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Distribution of Copper and Zinc in the Eyes of Fresh-water Fishes and Frogs. Occurrence of Metals in Melanin Fractions from Eye Tissues

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The results of Leiner & Leiner (1944) showed zinc to be present in the choroid, and other pigmented tissues, of the eyes of fresh-water fishes from Lake Constance, in amounts as much as one hundred times those in similar tissues from mammalian eyes (figures for which are given by Bowness, Morton, Shakir & Stubbs, 1952). Very high concentrations of zinc in the pigmented eye tissues were reported by Leiner & Leiner for each of nine species of fish from Lake Constance; indeed, many were higher than any previously reported to occur normally in living matter (cf. Monier-Williams, 1949). The highest recorded concentration of zinc in animal products, prior to the work of Leiner & Leiner (1944), was for serpent venom, namely 0.56 % of the dry weight (Delezenne, 1919). Monier-Williams (1949) attributes even higher concentrations to tench and herrings (Bertrand & Vladesco, 1921). The original paper does not in fact record very high values, and the mistake arose presumably from an erroneous abstract (*Analyst* (1921), **46**, 244). The range of zinc concentrations found by Leiner & Leiner in the dry choroids of fresh-water fishes was from 0.44 to 2.96 %, and the average for all the species examined was 1.18 %.

It was possible that the accumulation of zinc might be a peculiarity arising only in Lake Constance, or alternatively, that very high concentrations of zinc in some eye tissues are common to fresh-water fishes everywhere.

The present investigation had two main objects: (i) to ascertain whether the results obtained by Leiner & Leiner can be confirmed by studies on fishes from Lake Windermere, and (ii) to determine whether the association between zinc concentration and melanin pigmentation found in mammalian eyes (Bowness *et al.* 1952) can be demonstrated for fresh-water fishes. Leiner & Leiner (1944) fractionated a powdered dry homogenate of whole fish eyes by differential centrifugation in an attempt to discover the nature of the material to which most of the zinc was bound. They found that black and grey fractions contained most of the zinc, but, as these accounted for the greater part of the original material, they did not achieve any significant increase in the concentration of zinc, and were unable to indicate the nature of the material to which the metal was bound. In the present work, the trypsin digestion technique (Bowness *et al.* 1952), by which a pigment fraction was separated from mammalian

irises, was found to be effective in separating an analogous black material, rich in zinc, from fish and frog choroids.

MATERIALS AND METHODS

Materials. The heads of trout (*Salmo trutta*, Regan) and whole perch (*Perca fluviatilis*, C.) were obtained through the courtesy of the Freshwater Biological Association at Lake Windermere. The fish were despatched packed in cloth, surrounded by ice in fish tins; the eyes were removed from the heads and dissected at Liverpool within 2 days of the death of the animals. The first batch of perch obtained were young and small, the second batch varied from small to fully grown and aged fish.

Edible frogs (*Rana esculenta*) were obtained alive from Holland through Nederlandsche Heidemaatschappij, Arnhem. The animals were guillotined and the eyes dissected within, at most, a few hours; the retinas were utilized for other work in this Department.

English frogs (*R. temporaria*) were obtained alive from Mr H. F. Ashton of Norwich. The animals were chloroformed, and the eyes dissected, at most within a few hours.

The size of both types of frog varied, but most of them were probably hatched during the previous season.

Dissection of eyes. Certain differences between fish eyes and mammalian eyes should be noted here. In many fish, the inside of the sclera is covered by the silver membrane (argentea). This contains crystals of guanine which produce a silvery-white reflecting surface. In the posterior portion of the eye, between the silver membrane and the choroid, are pockets of blood, which, when the eyes come to be dissected, has clotted. These pockets are associated with the choroid body or 'gland'.

The technique of dissection used was similar to that described by Bowness *et al.* for mammalian eyes. The retinas from fish or frog eyes could be detached without adhering pigment by pulling the optic nerve while exerting a gentle pressure on the eyeball. The retina could then be carefully drawn out of the eye, attached to the optic nerve. With fish eyes, after the separation of the choroid from the blood clot, small pieces of silver membrane were found still to adhere to the tissue; these could only be removed by teasing the choroid with tweezers and a small scalpel. The irises of the fish were never completely separated from argentea.

