lack the appropriate enzyme, and the adult frog may have the enzyme but lack the dietary provitamin. This argument does not depend upon astaxanthin being in fact a provitamin A_2 ; it would apply equally well to an as yet unknown precursor. Wald's picture of the roles of vitamins A_2 and A_1 in biochemical evolution is simple and attractive. If valid it is of great significance (cf. Florkin, 1949). In testing the idea, many apparently discrepant observations have been made. Although they do not decisively invalidate the theory, they make it necessary to restate the case, with a clearer distinction drawn between the genetic and environmental (dietary) factors. The evidence of vitamin A_1/A_2 ratios in carnivorous amphibians and fishes is of uncertain value in relation to the fundamental issue. If there should be more than one provitamin A_2 , e.g. β -carotene and an animal carotenoid, each the substrate of a specific enzyme, the problem will be further complicated. It therefore seems wise to suspend judgement for the present.

SUMMARY

1. It has been reported (Wald, 1946) that tadpoles of *Rana catesbeiana* contain vitamin A_2 but that after metamorphosis the adults contain vitamin A_1 only.

2. Tadpoles of *R. esculenta* and *R. temporaria* have been shown to have vitamin A_1 only. The visual pigment in both cases was rhodopsin.

3. The occurrence of vitamin A_2 has been reviewed and an endeavour has been made to reconcile the many apparently conflicting reports. It is concluded that vitamin A_2 may result from (a) preformed vitamin A_2 in the diet, (b) the conversion of β -carotene and other provitamins to vitamin A_2 , and (c) the conversion of a specific provitamin A_2 which might be an animal carotenoid such as astaxanthin. So far there is no direct evidence for this last suggestion.

We wish to thank the Medical Research Council for financial support.

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Metabolism of Polycyclic Compounds

7. THE METABOLISM OF NAPHTHALENE, 1-NAPHTHOL AND 1:2-DIHYDROXY-1:2-DIHYDRONAPHTHALENE BY ANIMALS

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(Received 25 August 1952)

The biological oxidation of naphthalene to 1-naphthol was first shown when Lesnik (1888) isolated 1-naphthylglucuronide from the urine of men and of dogs dosed with naphthalene. Almost 60 years later another type of oxidation product, the *laevo* form of 1:2-dihydroxy-1:2-dihydro-

naphthalene ('naphthalenediol'), was isolated from the urine of rats (Young, 1947) and the *dextro* form of the same compound from the urine of rabbits (Booth & Boyland, 1949). The suggestion has been made that such dihydroxydihydro derivatives might be the precursors of phenols in metabolism of hydrocarbons (Boyland, Levi, Mawson & Roe, 1941; Weigert & Mottram, 1946; Young, 1950). The

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present experiments and other experiments on the dynamics of naphthalene metabolism (Boyland & Wiltshire, 1953) are in agreement with the hypothesis that the dihydroxydihydro derivative or diol is the normal precursor of naphthol, but the experiments are not yet conclusive on this point.

METHODS

Glucuronic acid in urine was measured by a modification of the method of Hanson, Mills & Williams (1944) using a pure sample of menthol glucuronide (Sigma Chemical Co., St Louis, Mo.) as a standard.

Phenolic compounds such as 1-naphthol in urine were determined (a) by use of the reaction with 2:6-dichloroquinonechloroimide to give blue-coloured indophenols developed by Gibbs (1927) or (b) by coupling with diazotized *p*-nitroaniline.

Estimation of 1-naphthol. (a) Samples containing 10–100 μ g. 1-naphthol were placed in 17 $\times$ 150 mm. tubes, and diluted to 1.1 ml. with water and 0.1 ml. 10*N*-HCl. The solution was then neutralized by dropwise addition of 2*M*-Na₂CO₃, and 1 ml. 0.1*M*-Na₂B₄O₇, and 10 ml. of butanol were added. Finally 0.2 ml. of phenol reagent (2:6-dichloroquinonechloroimide (0.5%) in ethanol) was added, and the tubes shaken vigorously. After standing for between 30 and 60 min., the butanol layer was decanted into a 15 ml. centrifuge tube, and centrifuged for 5 min. The butanol layer was transferred to the cell of a Spekker colorimeter and read against a blank, using filter OY2. (b) To samples containing 5–40 μ g. 1-naphthol contained in 3 ml. *N*-HCl and 4 ml. ethanol, 1 ml. of 0.005*M* diazotized *p*-nitroaniline in *N*-HCl was added. The solution was shaken and neutralized with 2 ml. of 2*N*-NaOH, and the colour of the azo compound which develops immediately was read in a colorimeter using an orange filter.

