drawing freehand a continuation of the slowly rising absorption on the long-wave side of 300 m μ . and the more steeply rising absorption below 270 m μ ., the end absorption between 270 and 300 m μ . can be interpolated (cf. Glover, Glover & Morton, 1952). If this approximation is subtracted from the original spectrum, the extinction due to 7-dehydrosteroid can be obtained. Making this correction the amounts of 7-dehydrosteroids calculated from the corrected absorption at 282 m μ . were 15.5 mg./100 g. lipid for the ovary, 0.42 mg./100 g. lipid for the male fat body, 21.8 mg./100 g. lipid for the carcass and 0.39 mg./100 g. lipid for the liver (from the results of chromatography). The carcass included the skin and the relatively high proportion of 7-dehydrosteroid is not out of line with experience on other animals in which skin 7-dehydrosteroid is the presumed source of vitamin D. As 7-dehydrosteroids were not especially looked for it is not possible to say if they were present in all tissues, but qualitative evidence for their presence was obtained with female fat bodies and the alimentary tract.

The presence of vitamin A₂ was established beyond doubt. In all the organs where enough vitamin was present for quantitative analysis there was more vitamin A₂ than vitamin A₁. Wald's (1947) observations on *T. viridescens* were made on eyes only. This animal passes its early life in fresh-water as a gilled larva and after several months metamorphoses to the land-living red eft. At this

stage both A₁ and A₂ are found in the eyes, A₁ predominating. After 1-2 years of growth the animal undergoes a second metamorphosis and returns to water for the rest of its life. At this stage vitamin A₂ preponderates over A₁, although both vitamins are present. There is a clear need to study the distribution of vitamins A₁ and A₂ in various tissues of this species at different stages in its life history. Wald's observations are consistent with the idea that during its period as a land animal the red eft is not obtaining dietary vitamin A₂ or synthesizing it. The larva may have acquired a store of A₂ which is not augmented until the final return to water.

The present observations confirm, for the two species studied, the preponderance of vitamin A₂ over vitamin A₁ in the main storage depots. The bearing of the work on theories of the origin of vitamin A₂ is discussed by Collins, Love & Morton (1953b).

SUMMARY

1. The lipids from two species of newt (*Triturus cristata* and *T. carnifex*) have been examined by spectroscopic methods.

2. Vitamins A₁ and A₂ are both present as well as carotenoids, 7-dehydrosteroids and unidentified substances with selective absorption in the ultra-violet region.

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

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Studies in Vitamin A

25. VISUAL PIGMENTS IN TADPOLES AND ADULT FROGS

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(Received 18 July 1952)

Until recently vitamin A₂ was thought to occur only in fishes. The recognition by Wald (1942, 1946) of vitamin A₂ in amphibians has important consequences for the comparative biochemistry of vitamins A₁ and A₂ without which no assessment of possible evolutionary significance can be reached. The two previous papers in this series (Collins, Love & Morton, 1953a, b) described experiments bearing

on the problem. Wald (1946) discovered that tadpoles of *Rana catesbeiana* (the American bullfrog) contained vitamin A₂ only, and that it was replaced by vitamin A₁ in the adults. This observation prompted the present work which was undertaken to ascertain whether a similar state of affairs obtained in other species such as *R. temporaria* and *R. esculenta*.

EXPERIMENTAL

Animals. Tadpoles of *R. temporaria* were caught near Liverpool. Tadpoles and adults of *R. esculenta* were bought. Adult toads (*Bufo vulgaris*) were also caught locally.

Dissection and extraction. The tadpole eye (*Rana temporaria*) weighs about 1.2 mg.; this makes it scarcely feasible to dissect out retinas on a scale adequate for the study of visual pigments.

