulate fractions. Incubation of the amine with the supernatant fraction, however, led to a 25 % decrease in the loss of amine under the conditions used. This suggests that the enzyme systems of the supernatant fraction differ from those associated with the cellular residue and, by extension, from monoamine oxidase.

According to Weissbach, Redfield & Udenfriend (1957) the liver of guinea pig contains a soluble monoamine oxidase located in the supernatant fraction. This preparation has the advantage that it contains an excess of aldehyde dehydrogenase capable of increasing formation of 5-hydroxyindolylacetic acid and inhibiting pigment production. This tissue was used to determine whether the two systems acting in rat liver could be distinguished

Table 4. *Effect of monoamine oxidase on excretion of 5-hydroxyindolylacetic acid by rats dosed with 5-hydroxytryptamine*

Dose of 5-hydroxytryptamine (2 mg./100 g. body wt.) was administered by gastric tube. Other compounds were given by gastric tube (p.g.) or intraperitoneally (i.p.).

Treatment	Dose (mg./100 g. body wt.)	Route of administration	5-Hydroxyindolylacetic acid in urine	
			(mg./100 g. body wt./24 hr.)	(% of control)
Control			0.18	100
Iproniazid	15	p.g.	0.02	11
Control			0.17	100
Iproniazid	15	p.g.	0.05	34
Control			0.41	100
Iproniazid	7.5	p.g.	0.22	54
Control			0.32	100
Iproniazid	7.5	p.g.	0.06	19
Control			0.22	100
Harmaline	1	i.p.	0.02	9
Tranylepromine	1	p.g.	0.01	4
Control			0.32	100
Harmine	0.5	i.p.	0.21	66

Table 5. *Effect of riboflavin deficiency on disappearance of 5-hydroxytryptamine in vitro*

Rat-liver preparations were used. The conditions are described under Methods.

Days on diet	5-Hydroxytryptamine disappearing (mg./g. wet wt. of liver)			
	Particulate		Supernatant	
	Riboflavin- supplemented	Riboflavin- deficient	Riboflavin- supplemented	Riboflavin- deficient
14	1.89	1.72	1.42	1.26
20	1.44	1.22	1.23	0.77
21	1.64	1.73	1.38	1.00
Mean	1.66	1.56	1.34	1.01

here. For this purpose, the supernatant fraction of fresh guinea-pig liver was incubated with 5-hydroxytryptamine and both disappearance of the amine and formation of 5-hydroxyindolylacetic acid were estimated in each sample. The difference (calculated as 5-hydroxytryptamine) served to estimate the extent of metabolism of the amine along pathways not involving monoamine oxidase. By varying the substrate concentration it was found that the enzymes of the monoamine-oxidase pathway (monoamine oxidase itself and an aldehyde dehydrogenase) were saturated by about $575 \mu\text{M}$ -5-hydroxytryptamine and that metabolism of the amine along other pathways continued at a linear rate beyond this concentration. Mepacrine, which had been shown in rats to increase the proportion of administered amine appearing in the urine as 5-hydroxyindolylacetic acid, was now tested on this system. Guinea pigs were treated with mepacrine, 20 mg./kg., for 4 days. A final dose was given 1 hr. before the animals were killed and their livers excised. The results are shown in Fig. 1. It can be seen that the treatment

of the animals with mepacrine led to a decrease in the rate at which 5-hydroxytryptamine was metabolized along the other pathways, but had no effect upon the formation of 5-hydroxyindolylacetic acid *in vitro*. In experiments with the supernatant fraction of guinea-pig liver pigment formation was not observed.

DISCUSSION

The action of monoamine oxidase converts 5-hydroxytryptamine into 5-hydroxyindolylacetaldehyde, a compound which has not been isolated. Its semicarbazone (prepared from the aldehyde, as it is formed enzymically) turns yellow with hydrochloric acid, and measurement of the specific absorption of this derivative has been used in the assay of monoamine oxidase (Weissbach *et al.* 1957). In the present work the test of monoamine-oxidase activity *in vivo* has depended upon the further oxidation of this aldehyde to 5-hydroxyindolylacetic acid in the tissue. On this basis the findings demonstrate that in the rat the monoamine-oxidase pathway is not the major metabolic

Fig. 1. Effect of mepacrine *in vivo* on metabolism of 5-hydroxytryptamine by guinea-pig-liver supernatants. (a) Formation of 5-hydroxyindolylacetic acid; (b) loss of amine by pathways not involving monoamine oxidase. ○, Control; ●, with mepacrine. For conditions, see Methods section.

route of 5-hydroxytryptamine when given intragastrically, for less than one-third of the amine appeared in the urine as 5-hydroxyindolylacetic acid. It is possible that the action of monoamine oxidase is actually greater than revealed by these measurements. For example, a portion of the aldehyde formed may be converted, not into the acid, but into other products not determined by the method used. Furthermore, it is possible that reduction of the aldehydic product of oxidation to the corresponding alcohol occurs, as has been observed with *isoamylamine*, *phenylethylamine*, *tyramine* and, possibly, *tryptamine* (Guggenheim & Löffler, 1916) and *catecholamines* (Kopin & Axelrod, 1960). However, this reaction has not been demonstrated with 5-hydroxytryptamine. Diversion of aldehyde into pigment formation through condensation reactions (Blaschko & Hellman, 1953) also occurs; as yet there is no accurate method for estimating the immediate product of the action of monoamine oxidase upon the amine. The aldehyde dehydrogenase responsible for the conversion of 5-hydroxyindolylacetaldehyde into 5-hydroxyindolylacetic acid is a flavoenzyme and would presumably undergo some diminution in activity in the course of riboflavin deficiency. However, this result would be measured

in the present studies only as a net effect on the formation of the acid and would not be reckoned among the other enzyme systems.

