melanins. In this sense, therefore, those metals which appear to be frequent, if not universal, constituents of tissues containing melanin pigment, could be regarded as having a physiological function.

SUMMARY

1. The changes in the absorption spectrum obtained by adding various metals to a 3:4-dihydroxyphenyl-L-alanine- (dopa-)melanin solution have been investigated.

2. An enzyme preparation from cattle irises and ciliary bodies, which catalyses the oxidation of tyrosine to melanin, has been extracted and partially purified.

3. In solutions adjusted to pH 6.8 with sodium

carbonate, zinc and cobalt were found to enhance the light absorption of melanin formed from tyrosine in the presence of the ocular enzyme extract. In solutions at pH 7.8 calcium acted similarly. In solutions buffered with sodium citrate and sodium hydroxide, ferrous iron enhanced the light absorption of the melanin formed.

4. Zinc was found not to combine at all with melanin formed in the absence of protein; it combines to some extent with the protein in the ocular enzyme extract, but is bound to a much greater extent when melanin is formed in the presence of this protein.

5. The possible existence of a physiological association between melanin, protein and various metals is discussed.

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Studies in Vitamin A

23. VITAMIN A AND ITS OCCURRENCE IN *AMBLYSTOMA TIGRINUM*

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Vitamin A₂ has been generally regarded as characteristic of freshwater fishes in the same way as vitamin A₁ belongs to saltwater fishes and higher vertebrates. This view can easily be over-simplified; many saltwater fishes contain both vitamins although vitamin A₁ predominates.

Until recently, vitamin A₂ had only been found in fishes and in a few mammals whose diet consisted of freshwater fish, but in 1942 Wald discovered

vitamin A₂ and porphyropsin in the newt *Triturus viridescens*. He later found (Wald, 1946) that vitamin A₂ and the porphyropsin visual system also occurred in the tadpoles of the bullfrog (*Rana catesbiana*). When metamorphosis began to take place, vitamin A₁ was detected, and the proportion of vitamin A₁ to vitamin A₂ increased as metamorphosis progressed, until in the adult frog all the vitamin A₂ had been replaced by vitamin A₁. Since

vitamin A₂ is found in freshwater fishes and vitamin A₁ in terrestrial vertebrates, Wald concluded from this observation that the tadpoles were re-enacting their evolutionary history and were altering the relative proportions of the two vitamins as they migrated from water to land.

The purpose of the present work was to investigate the vitamin A₁/vitamin A₂ ratio of the urodelan amphibian *Amblystoma tigrinum*, both as an aquatic larva and as a terrestrial adult. In this animal sexual maturity can be attained in the larval state which normally persists throughout life. The larvae are known as axolotls, but under certain conditions metamorphosis can be induced. It was to be expected that after this had occurred a change in the vitamin A₁/vitamin A₂ ratio would be detected if the change recorded for *R. catesbiana* was a particular example of a general phenomenon.

EXPERIMENTAL

Rearing the animals. Spawn was obtained from black and albino axolotls. The spawn hatched out in 2 weeks and the young animals commenced feeding a fortnight later. For the ensuing 4 months they were fed live *Daphnia pulex*, and then small earthworms were given for 2 months. From then until the animals were killed the diet consisted of chopped raw meat given by hand. The weed *Elodea canadensis* which was frequently put in the aquaria was eaten by the axolotls before it had time to establish itself. The axolotls, from the time they are a few inches long, can easily be held and fed by hand. Vitamin A or β -carotene was dissolved in arachis oil and administered by means of a teat pipette. In feeding the metamorphosed animals generous amounts of β -carotene were dissolved in the arachis oil, and pieces of meat were dipped in the solution, after which they were introduced into the mouth with forceps.

Metamorphosis. Desiccated thyroid powder (British Pharmacopoeia) (5–10 mg.), enclosed in a folded piece of meat, was administered twice a week to each animal. The doses were kept small at first as otherwise the animals vomited. In addition, the water level in the aquaria was lowered until only about half the animal was submerged and this amount of water was decreased as metamorphosis progressed. The successive stages were as follows:

- Mature axolotls: water level reduced and thyroid administered;
- 8 weeks later gills noticed to be smaller in size;
- 11.5 weeks later buccal respiration began;
- 14 weeks later eyes bulging in one animal;
- 16 weeks later β -carotene administration began;
- 17 weeks later all had bulging eyes and preferred land to water;
- 19 weeks later the metamorphosed animals were killed.

Four axolotls were obtained from the Zoological Society of London in order to have some animals which had been fed differently from those reared in the laboratory.

Materials. Synthetic vitamin A acetate (Hoffmann-La Roche) was used; β -carotene was crystalline material (British Drug Houses Ltd.).

Analysis of tissues. Each tissue was ground with anhydrous Na₂SO₄ and extracted with light petroleum. The ultraviolet absorption spectra were measured in cyclohexane in a 1 cm. cell using a Beckman photoelectric spectrophotometer. The absence or presence of vitamin A was also investigated by means of the Carr-Price (SbCl₅) colour reaction using a Hilger-Nutting visual spectrophotometer.