All operations were carried out in red light. The tadpoles were killed, after dark adaptation, by placing them in a strong solution of NaCl. The eyes were removed by a fine scalpel (made from a dissecting needle). The salt solution rendered the lens opaque, enabling the eye to be orientated so that the lens and cornea could be cut away with a pair of fine scissors. The opened eyes were then shaken vigorously in saline (0.9%, w/v, NaCl) and the material was poured through a fine wire gauze. The resulting suspension was centrifuged until the supernatant liquid was clear. The sediment was resuspended in 4% (w/v) alum solution ($K_2SO_4 \cdot Al_2(SO_4)_3 \cdot 24H_2O$) and left for 1 hr. The mixture was then centrifuged and the precipitate washed with saline and recentrifuged. The hardened and washed tissue was then extracted with 1 ml. of 1% (w/v) aqueous digitonin (for preparation see Collins, Love & Morton, 1952) for 1 hr. After centrifuging, the solution was mixed with an equal volume of buffer solution (pH 9.3; 19 g./l. of $Na_2B_4O_7 \cdot 10H_2O$) and its absorption spectrum measured between 400 and 600 m μ . in a photoelectric spectrophotometer. The solution was then exposed to a bright light (tungsten filament lamp) and the absorption remeasured. The difference between these two spectra represents the absorption due to the visual pigment.

In some instances other organs of the animals were examined for vitamin A. The tissue was saponified by heating a mixture of 1 part of tissue, 2 parts 60% (w/v) KOH and 5 parts of ethanol on a steam bath until a clear solution was obtained (about 15 min.). After dilution with water, the unsaponifiable material was extracted with diethyl ether. The ultraviolet absorption spectrum was recorded for the unsaponifiable material. In addition, the $SbCl_5$ reaction was investigated, using a visual spectrophotometer; vitamin A_1 has λ_{max} at 620 m μ . whilst vitamin A_2 has λ_{max} at 693 m μ .

RESULTS

The results of a preliminary investigation with tadpole eyes (*R. temporaria*) were disappointing. No visual pigments could be detected in an extract from thirty pairs of eyes. It was clear that either more animals would have to be used or else larger tadpoles obtained. The latter possibility was suggested by the work of Gordon, Goldsmith & Charippa (1943) who showed that metamorphosis of the tadpoles of *R. pipiens* was very considerably retarded by aqueous thiourea at a concentration of 0.033%. The tadpoles increased in size and weight but did not develop the characteristics of adult frogs.

Tadpoles of *R. temporaria*, with rudimentary hind limbs, were kept in aerated aquaria containing

0.04% thiourea. Toxic effects were observed at higher concentrations after 1 day; the animals appeared to be paralysed and floated upside down. The tadpoles were fed on green algae (*Spirogyra*) supplemented with dried fish powder and Bemax. After 3 weeks there was a marked difference between the animals treated with thiourea and those retained as untreated controls. The well-developed hind limbs of the latter were used in squatting, while those of the former were still rudimentary. After 30 days the untreated animals had four legs and a partly resorbed tail. The treated tadpoles though mostly having well-developed hind legs had neither front legs nor shortened tail. The weight of each animal had increased from about 0.5 g. to nearly 1 g.

Fig. 1. Visual pigment from tadpoles of *R. temporaria*. The curves shown were measured before exposure (top curve) to light and again afterwards (lower curve). —, obtained from tadpoles treated with thiourea; ----, obtained from tadpoles not treated with thiourea.

The tadpoles were killed after having been treated with thiourea for 37 days. The visual pigment was extracted from the twenty treated with thiourea. In order to make sure that enough material was available ten of the largest were selected from a batch of eighty which were at about the same stage of metamorphosis as the thiourea-treated animals. The absorption spectrum of the visual pigment is shown in Fig. 1 and is quite clearly that of rhodopsin, corresponding with vitamin A_1 . The hepatopancreas and the intestines (including

contents) from these animals were examined for vitamin A; no vitamin A₂ could be detected, but vitamin A₁ was clearly present in both these organs.

It seemed to be desirable to confirm these results on tadpoles not treated with thiourea. Accordingly, 140 tadpoles of *R. temporaria* with rudimentary hind limbs were obtained. They were dissected at the rate of thirty a day, the eye tissue being stored at -78° until required. The combined eye tissues were extracted and the absorption spectrum of the visual pigment is shown in Fig. 1.

Fig. 2. Visual pigment from tadpoles of *R. esculenta*. The curve shown was measured before exposure to light (top curve) and again afterwards (lower curve).

Tadpoles of *R. esculenta* were next examined. At first the whole animals were extracted and the unsaponifiable fractions examined for vitamin A. There was a small absorption maximum at 330 mμ., but the colour test was inconclusive because of interference by the large amounts of carotenoid present.