Analyses for copper and zinc. The eye tissues from the frog *R. temporaria* and from perch were very small; after washing them with twice-distilled water, it was not found practicable to remove the excess water with filter paper. Most of the concentrations are therefore expressed only on a dry-weight basis, wet-weight concentrations having been obtained only for trout and some of the larger tissues of *R. esculenta*.

The analytical method has already been described (Bowness *et al.* 1952). The accuracy of the method as applied to fish-eye tissues has not been assessed experimentally, but it should not be less than that obtained with mammalian material. The ratio of the amount of Zn to a given weight of organic material is much greater for fish eyes than for mammalian eyes; the error in estimating Zn concentration which is due to loss in the course of ashing the organic material is therefore much less important for fish tissues. The generally smaller size of the fish eyes, and the consequent increased greater difficulty of dissection,

resulted, however, in increased uncertainty due to adhering extraneous tissue. It is therefore difficult to assess the extent of uncertainty arising from experimental procedure for the figures given in Tables 1 and 2; in general, it is mainly the third figure which is doubtful.

Separation of pigment fractions. Initially, an attempt was made to separate a pigment fraction from irises, as was done for cattle. However, it was found impossible to remove all the adhering silver membrane. The crystals of guanine accompanied the pigment in the fractionation and could not be removed without the use of hydrolysing agents (e.g. HCl), which are liable to split off the metals from the pigment material.

Attention was then transferred to the choroid. By careful dissection, this could be completely freed from guanine-containing material. It was found to be softer and less fibrous than the choroids of cattle and sheep, and therefore might be expected to be more readily digested by trypsin. Choroid tissue from perch and frogs was, in fact, split up entirely in 3-7 days by digestion with a pancreatin and trypsin suspension, but the process was not successful for trout. At the end of the time required for the choroids to be disintegrated, the digest was filtered through glass wool to remove any undigested tissue, and the filtrate centrifuged until no pigment remained in suspension. The supernatant was poured off, and the black residue stirred up with twice-distilled water and recentrifuged. This procedure was repeated four times. The final residue is the pigment-protein fraction.

Emission spectroscopic technique. Emission spectra of the ash of the perch melanin-protein fraction were recorded by the use of a carbon arc and a high-dispersion Littrow spectrograph (Hilger E. 1301). Details of the technique used for identifying the elements present are given by Bowness *et al.* (1952).

RESULTS

The main findings are displayed in Tables 1 and 2. The recorded results have been calculated directly from the experimental readings.

In order to test whether high concentrations of zinc, such as those in some of the eye tissues, are of general occurrence in the body of the perch, analyses were carried out on liver, muscle and skin.

Proportion of the copper and zinc in the perch choroid bound to the insoluble pigment-protein fraction. The dry matter in 73.3 mg. of fresh perch choroid was found to weigh 8.6 mg. When 309.2 mg. of fresh choroids, corresponding to 36.2 mg. dry weight, were digested with trypsin and a pigment-protein fraction isolated from the supernatant, the dry weight of this fraction was 14.9 mg. The weights of zinc and copper present in these amounts of choroid and pigment-protein material can be calculated by using the appropriate concentrations of the metals given in Tables 1 and 2. The ratio of the weight of zinc or copper in 14.9 mg. pigment-protein to that in 36.2 mg. choroid shows the proportion of the total weight of these metals in the choroid which is in non-ionic combination with the pigment-protein material. For zinc, this proportion

Table 1. *The concentrations of copper in the eye tissues of trout, perch and frogs*

(The ordinary figures show the concentrations in $\mu\text{g./g.}$ dry tissue; the figures in brackets underneath (when given) show the concentration in $\mu\text{g./g.}$ wet tissues corresponding to the figures immediately above. A dash means that the tissue was absent, or not available for analysis.)

Tissue	Trout (fresh-water)	Perch	Frog	
			<i>R. temporaria</i>	<i>R. esculenta</i>
Lens	5.62 (2.42)	0.85	29.8	27.4 (13.6)
Cornea	18.4 (3.5)	21.0	104	—
Sclera	8.65 (2.05)	27.3	120	199 (8.87)
Retina	35.8 (4.85)	18.1	117	—
Argentea	199.0	17.1	—	—
Iris plus ciliary body	105.0 (26.5)	18.8	—	—
Choroid	87.8 (18.0)	30.5	133	—
Pigment-protein fraction of choroids	—	49.3	—	985

Table 2. *The concentrations of zinc in the eye tissues of trout, perch and frogs*

(The ordinary figures show the concentrations expressed as $\mu\text{g./g.}$ dry tissue; the figures in brackets underneath, when given, show the concentrations in $\mu\text{g./g.}$ wet tissue corresponding to the figures immediately above.)

