Distribution of Copper and Zinc in Mammalian Eyes. Occurrence of Metals in Melanin Fractions from Eye Tissues

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Several methods have been used in the investigation of trace elements in eye tissues. Burdon-Cooper (1928) and Burdon-Cooper & Lewis (1929) give the results of emission spectrographic analysis of the mineral constituents of normal and cataractous human-eye lenses. The technique failed to demonstrate the presence of zinc, iron and manganese, which Tauber & Krause (1943) were later able to detect (and estimate) by other methods. The latter authors pointed out that the persistent spectral lines of the three elements could easily be obscured by lines due to elements such as calcium and phosphorus, which are present in the ash as major constituents.

The first stage in the present investigation was the application of modern methods of emission spectrum analysis to the problem of detecting and determining trace metals in eye tissues (A.L.S. and M.H.S., see Shakir, 1948). So far as detection was concerned, excellent results were obtained, but the range covered by zinc, copper and manganese contents of various eye tissues proved to be too narrow for emission spectra to give quantitative information of sufficient accuracy. Accordingly, a good deal of exploratory work was carried out using organic reagents forming metallic complexes which could be estimated by spectrophotometric methods. Selected procedures have now been improved and the results revised and extended (J.M.B.).

In the course of the work based on emission spectroscopy, semi-quantitative estimations of trace elements were made on dialysed and undialysed tissues from fish eyes (Shakir, 1948). The presence of comparatively large quantities of dialysable inorganic material results in a cloud of vaporized salts around the electrodes, and this has a marked effect on the recorded intensities of the spectral lines shown by the 'trace' elements. This effect introduces uncertainty into the validity of comparing the results with dialysed and undialysed preparations. A fivefold difference in trace metal concentration is certainly demonstrable, but it is difficult to be sure about small differences. The emission spectrum technique is thus not sufficiently sensitive for further study of the rather small differences recorded by Tauber & Krause (1943) in respect of the concentrations of zinc, copper and manganese in cattle-eye tissues. In spite of such limitations, it seemed clear that tissues from fish eyes contained non-dialysable zinc and copper in excess of the retained amounts of other trace metals. The preliminary work with dithizone zinc and copper complexes indicated that it might be possible to establish significant differences between the various eye tissues in mammals. Repetition and extension of the work shows this to be the case.

Leiner & Leiner (1944) had shown for zinc in fish eyes that such differences occur. Although the absolute concentrations found in each tissue varied greatly from species to species, a list showing the different types of tissues in order of decreasing zinc content was practically the same for all the fishes studied.

The results of Tauber & Krause (1943) on cattle eyes do not show a similar distribution of zinc, but the analytical method described in their paper seems inadequate. Their determinations of copper concentrations in eye tissues appear to be the only ones recorded in the literature.

It was therefore decided that the whole problem of zinc and copper in eye tissues deserved further study. The first part of this paper embodies the experimental details and results of determinations of zinc and copper concentrations in all the eye tissues of several mammals. The results of these determinations show that the iris and choroid tissues contain the highest concentrations of zinc and copper. It therefore became necessary to investigate the more precise location of these metals in irises and choroids. The second part of the paper deals with the experimental methods and results of a fractionation of irises, and a study of a pigment fraction obtained from irises and choroids.

1. ZINC AND COPPER CONCENTRATIONS IN THE VARIOUS EYE TISSUES

Experimental

Materials

Cattle and sheep eyes were obtained from the abattoir and dissected within 12 hr. of the death of the animals.

Whale eyes were obtained from sperm whales caught in the Antarctic region, and had been stored (whaling ship

'*Balaena*') in tins at a temperature below 0° for several months before dissection.

Rabbit eyes from animals of Dutch, cross-bred, Himalayan and albino varieties, were obtained through the courtesy of Dr A. U. Smith, from the National Institute for Medical Research, Mill Hill, London, N.W. 7, and were dissected 2–3 days after the death of the animals.

Analytical methods

General. Concentrations of Zn and Cu were determined mainly on a dry weight basis, for the tissues can be lightly washed, to remove adhering fluids and particles, with very little alterations in solid content, though the natural wet weight cannot then be accurately determined. Approximate wet weights of sheep and cattle tissues were obtained by drying the tissues, after washing, with ashless filter paper. A comparison of wet and dry weight concentrations of the trace metals then enables the effect of the loss of water from liquid tissues, such as the vitreous humour, to be gauged.

Preparation of tissues for analysis. Dissection was carried out with stainless steel instruments. An incision in the sclera was made with a scalpel and the front portion of the eye parted from the back by cutting round the outer margin of the iris. The tissues of the two portions were then separated and the two parts of the sclera combined, after removing the adhering muscle and fatty tissue. Each individual tissue was then washed with twice-distilled water. All manipulations after washing were carried out with glass rods.

The tissues were dried to constant weight in an oven maintained at 110°.

Tissues were normally incinerated in translucent silica crucibles at 450–550° in an electric muffle furnace with a silica lining. If the last trace of carbon residue was difficult to burn away it was found that addition of a few drops of twice-distilled water to the cooled ash permitted easy oxidation of the carbon on reheating. Lens tissue must be very slowly heated to 450°, otherwise the contents of the crucible froth over.