The eye tissues of fifty tadpoles were extracted and Fig. 2 shows the absorption spectrum of the visual pigment which again is clearly rhodopsin.

For comparison, the visual pigments from eleven toads (*Bufo vulgaris*) and twenty edible frogs (*Rana esculenta*) were obtained. Table 1 shows the position of the absorption maximum of preparations from a number of species.

DISCUSSION

There are two possible ways of accounting for the distribution of vitamin A₂ in animals. Stated in the extreme form they are as follows:

(1) The first would ascribe the occurrence of vitamin A₂ to a genetical difference. Thus, animals having vitamin A₂ would possess an enzyme system absent from animals with vitamin A₁ only. This hypothetical enzyme would either convert vitamin

A₁ to vitamin A₂, or β-carotene to vitamin A₂, or both processes might occur.

(2) The second possibility would ascribe all differences in the proportions of vitamin A₁ and vitamin A₂ to differences in food. This assumes the presence in the diet of either preformed vitamin A₂ or of a specific provitamin A₂ or both.

Table 1. Position of the visible absorption maximum of the visual pigments in a number of species

Species	$\lambda_{\max}$ (obs.) (mμ.)	$\lambda_{\max}$ (corr.)* (mμ.)
<i>Bufo vulgaris</i>	501.5	502.5
<i>R. temporaria</i> (adult)	—	503*
<i>R. esculenta</i> (adult)	502.0	503
<i>R. temporaria</i> (larval)	502.2	—
<i>R. esculenta</i> (larval)	500.2	—
Rat	—	498*
Ox	499†	—
Sheep	—	499*
Char (<i>Salvelinus willoughbyi</i>)‡	—	514*
Perch (<i>Perca fluviatilis</i>)§	520-528*	—

* Method of correction and data given by Collins & Morton (1950).

† Collins *et al.* (1952).

‡ This species has both vitamin A₁ and A₂ in its eye.

§ This species has vitamin A₂ in its eye.

Wald (1946) made out a closely reasoned case for the first view. He showed that vitamin A₂ is associated either with freshwater fishes, or, more fundamentally, with spawning in a freshwater environment. In a survey of the visual systems of marine and freshwater fishes Wald (1937, 1939) found that the marine fish examined had vitamin A₁ only, with the exception of the *Labridae* which had a predominance of vitamin A₂. Permanently freshwater fishes, he claimed, had vitamin A₂ only, while those which migrate from fresh water to the sea or vice versa showed much variation. The deciding factor seemed to be the spawning environment but many species had a mixture of the vitamins. Wald points out that vertebrates probably originated in fresh water and that the switch to the vitamin A₁ system is associated with the evolutionary change to another environment. In support of this he cites the case of a cyclostome, the sea lamprey (*Petromyzon marinus*) which spawns in fresh water but otherwise lives in the sea: he found that this animal contains predominantly the vitamin A₂ system. Wald predicted that amphibians, which usually spawn in fresh water, but are capable of a terrestrial existence, would possess vitamin A₂, although the adult frog certainly contains vitamin A₁ only. However, two discoveries seemed to support Wald's suggestion: Wald (1942) found vitamin A₂ in a newt (*Triturus viridescens*) and this has now been confirmed by the present writers (Collins *et al.* 1953b) in

T. carnifex and *T. cristata*. Wald (1946) found vitamin A₂ in the tadpoles of *Rana catesbiana*; this he regarded as an example of recapitulation. Thus in 1946, the evidence in favour of the phylogenetic distinction between animals containing vitamin A₁ and those containing vitamin A₂ appeared to be strong.

Two considerations are hard to reconcile with such a view. First, the present work establishes that at least two species of frogs contain vitamin A₁ in the tadpole stage. Secondly, the widespread occurrence of vitamin A₂ in marine fish-liver oils (often 4–20% of the total vitamin) is often forgotten. Thirdly, earlier work on the freshwater lamprey (*Petromyzon fluviatilis*) showed that vitamin A₁ was greatly preponderant over vitamin A₂ (Edisbury, Lovern & Morton, 1937; Lovern, Morton & Ireland, 1939).