Tissue	Trout (fresh-water)	Perch	Frog	
			<i>R. temporaria</i>	<i>R. esculenta</i>
Lens	15.7 (6.75)	15.6	78.5	16.3 (8.1)
Cornea	81.3 (11.5)	189	278	—
Sclera	457 (95.6)	343	326	266 (132)
Retina	580 (74.2)	728	500	—
Argentea	7020	2010	—	—
Iris plus ciliary body	5990 (1560)	6380	—	—
Choroid	15100 (3100)	23800	14400	—
Pigment-protein fraction of choroids	—	43800	—	28100

is $\frac{43800}{23800} \times \frac{14.9}{36.2} \times 100 = 74.7\%$ (see Table 2); for copper, $\frac{49.3}{30.5} \times \frac{14.9}{36.2} \times 100 = 53.0\%$ (see Table 1).

The pigment-protein material, to which these fractions of the total copper or zinc are bound, constitutes 41.6% of the dry weight, or 4.82% of the wet weight of the choroid.

Table 3. *Concentrations of zinc in perch skin, muscle and liver*

Tissue	Zinc concentration ($\mu\text{g./g.}$ dry tissue)
Skin (white)	31.0
Muscle	57.7
Liver	59.6

Absorption spectra of pigments. Light passing through a suspension of particles in water is partly scattered and partly absorbed by the particles. The scattering of light by a particle depends on its size. The absorption curve of a suspension will, therefore, be governed by two factors, the light absorption of the substance suspended (itself dependent on the structure of the compound), and the size and number of particles suspended.

The absorption curves of suspensions in water of melanin-protein fractions from perch and frogs are given in Fig. 1.

Hydrolysis of pigment-protein fraction. Prolonged trypsin digestion of melanin-protein fractions from perch and frogs was found gradually to lower their zinc and copper contents. The zinc content on dry

weight of the perch material was reduced from 4.38 to 4.1% by 3 days' incubation with trypsin suspension. It is possible, therefore, that the original figure of 4.38% is too low by virtue of the incubation with trypsin during the separation procedure.

Fig. 1. Absorption spectra of melanin fractions from fish and frog eyes. —, perch pigment; ---, frog (*R. esculenta*) pigment.

Acid hydrolysis of the pigment was found to have an effect analogous to that obtained with cattle pigment. Part of the dry melanin-protein fraction (8.3 mg.) from perch was treated with 10 ml. 0.1N- H_2SO_4 for 1 hr. at 100°. The resulting mixture was centrifuged, the residue being washed with twice-distilled water and recentrifuged twice. The final residue was dried, weighed, ashed and analysed for zinc.

choroid will include much dialysable material, so that there is evidently a higher concentration of bound metals in the pigment-protein fraction than in the original choroid.

Emission spectroscopic analysis of the ash from perch material showed that the main metallic constituents of the pigment-protein fraction are zinc, calcium, and iron. Minor constituents were aluminium, copper, magnesium and strontium. No trace was found of barium, which was identified in the ash of cattle pigment-protein fraction.

DISCUSSION

It is evident from Table 4 that the high concentrations of zinc obtained by Leiner & Leiner (1944) are not due to Lake Constance as a special habitat.

In all seven of the other species of fish from Lake Constance examined by Leiner & Leiner, the iris, choroid, and silver membrane had the highest zinc contents of any of the eye tissues. Of these three, the choroid usually had the highest concentration, but in the Burbot the iris was higher in zinc; in no case was the silver membrane highest.