Estimation of 1-naphthylsulphuric acid and 1:2-dihydroxy-1:2-dihydronaphthalene. 1-Naphthylsulphuric acid is completely hydrolysed by *N*-HCl at 100° in 15 min. Naphthalenediol is quantitatively dehydrated to naphthol by the same treatment (Young, 1947). The sum of these two compounds was determined as follows. Samples containing 10–100 μ g. of naphthol precursors in 1 or 3 ml. *N*-HCl were heated in test tubes immersed in a boiling-water bath for 15 min., and the naphthol estimated. Standard curves were prepared using 1-naphthol, naphthalenediol and 1-naphthylsulphuric acid. No significant amount of 1-naphthol was lost after 15 min. heating in *N*-HCl.

Trials showed that continuous ether extraction of a neutral solution for 24 hr. did not remove any synthetic 1-naphthylsulphuric acid but extracted 1:2-dihydroxy-1:2-dihydronaphthalene quantitatively. The hydrolysis of pure synthetic potassium salt of 1-naphthylsulphuric acid yielded only 82% of the theoretical amount of 1-naphthol. This loss is not corrected for in the calculation of the experimental results.

Estimation of 1-naphthylglucuronide. Phenylglucuronides differ in acid stability (Hanson *et al.* 1944), but they are generally less readily hydrolysed than ethereal sulphates. Urine from animals injected with 1-naphthol, which contained more glucuronic acid than normal urine, yielded a maximum of 1-naphthol if hydrolysed with either *N*- or 6*N*-hydrochloric acid at 100° for 2 hr. In control experiments using 1-naphthol or naphthalenediol, under these

conditions, losses amounting to 37% occurred, and using 1-naphthylglucuronide losses of 45–50% occurred. A second standard curve was therefore prepared using 1-naphthol which had been heated with acid under the same conditions. Two methods of estimation, (a) and (b), gave very similar results. (a) Samples containing 20–100 μ g. 1-naphthylglucuronide were diluted to 0.3 ml. with water, and 0.3 ml. 10*N*-HCl added. After heating for 2 hr., the samples were neutralized with 2*M*-Na₂CO₃ and naphthol estimated with the 2:6-dichloroquinonechloroimide reagent. (b) Suitably diluted urine (1 ml.) was heated with 1 ml. 2*N*-HCl for 2 hr. at 100° and the phenol estimated with the diazotized *p*-nitroaniline reagent.

The methods described provide four analytical values: (1) total glucuronic acid, (2) free naphthol, (3) free naphthol plus naphthol formed from naphthylsulphuric acid and naphthalenediol after 15 min. hydrolysis in *N*-HCl, and (4) the amount in (3) plus further naphthol released after 2 hr. hydrolysis in acid, which may provisionally be assigned to naphthylglucuronide. By appropriate deductions, (1) = glucuronic acid, (2) = free naphthol, (3) – (2) = '15 min. naphthol', (4) – (3) = '120 min. naphthol or glucuronide'. The '15 min. naphthol' was differentiated by solvent extractions between ether and neutral aqueous solution as '15 min. naphthol extractable by ether' which is attributed to naphthalenediol, and '15 min. naphthol not extractable by ether' which is probably naphthylsulphuric acid.

Animals

Rabbits. Rabbits weighing about 2 kg. were kept in metabolism cages on a diet of bread, oats and cabbage with unrestricted water and injected with naphthalene, with synthetic naphthalenediol, m.p. 103° (Booth, Boyland & Turner, 1950) or with 1-naphthol dissolved in arachis oil or water. The urine was collected daily and stored in the refrigerator.