The least amount of vitamin A that can be detected by the Carr-Price reaction can be calculated as follows. If all the vitamin from several axolotl livers is transferred to a test tube, the solvent removed and 4 ml. of SbCl₅ reagent added, it should be possible to detect, in a 2 cm. cell, a faint blue colour corresponding to an absorption at 620 m μ . of 0.1 or more. This corresponds to about 1 μ g. of vitamin A in the 4 ml. of solution ($E_{1\%}^{1\text{cm.}}$ 620 m μ . = 5070). In the presence of carotenoids the least amount of vitamin A that can be detected is greater than this.

Preliminary analysis of *Daphnia pulex*. Two batches of *Daphnia* were obtained from a pond in the Wirral Peninsula, Cheshire. Vegetable matter and small animals were removed by hand until the *Daphnia* sample was substantially free from other species. In the first batch about 300 000 *Daphnia* gave 45.9 g. of tissue. On examining the ether extract of this tissue neither vitamin A₁ nor A₂ could be detected. The second batch (approx. 725 000 *Daphnia* weighing 76.1 g.) yielded 0.901 g. of lipid. The lipid was examined spectroscopically before and after chromatography on alumina weakened by the addition of 10% of water. No vitamin A₁ or A₂ could be detected in any of the fractions and the SbCl₅ test was also negative. Carotenoids were present and it is hoped that they will be the subject of further study.

RESULTS AND DISCUSSION

The experimental results are summarized in Table 1. When the liver contained no detectable amount of vitamin A the diet consisted of either *D. pulex* or chopped meat with or without β -carotene. A preliminary analysis of *Daphnia* failed to reveal the presence of preformed vitamin A. The amount of vitamin A in meat is negligible. One must therefore conclude that either *Amblystoma* has no need for vitamin A or that its requirements are so low as to be beyond the present limits of detection.

That *Amblystoma* is able to assimilate vitamin A and to store it in its liver is shown by feeding either vitamin A acetate in arachis oil or mammalian liver which contains fairly large amounts of the vitamin. The animals which contained no detectable vitamin A (<0.3 i.u./g. of liver) showed none of the recognized signs of avitaminosis A; they grew well, the retinas had well-formed rods, the animals were not blind and their visual responses were similar to those of animals given vitamin A. The animals which had been fed large amounts of arachis oil showed some increase in the lipid content of the liver (from about 2.6 to 3.1%). Although axolotls are able to store vitamin A in easily detectable amounts when preformed vitamin is given with the food, no appreciable

Table 1. *Vitamin A content of livers from Amblystoma tigrinum at various stages of development and on various diets*

No. of animals	Average length (cm.)	Diet	Average wt. of livers (g.)	Lipid (%)	Carotenoid*		Vitamin A†	
					(mg./100 g. of lipid)	(µg./animal)	(mg./100 g. of lipid)	(µg./animal)
64	1.3	<i>Daphnia pulex</i>	—	—	Negligible		‡	‡
12	12.5	Chopped meat only	0.92	2.48	1.9	0.43	< 0.2	< 0.05
8	16.5	Vitamin A concentrate three times a week for 4 weeks	1.25	3.60	4.0	1.8	35	16
5	16.5	β-Carotene in oil three times a week for 8 weeks	1.78	3.10	3.8	2.1	< 0.2	< 0.1
4§	17.0	When young, <i>D. pulex</i> ; later, earthworms and liver	2.12	2.60	11.2	6.2	40	22
5	17.3	β-Carotene in oil twice a week for 3 weeks	1.36	3.35	11.6	5.3	< 0.2	< 0.1

* Calculated from the absorption at 450 mµ. assuming $E_{1\text{cm}}^{1\%} = 2500$ as an average value for the mixed carotenoids.

† Calculated from the Carr-Price reaction; $E_{1\text{cm}}^{1\%}$ for vitamin A at 620 mµ. taken as 5070.

‡ The ultraviolet absorption curve had a slight inflexion at 350 mµ., but no colour was obtained with the Carr-Price reagent.

§ From Zoological Society of London.

|| Metamorphosed animals.

storage of β-carotene occurred when it was added to the diet and large amounts of β-carotene were present in the faeces. Even more significantly no detectable conversion of β-carotene to vitamin A occurred.

Cama & Goodwin (1949) showed that thyroxine decreased, and thiouracil increased, faecal excretion of carotene in the rat. A similar effect has been shown to occur in cows (Chanda, Clapham, McNaught & Owen, 1951). Axolotls presumably have hypoactive thyroids as administration of thyroxine will induce metamorphosis. It could be argued that because they had hypoactive thyroids this would result in a decreased absorption of carotene and possibly in an impaired conversion to vitamin A. However, the experiment on the metamorphosed *Amblystomas* still showed little absorption of carotene and no detectable conversion to vitamin A.

It is interesting to compare the vitamin A content of axolotls with that of other animals. Rats with xerophthalmia are said to have in the liver not more than 30 i.u./g. of vitamin A (Lewis, Bodansky, Falk & McGuire, 1942). In the present work axolotls specially fed with vitamin A had about 36 i.u./g. of liver, but the apparently normal animals not so treated had not more than 0.3 i.u./g. of liver.