In comparing the results for fish from Lake Constance and Lake Windermere there are several points to note. Leiner & Leiner give the name of their trout as 'Sea-trout' (*Trutta lacustris* L.). There is some dispute as to whether the trout all belong to the same genus, but in the present work the view of Regan (1911), who classified all the English trout as *Salmo trutta*, is followed. There must remain some doubt, however, as to whether both sets of fish are of the same species. Secondly, in considering the

Table 4. A comparison of the results of Leiner & Leiner (1944) for perch and trout eye tissues with those obtained in the present work

Tissue	Trout ($\mu g.$ Zn/g. dry tissue)		Perch ($\mu g.$ Zn/g. dry tissue)	
	Leiner & Leiner	Present work	Leiner & Leiner	Present work
Lens	16.0	15.7	20	15.6
Retina	540-990	580	1090-1780	728
Iris plus ciliary body	6960-8700	5990	6200-7710	6380
Silver membrane	6360	7020	8200	2010
Choroid body	980-1490	—	510-1730	—
Choroid	9200-11350	15100	24000-29600	23800

The dry weight of the residue was 4.1 mg. and the zinc content 2.4 $\mu g.$ The estimated zinc content of the original melanin-protein fraction would be 365.2 $\mu g.$

Acid hydrolysis has thus removed nearly all the zinc and half the dry weight of the original material.

Ash content of the melanin-protein fraction. The amount of dry material remaining as ash after incineration was found to be just over 10% for the whole choroid, and just under 10% for the pigment-protein fraction from perch. The ash from the

silver membrane, it should be noted that the tissue is very small and it is not possible to separate it entirely from pigmented material. The amount of pigment remaining in attachment is partly a matter of chance, and there is therefore much latitude for variation in the results. Thirdly, the choroid body was not analysed in the present work because it appeared to be mainly composed of blood; it was completely dissected away from the choroid and silver membrane and thus does not enter into any of the present results.

The zinc concentrations in the eye tissues of the frog fairly closely parallel those of the fresh-water fish. Leiner & Leiner (1942) studied the concentrations of zinc in the eye tissues of many salt-water Teleosts. The general range of zinc concentrations was lower than that in fresh-water fish and frogs, but higher than that in mammals; for example, the range of zinc concentrations for the dry choroids of all the species of salt-water fish studied was 530–9800 $\mu\text{g./g.}$ (Leiner & Leiner, 1944). In all the fish examined, whatever the absolute amounts of zinc present, the same tissues contain the highest concentrations of zinc. Zinc concentrations in the eye tissues decrease in the following order: choroid or iris, retina, sclera, cornea, lens. The argentea concentrations are rather variable. Excluding this last-named tissue, which does not occur in mammals, and excluding the mammalian lens, the same order is true for results obtained on cattle, sheep, whale and rabbit eyes (Bowness *et al.* 1952). The range, from highest to lowest zinc concentration, is very much smaller in mammals than in fish. The concentration in the lens of mammalian eyes is generally slightly higher than that of fish eyes, yet the concentration in the choroid may be only 1/100th that of the same fish tissue.

Table 3 shows that, despite the high concentrations of zinc in the eye tissues, other parts of the perch have much the same concentrations as those given by Lutz (1926) for various mammalian species.

The results for copper concentrations in various eye tissues do not follow any clear-cut general pattern. The variation in the order of eye tissues, arranged according to copper concentrations, is much greater than is the case for zinc. The physico-chemical and biochemical agencies which accumulate zinc appear, in all the vertebrate eyes examined, to be active in one tissue more than another, and most active in the same tissues in all species. The same cannot be true for copper. In general, pigmented tissues and pigment-protein fractions from all the vertebrate eyes examined by us contain relatively high copper concentrations. In some species, other tissues may have concentrations as high, or higher. In trout, the silver membrane has a higher copper content than either choroid or iris. In connexion with this, it was observed that the debris from an attempted digestion of mixed choroids and silver membranes of trout, which consisted largely of guanine, had a high copper content (672 $\mu\text{g./g.}$ dry tissue). Frog-eye tissues seem to have a relatively high copper content, the pigment-protein fraction of *R. esculenta* being especially high in copper.

The very high concentrations of zinc in the pigment-protein fractions of perch and frogs, and the observation that at least 74% of all the zinc in

perch choroids is bound in non-ionic form to this fraction, show that the high zinc contents of the pigmented eye tissues can largely be accounted for by an association with melanin pigmentation.