Rats. Rats weighing 150–250 g. were fed rat cakes and bread with unrestricted water. The pooled urine of groups of rats was collected daily and stored in the refrigerator. Some rats were kept individually in metabolism cages of the design used by Elson & Lamerton (1951). In these cages urine was collected by absorption on a filter-paper cone, from which faecal pellets and food droppings could be removed after drying. The paper was then cut into small pieces and extracted successively with 10 ml. 2% NaHCO₃ solution, 20 ml. water and 20 ml. ethanol. The extracts were filtered into a 50 ml. flask and the volume adjusted to 50 ml. with water. Control experiments showed that recovery of 1-naphthol, naphthylsulphuric acid, naphthalenediol and glucuronide was substantially complete in this process.

Faeces were collected and analysed in some experiments. The whole sample of faeces was weighed and extracted by vigorous shaking with 10 vol. of water and 10 vol. of ether. The free and hydrolysable naphthol were estimated in both the ether extract and the filtered aqueous layer.

RESULTS

Estimation of metabolites in urine samples. Pooled samples of urine and individual samples from rabbits and rats were analysed by the methods described. Normal urines contain glucuronic acid and small amounts of material which reacted with the 2:6-

Table 1. *Excretion of products by animals injected with 1-naphthol*

(Rats were given approx. 200 mg. 1-naphthol/kg. body weight and rabbits were given approx. 100 mg. 1-naphthol/kg. body weight.)

		Excretion products expressed as mg. naphthol/100 mg. naphthol injected					
		In urine					
Species and no. of animals	Sex	1-Naphthol	Naphthalene- diol	Naphthyl- sulphuric acid	Naphthyl- glucuronide	In faeces. 1-Naphthol	Total as % of dose
Rats, 6	M.	15.7	0	17.0	20.6	3.6	56.9
Rat, 1	M.	13.6	—	35.2	14.0	—	62.8
Rat, 1	M.	4.2	—	15.0	6.4	—	25.6
Rats, 6	F.	14.7	0	18.8	18.0	3.4	52.9
Rat, 1	F.	5.2	—	10.2	9.9	—	25.3
Rat, 1	F.	6.2	—	9.2	12.3	—	27.7
Rabbits, 2	M.	5.2	0	31.8	28.6	—	65.6
Rabbits, 2	F.	18.4	0	28.8	25.0	—	72.2

Table 2. *Excretion products from rats injected with DL-trans-1:2-dihydroxy-1:2-dihydronaphthalene (naphthalenediol)*

(Groups, each of six rats, injected with 1% aqueous solution.)

Excretion products expressed as mg. 1-naphthol								
Dose injected			In urine				In faeces. Naphthalene- diol	Total as % of dose
			1-Naphthol	Naphthalene- diol	Naphthyl- sulphuric acid	Naphthyl- glucuronide		
Sex	(mg./kg.)	Total (mg.)						
M.	100	130	4.4	8.9	0.8	5.0	1.4	15.8
M.	100	101	1.6	7.5	1.8	5.2	1.9	17.8
M.	150	155	3.6	10.8	1.1	15.7	2.1	20.1
M.	225*	313	3.2	11.5	2.4	11.9	1.9	9.3
F.	100	95	1.1	2.7	2.9	9.3	—	17.0
F.	150	146	1.1	4.2	2.9	9.0	—	17.1
F.	200	197	1.1	4.5	4.5	13.1	—	23.4

* Two of the rats in this group died within 24 hr. of injection.

dichloroquinonechloroimide phenol reagent, but gave no colour with diazotized *p*-nitroaniline.

Injection of 1-naphthol. After injection of 1-naphthol, increased amounts of free phenol (probably 1-naphthol (cf. Berenbom & Young, 1951)) and of conjugated phenols were excreted. In both rats and rabbits most of the naphthol was conjugated (Table 1) and an increased excretion of glucuronide was also found. No naphthalenediol was excreted by naphthol-treated animals.