CONCLUSIONS

This investigation has shown that it is possible to rear axolotls to a normal size on a diet which contains no detectable amounts of vitamin A. The animals, moreover, fail to form recognizable vitamin A from ingested provitamins.

This observation is significant because the distri-

bution of vitamin A in vertebrates is so widespread as to strengthen the general belief in its indispensability. Karrer, Euler & Schöpp (1932) reported the absence of vitamin A from the livers of two lions, one tiger, a racoon, a harbour seal, a cormorant, a leopard adder and a single axolotl, and only a trace was found in the liver of a polar bear. The animals had all been in a zoo and may not have been normal from the point of view of vitamin A. Certainly the case of the polar bear must be regarded with caution, because Rodahl & Davies (1949) have shown that, in general, polar bear liver is extremely rich in vitamin A. It is perhaps more important that certain elasmobranchs with livers rich in squalene consistently exhibit negligible stores of vitamin A. It would therefore appear that the only parallel to the present consistent series of observations in axolotls is to be found in some elasmobranchs. It cannot be denied, however, that the possibility remains open that some vertebrate species may be able to dispense with vitamin A. An animal with no apparent need for vitamin A may either lack functions which involve vitamin A in other vertebrates, or have an alternative mechanism, or require extremely small quantities. In the last case provitamins may be converted, as required, to vitamin A without any surplus being available for storage in the liver.

Still another possibility needs to be considered, namely, that vitamin A may not be the active form but must be converted to another compound which has eluded recognition. This idea is not inconsistent with the work of Kaunitz & Slanetz (1951) on the vitamin A activity of vitamin A-free lard. If this hypothesis were tenable it is conceivable that an animal such as *A. tigrinum* might convert β-carotene

and other provitamins A directly to a hypothetical compound having vitamin A activity without a recognizable absorption spectrum or reaction with antimony trichloride.

It was hoped that a study of this species both as aquatic larvae and as terrestrial adults would prove a fair test of Wald's (1946) hypothesis, particularly since Wald (1947) recorded that 'such a permanently larval salamander as the mudpuppy (*Necturus maculosus*) contains only vitamin(s) A₂ in its retina as an adult'. As no vitamin A was found in axolotls, the original aim was defeated, but new problems have arisen out of the apparent dispensability of the vitamin for this species.

SUMMARY

1. The purpose of the present work was to investigate the proportions of vitamins A₁ and A₂ in an animal (*Amblystoma tigrinum*) which can exist both as sexually mature aquatic larvae and as terrestrial adults.

2. The vitamin A and carotenoid contents of the livers of *A. tigrinum* on various diets have been determined.

3. No conversion of β -carotene to vitamin A could be observed. Unless preformed vitamin A had been added to the diet, no vitamin A could be detected in the livers.

4. It is concluded that the requirements of this species for vitamin A must be either extremely small, or non-existent, or, alternatively, that an unrecognized compound other than vitamin A₁ is present and can replace the vitamin in physiological processes.

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Studies in Vitamin A

24. SPECTROSCOPIC EXAMINATION OF THE LIPIDS OF TWO SPECIES OF NEWTS

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In a previous paper (Collins, Love & Morton, 1953a) the history of the discovery of vitamin A₂ in amphibians is briefly recounted. So far, the only amphibians which have been found to contain vitamin A₂ are the newt, *Triturus viridescens* (Wald, 1942), and the bullfrog tadpole, *Rana catesbiana* (Wald, 1946). It is the purpose of the present paper to report on an examination of two species of newts, *Triturus cristata* and *T. carnifex*, for the presence of vitamins A₁ and A₂.

EXPERIMENTAL

Animals. Great newts (*T. cristata*) and Italian great newts (*T. carnifex*) were bought from a dealer. Some newt larvae (*T. cristata* and *T. vulgaris*) were caught near Speke, Liverpool.

Extraction. The tissues were ground up with Na₂SO₄ (anhydrous) and silver sand and extracted with light petroleum (b.p. 40–60°).

Chromatography. Alumina (P. Spence and Co., Widnes, grade O) was weakened by adding 10% (w/w) of water. This material was used for all chromatographic separations in conjunction with light petroleum (b.p. 40–60°) or diethyl ether or mixtures of the two solvents.

Estimation of vitamin A. This was accomplished either from the ultraviolet absorption using the data given by Cama, Collins & Morton (1951) or from the SbCl₃ colour test. In the latter case the absorptions at 620 m μ . ($E_{620}^{1\%}$) and at 693 m μ . ($E_{693}^{1\%}$) were measured in a visual spectrophotometer (Hilger-Nutting). In order to calculate the amounts of vitamin A₁ and vitamin A₂, use was made of the data given by Shantz (1948) and by Cama *et al.* (1951). For the pure substances, $E_{1\text{cm}}^{1\%}$ vitamin A₁ at 620 m μ . = 5070, $E_{1\text{cm}}^{1\%}$ vitamin A₂ at 693 m μ . = 4100. As vitamin A₂ absorbs at 620 m μ . the