As compared with up to 2.6% found in material from cattle, the ash content of dry perch pigment-protein fraction was about 10%, of which calcium and iron were shown to be fairly abundant constituents. Zinc, however, constituted nearly half of this ash, as compared with only 3.21% of the cattle material. Iron appeared to be the major metallic constituent of the cattle pigment-protein ash, and barium, which was not found in the perch material, was present as a minor constituent. It seems, therefore, that the absolute amount of the metallic elements associated with the pigment-protein material, and the relative proportions of each, vary from one vertebrate species to another.

The absorption curves of pigment-protein fractions from perch and frog eyes differ from those obtained from cattle (Bowness *et al.* 1952) and from those of other melanins (Edwards & Duntley, 1939; Serra, 1945; Zwicky & Almasy, 1935). The ratio of the light absorption in the ultraviolet region to that at higher wavelengths is much lower for the fish material than for the others. This may be caused partly by the different size of the particles in suspension, but such a large difference probably also indicates a difference in chemical structure.

Though it appears established that there is a connexion between melanin pigmentation and the presence of high concentrations of zinc (and possibly other metals) in the pigmented eye tissues, and perhaps in other pigmented tissues (cf. Bowness *et al.* 1952), the function of such metallic accumulations remains an open question. Leiner & Leiner (1941) showed that the zinc contents of the tissues of fish and mammals could not be accounted for solely by the amount of metal bound to carbonic anhydrase. They showed that zinc could lessen the inhibition of carbonic anhydrase by various tissue constituents. There seems no reason, however, why such a function should require a great accumulation of zinc in the pigmented eye tissues of fish, and especially in fresh-water fishes.

As stated previously (Bowness *et al.* 1952), the accumulation of copper in pigment fractions cannot be explained by the presence of free tyrosinase. The only other organic copper compounds known to occur in vertebrates are haemocuprein and hepatocuprein (Keilin & Mann, 1938), but these also are relatively water-soluble and could not account for the copper in the pigment fraction unless they were combined in some insoluble form.

Possible functions of the metals associated with the melanin-protein fractions of pigmented tissues will be discussed in a further paper.

SUMMARY

1. Results for the copper and zinc contents of eye tissues of perch, trout, and frogs are presented. The results of Leiner & Leiner (1944) for zinc in perch and trout are confirmed.

2. Most of the zinc, and half the copper, of the perch choroids is bound in non-ionic form to the pigment-protein fraction of the tissue.

3. The absorption spectra of pigment-protein suspensions from perch and frog eyes differ from those of cattle eyes.

4. On incineration, the perch pigment-fraction was found to leave about 10% of the dry weight as ash. Calcium, iron and strontium were constituents of this ash, but almost half is zinc (calculated as metal).

5. The association between zinc and melanin pigmentation is discussed.

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Effects of High-voltage Cathode Rays on Aqueous Solutions of Tryptophan, Tyrosine, Phenylalanine and Cystine

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The effects of irradiation by high-voltage cathode rays on amino-acids in fish muscle and on aqueous solutions of histidine monohydrochloride have already been reported (Proctor & Bhatia, 1950; Bhatia & Proctor, 1951). The irradiation caused no significant destruction of any one of ten amino-acids in fish. It was shown, however, that histidine in aqueous solution is decomposed by irradiation with cathode rays through deamination and fission of the glyoxaline ring.

Stenström & Lohmann (1928) concluded from their experiments on X-ray irradiation of aqueous solutions of tyrosine, that the phenolic structure of this amino-acid is changed by the irradiation. They also irradiated solutions of cystine and noticed no measurable change when the colorimetric method of Folin & Looney (1922) was used to determine cystine. Stenström & Lohmann (1931) also re-

ported that ammonia was given off from tyrosine and that tryptophan was destroyed upon irradiation with roentgen radiations. Buckhman & Manoilov (1949) found that cysteine evolves hydrogen sulphide on irradiation with ultraviolet rays. Dale & Davies (1951) studied the liberation of hydrogen sulphide by X-radiation from aqueous solutions of cysteine and glutathione.

The present study was undertaken to ascertain the effects of various doses of cathode rays on aqueous solutions of DL-tryptophan, DL-phenylalanine, L-tyrosine and L-cystine in various concentrations.

EXPERIMENTAL

Stock solutions of the amino-acids were prepared in concentrations of 10 mg./ml. A weighed quantity of the amino-acid was dissolved in a minimum quantity of 20% (w/v) HCl, and the solution was made to volume with distilled water. From these stock solutions, test solutions were made as desired.

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