The free naphthol present in the urine of naphthol-dosed animals was completely extracted by ether by continuous extraction of the neutral (pH 7.8) urine. There is considerable variation in the amounts of products excreted but, if allowance is made for the 50% loss which occurs on hydrolysing naphthylglucuronide and the 20% loss on hydrolysing naphthylsulphuric acid, then, in some cases, over 80% of the administered naphthol would be accounted for in the excretion products. It would appear that only a relatively small amount of naphthol is degraded by metabolic processes in the animal body.

Injection of 1:2-dihydroxy-1:2-dihydronaphthalene. After injection of naphthalenediol the recovery of products estimated as naphthol (Table 2) was much less than following injection of 1-naphthol. The glucuronide fraction might be expected to contain both the 1-naphthylglucuronide and the naphthalenediol glucuronide, but the latter compound has not yet been isolated. Examination of the faeces showed only the diol to be present, free or conjugated 1-naphthol being absent. The low recovery of phenols or phenol precursors from the animals indicates that the diol is attacked more readily than 1-naphthol. The data also show that the diol is partially converted to 1-naphthol contrary to the earlier statement by the authors (Boyland & Wiltshire, 1952).

Injection of different doses of naphthalene. The estimation of metabolic products after single doses of naphthalene (Table 3) shows that the proportions of the different products excreted do not change with the dose except that the proportion of glucuronide increases with increase in dose given to female rats.

Table 3. *The metabolism of different doses of naphthalene*

(Groups of six rats dosed at each level.)

Dose of naphthalene		Amounts excreted expressed as mg. naphthol/100 mg. naphthalene injected				
(mg./kg.)	Total (mg.)	1-Naphthol	Naphthalene-diol	Naphthylsulphuric acid	Naphthylglucuronide	Total mg. naphthol/100 mg. naphthalene
A. Male rats						
100	112	5.0	3.2	2.0	4.0	13.4
200	249	8.0	3.1	3.2	4.1	17.1
400	490	6.9	2.2	2.4	3.9	15.5
800	1016	4.3	4.6	3.3	3.9	15.8
Mean		6.0	3.1	2.7	4.0	15.5
B. Female rats						
100	106	4.4	1.0	2.3	2.9	10.6
200	222	4.7	1.9	2.1	5.4	12.9
400	428	5.9	2.2	1.6	7.4	16.8
800	888	4.1	4.8	5.3	10.0	22.1
Mean		5.4	3.3	2.8	—	15.6

Figs. 1 (a) and (b). The change in the nature of the excretion products from male rats dosed repeatedly with naphthalene. Each arrow represents dosing with 50 mg. naphthalene per rat. $\circ$ - $\circ$, naphthalenediol; $\bullet$ - $\bullet$, naphthylglucuronide.

Repeated injections of naphthalene. Groups, each of twenty rats, were dosed daily so that each rat received 50 mg. of naphthalene dissolved in 0.5 ml. arachis oil. The urine and faeces were collected and analysed each day. The results of two such experiments with male rats, and one with female rats, in which estimations were made by method (b), are given in Figs. 1 and 2. In another experiment two male and two female rats were kept in individual metabolism cages and the products estimated separately by method (a); the mean values found in this experiment are shown in Fig. 3. The amounts of free 1-naphthol and naphthylsulphuric acid remained reasonably constant during the period of the

observations; the mean values only of these fractions are shown in Figs. 1 and 2. In the other experiment (Fig. 3) the naphthalenediol and naphthylsulphuric acid were not estimated separately.

In experiments with male rats and sometimes with female rats (e.g. in the experiment shown in Fig. 3), the proportion of the dose which was excreted as diol was small at the beginning but increased during the first week of injection (Figs. 1 and 2). During the period in which this increase in the amount of free diol excreted occurred there was a decrease in the amount of the glucuronide fraction.

In some experiments with female animals the proportion of the initial dose excreted as diol was

greater than with males, and this proportion decreased (Fig. 2) or varied in a random fashion about a mean. In the case where the amount of diol decreased with repeated dosing (Fig. 2) the glucuronide fraction increased during this period. In general, the total naphthol present as diol and glucuronide remained constant.

Fig. 2. Experiments as in Fig. 1 but with female rats.