The present experiments do not provide evidence that riboflavin is involved in the action of monoamine oxidase in the same sense as it is in that of D-amino acid oxidase. Riboflavin-deficient rats did not show a decreased excretion of 5-hydroxyindolylacetic acid after administration of 5-hydroxytryptamine as might have been expected from the decreased oxidation of this amine by the livers of such animals (Hawkins, 1952*b*; Sourkes, 1958). The fact that excretion of the acid could be increased by two riboflavin antagonists, mepacrine and galactoflavin, suggests that a more complex mechanism than monoamine oxidase alone is operative and that these drugs bring about the diversion of a greater proportion of the exogenous 5-hydroxytryptamine into the monoamine-oxidase pathway than normally, through blocking some other route of degradation of the amine. This other pathway can be assumed to be a 'normal' one, the activity of which is diminished by flavoenzyme inhibitors, as well as by riboflavin depletion (seen in the supernatant fraction of deficient rat livers).

This hypothesis of a second riboflavin-dependent enzyme involved in the metabolism of 5-hydroxytryptamine explains the results of Tables 2 and 3. Thus when the metabolism of the amine has already been diverted from this enzyme into the monoamine-oxidase route, because of riboflavin deficiency, then the effect of mepacrine or of galactoflavin is less than that seen in the riboflavin-fed controls. The disappearance of 5-hydroxytryptamine from supernatant fractions of rat liver was decreased by 25% as a result of riboflavin deficiency, whereas the decrease in particulate fractions containing the major portion of monoamine oxidase (Hawkins, 1952*a*) amounted to but 6%. Because clear supernatant fractions are not obtained with stored tissues later homogenized in phosphate buffer, some activity measured in this fraction was undoubtedly due to monoamine oxidase, but hardly enough to account for the observed change in activity. The evidence adduced from studies of liver supernatant obtained from guinea pigs treated with mepacrine indicates that mepacrine is acting under these conditions as an inhibitor of an enzyme or of a system of enzymes apart from monoamine oxidase.

SUMMARY

1. The effect of mepacrine and of riboflavin deficiency on 5-hydroxytryptamine metabolism has been studied *in vivo* and *in vitro*.

2. In rats dosed with the amine, prior treatment with mepacrine increased excretion of 5-hydroxy-

indolylacetic acid. Riboflavin deficiency frequently had the same effect.

3. Mepacrine did not affect formation of the acid in the supernatant fraction of homogenized guinea-pig liver but it decreased the disappearance of the amine along other pathways.

4. Riboflavin deficiency resulted in decreased disappearance of the amine in supernatants of rat liver but not in the particulate fractions.

5. A hypothesis has been proposed that in mammalian tissue there is a pathway of metabolism of 5-hydroxytryptamine, other than the one catalysed by monoamine oxidase, which is sensitive to flavoenzyme inhibition and to riboflavin deficiency.

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Evidence for the Occurrence of Uridine Diphosphate N-Acetylglucosamine in Crustacean Tissues

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The polysaccharide chitin, which, from available evidence, is believed to consist of unbranched chains of N-acetylglucosamine residues, is a prominent constituent of the arthropod exoskeleton. Although information about chitin biosynthesis in arthropods is very sparse, its formation in *Neurospora crassa* involves uridine diphosphate N-acetylglucosamine as a glycosyl donor (Glaser & Brown, 1957). Although this and other uridine coenzymes are utilized extensively in the tissues of higher animals, relatively little is known of their biochemistry in invertebrates. This paper describes an investigation of the nucleotide content of the hypodermis and hepatopancreas of three species of Crustacea, with particular reference to the possible function of uridine diphosphate derivatives in polysaccharide biosynthesis.

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MATERIALS AND METHODS

Animals. Live specimens of the shore crab *Carcinus maenas* and of the spider crab *Maia squinado* were obtained from the Marine Biological Laboratory, Plymouth. Live lobsters (*Homarus vulgaris*) were obtained from local dealers.

Extraction of nucleotides from hepatopancreas and hypodermal tissue. (i) Perchloric acid method. The freshly excised tissue was weighed, frozen in liquid oxygen, powdered in a cold mortar and then dispersed in 2 vol. of cold 0.6N-perchloric acid. The insoluble residue was collected by filtration and re-extracted with cold 0.6N-perchloric acid. The combined filtrates were neutralized with 5N-KOH and the precipitated KClO₄ was removed by centrifuging, the neutralized extract being diluted to 5 ml. final volume for every gram of tissue used.

(ii) Aqueous extraction. The tissue was cooled on ice, weighed and minced in a chilled blender (a MSE Nelco 10 homogenizer or a Waring Blendor was used). The suspen-