It is agreed that most vertebrates are able to convert β -carotene to vitamin A₁ and that they do not convert vitamin A₁ to vitamin A₂. Thus vitamin A₂ is not present in whale-liver oil. Some species of freshwater fishes are also known not to convert vitamin A₁ to vitamin A₂ when the former is given orally (Marcos & Salah, 1951). On the other hand, some freshwater fishes are known to be able to use β -carotene as a provitamin for A₁ and A₂ (Morton & Creed, 1939). It thus seems fair to conclude that a particular enzyme (with presumably a corresponding gene) plays a decisive part in the occurrence of vitamin A₂ in some species. If it be granted that not all freshwater fishes nor all saltwater fishes possess this enzyme, it is highly probable that those which do not may obtain preformed vitamin A₂ or a provitamin A₂ in their diet, which might be other fish, invertebrates or vegetable matter, with consequent wider distribution of vitamin A₂. It may then become difficult to distinguish between exogenous and endogenous vitamin A₂ or any specific provitamin which may exist. In a species capable of forming both vitamins from a common precursor a constant proportion of the two vitamins might result. It is thus conceivable that some species may live on a diet which results in the predominance of vitamin A₂.

In amphibians the evidence for a phylogenetic origin for vitamin A₂ is scanty. The newts contain a mixture of vitamins with a predominance of vitamin A₂, but of the species of frogs studied in the adult form all have vitamin A₁ only. In the larval stage only bullfrog tadpoles have vitamin A₂. The feeding habits of these species seem to provide a clue. One difference that may be significant is whether the diet is of vegetable or animal origin.

The food of frogs and other amphibians, in general, consists of any moving creature small enough to be swallowed. Newts (adults and larvae) have sufficiently large mouths to enable them to eat

small water creatures (e.g. *Daphnia*) but the tadpoles of *Rana temporaria* and *R. esculenta*, which are much smaller, probably live mostly on algae. Adult frogs feed on insects, spiders, worms, snails, caterpillars, slugs, etc., but in addition, *R. esculenta* is partly aquatic and may eat water creatures as well. This is supported by the fact that Lederer & Rathmann (1938) found a small proportion of vitamin A₂ in the liver oil of adults. The tadpole of the bullfrog (*R. catesbiana*) spends over a year in the water, and because of its relatively large size probably eats small water animals. If such creatures contained a provitamin A₂ or preformed vitamin A₂, the occurrence of this vitamin in amphibians could be explained.

It thus appears that to account for the distribution of vitamin A₂ in the animal kingdom it is necessary to assume that it may owe its presence in a given case either to the diet or to an inherited ability to convert a provitamin to vitamin A₂ (or mixtures of vitamins A₁ and A₂). The missing link in this argument concerns the nature of the immediate precursor of vitamin A₂, or the hypothetical existence of preformed vitamin A₂ in zooplankton. The first evidence that preformed vitamin might occur in an invertebrate was obtained by Morton & Creed (1939). They showed, by spectroscopic evidence, that vitamin A₁, with no signs of vitamin A₂, appeared to be present in the unsaponifiable material from the freshwater shrimp *Gammarus neglectus*. Later, vitamin A₁ was shown to be present in marine crustaceans (Kon & Thompson, 1949). However, no vitamin A₁ or A₂ could be detected in *Daphnia pulex* (Collins *et al.* 1953a). The difference between the carotenoids in vegetable and animal diets is that some (e.g. astaxanthin) occur only in animals.

It thus seems that vitamin A₂ may arise in three possible ways. First, it may occur preformed in the diet. This could be from another vertebrate containing vitamin A₂ or possibly an invertebrate, although vitamin A₂ has never been detected therein even though vitamin A₁ has been shown to be present in certain crustaceans. Secondly, vitamin A₂ may arise by the conversion of β -carotene and other provitamins A. This is the case with certain freshwater fishes. Thirdly, vitamin A₂ may arise from a specific provitamin A₂.

In the present state of knowledge, astaxanthin, which is the principal carotenoid of crustaceans (whether of freshwater or marine origin), must obviously be considered as a possibility. However, as some species, e.g. whales, ingest vast quantities of astaxanthin but do not form vitamin A₂, it is clearly not enough to have this possible dietary provitamin. Similarly, amphibians in their adult form may ingest little astaxanthin and much β -carotene. The whale may get a provitamin A₂, but

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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