Fig. 3. The mean values from an experiment in which individual samples from two male and two female rats were examined. In this experiment the variation between individual animals was not great. ●—●, glucuronic acid; □—□, naphthylglucuronide; ○—○, naphthalenediol and naphthylsulphuric acid; ●—●, free naphthol.

The estimation of the diol depends upon the difference between free 1-naphthol and naphthol liberated in 15 min. in the ether extracts of urine. The amount of free 1-naphthol extracted by ether

from the neutral urine varied considerably. In the experiment illustrated in Fig. 1 (b) only 12% of the apparent free naphthol in the urine sample following the first injection was ether-soluble. After the first sample the amount of ether-soluble 1-naphthol increased to between 20 and 33%. These figures show that the apparent 1-naphthol in urine of naphthalene-treated rats is largely present in some form not extracted by ether in contrast to the 1-naphthol in urine of rats dosed with 1-naphthol which was completely extracted by ether.

DISCUSSION

The comparison of the metabolism of naphthalenediol and 1-naphthol shows that the diol is partially converted to 1-naphthol and is thus a possible precursor of the naphthol in naphthalene metabolism. As 1-naphthol is not converted to diol, it is unlikely that naphthol is a precursor of the diol. The high recovery of injected 1-naphthol in free and conjugated forms indicates that the phenol is not readily broken down. The much lower recovery of phenolic products from injected diol suggests that this compound is liable to further degradation, probably by ring fission.

The experiments in which repeated doses of naphthalene were given to male rats showed a progressive increase in the amount of free diol excreted. At the same time there was a decrease in the estimated glucuronide fraction. Thus apparent increase in diol excretion might be due to (a) a real increase in production of the diol, (b) a decrease in the degradation of the diol by metabolism or (c) a decrease in conjugation with glucuronic acid. Although the naphthalenediol glucuronide has not yet been isolated and identified, it seems probable that it would be formed by analogy with anthracene metabolism (Boyland & Levi, 1936). This point cannot be decided until the diol glucuronide and naphthylglucuronide can be estimated separately, but the most probable explanation of the increase in apparent diol excretion would appear to be reduced conjugation with glucuronide.

In the experiments with female rats the decrease in diol excretion in some cases appears to be due to increased conjugation with glucuronide. The cause for the changes in the extent of glucuronide conjugation is not known, but it is remarkable that repeated dosing with naphthalene should decrease glucuronide excretion in male rats more readily than in female rats.

The fact that the apparent free 1-naphthol present in urine of naphthalene or naphthalenediol-treated animals is not extracted by ether suggests that it is present in the form of a complex. Caffeine and tetramethyluric acid form complexes with naphthalene and 1-naphthol (Booth, personal

communication) and the naphthol in urines may be combined in an analogous way to a urine constituent. The metabolism of naphthalene may involve complex formation and subsequent oxidation of the naphthalene complex. The naphthalene liberated from urine by treatment with acid may be present in a similar form (Chang & Young, 1943). The nature of such complexes is being investigated.

Some of the probable steps involved in naphthalene metabolism are shown in the following scheme.

SUMMARY

1. 1-Naphthol injected into animals is mostly excreted either as free naphthol, naphthylsulphuric acid or naphthylglucuronide. The naphthol excreted is readily extracted from the neutral urine with ether.

2. 1:2-Dihydroxy-1:2-dihydronaphthalene (naphthalenediol) injected into animals is largely broken down, partly excreted unchanged, partly converted to free 1-naphthol, naphthylsulphuric acid and a glucuronide liberating 1-naphthol on hydrolysis.

3. About 20% of injected naphthalene is excreted either as free 1-naphthol, naphthalenediol,

diol or with naphthalene was not extracted from neutral urine by ether.

5. These findings are discussed in relation to naphthalene metabolism.

We wish to thank Mr J. Marsh and Mr R. White for assistance in the experiments described. We are indebted to Prof. L. Young for a gift of 1-naphthylglucuronide. This investigation has been supported by grants to the Royal Cancer Hospital and Chester Beatty Research Institute 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, Public Health Service.

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