To each crucible containing ash, 0·1 N-H_2SO_4 (10 ml.) was added and the contents evaporated to dryness on a steam bath. More 0·1 N-H_2SO_4 (5 ml.) was then added and the crucible heated on the steam bath for a further 10–15 min. The contents were then washed into the beaker used to support the crucible in the extraction procedure, and the solution was made up to a standard volume ready for the estimation of Cu.

Analytical method. Both Cu and Zn were estimated colorimetrically by means of diphenylthiocarbazone (dithizone) solution in CCl_4. From an acid solution only the Cu complex is extracted; if this is first removed, Zn can then be estimated by buffering to pH 4·75, and again extracting with dithizone. Extinction coefficients of the CCl_4 solutions were measured by means of a Beckman photoelectric spectrophotometer.

The method given by Sandell (1944) was used for Cu, and a modification of the method of Vallee & Gibson (1948) for Zn.

The absorption curve of dithizone in CCl_4 solution (Fig. 1) has a minimum about 510 mμ., and at this wavelength the curve for copper dithizonate (Fig. 2) is near its maximum. Thus, extraction with a known excess of dithizone, and measurements of the extinction coefficient at 510 mμ., will indicate the amount of Cu present. This method is not practicable for Zn because a large excess of dithizone is needed for the Zn to be extracted quantitatively. The extinction coefficient of pure zinc dithizonate at 620 mμ. is very low indeed (Fig. 3) and thus, in a solution containing both dithizone and zinc dithizonate, measurement of the E value at 620 mμ. gives a measure of the excess dithizone present. Zinc dithizonate has a maximum at 535 mμ. (Fig. 3) and thus, if the extinction at this wavelength is measured, and the contribution made by excess dithizone to the total absorption at 535 mμ. is calculated and subtracted, the Zn can be estimated. (The relative E values for pure dithizone at 620 and 535 mμ. are known (Fig. 1), so that measurement at 620 mμ. allows the E value at 535 mμ. to be computed).

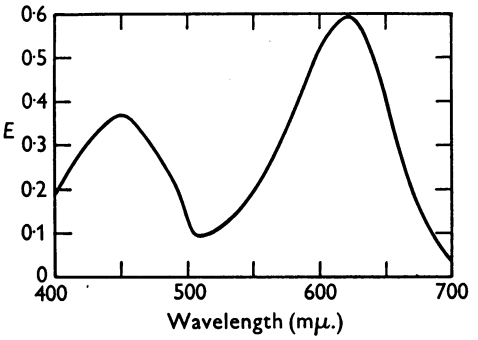

Fig. 1. Absorption spectrum of diphenylthiocarbazone in CCl_4. $E^{1\%}_{1\,cm.}$ at $\lambda_{620\,m\mu.} = 540$.

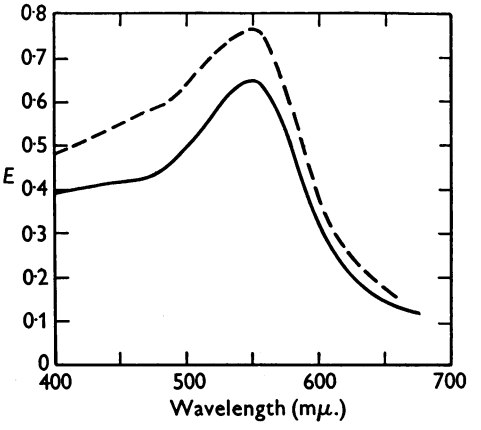

Fig. 2. Absorption spectra of copper dithizonate solutions in CCl_4. – – – –, ash from eye tissues dissolved in 0·1 N-H_2SO_4. Excess of this solution extracted with dithizone in CCl_4. ——, excess $CuSO_4$ in 0·1 N-H_2SO_4 extracted with dithizone in CCl_4 (about 0·1 μg. Cu/ml.).

Reagents and apparatus. Twice-distilled water (the final distillation being from, and into, Pyrex glass vessels) was used to make up all solutions, and for the final washing of the vessels. All glass vessels used were of Pyrex and, after washing, were dried in an oven at 110° and stored away from dust.

All reagents used were of standard A.R. grade except diphenylthiocarbazone (British Drug Houses Ltd.) which bore no special label:

(*a*) 0·1 N-H_2SO_4: 2·7 ml. A.R. H_2SO_4 made up to 1 l.

(*b*) Sodium thiosulphate solution: 25 g. $Na_2S_2O_3.5H_2O$ in 100 ml. water.

(*c*) Buffer (pH 4·75): 2 N-acetic acid (500 ml.) and 2 N-sodium acetate (500 ml.) were mixed together. Even when reagents of A.R. quality were used, this solution gave a substantial 'blank' reading in the Zn estimation. This was eliminated by shaking 1 l. of the buffer solution, for 5–10 min. each time, with successive portions of 0·01 % dithizone solution, and discarding the lower (CCl_4) layers until the colour of the dithizone remained unchanged.

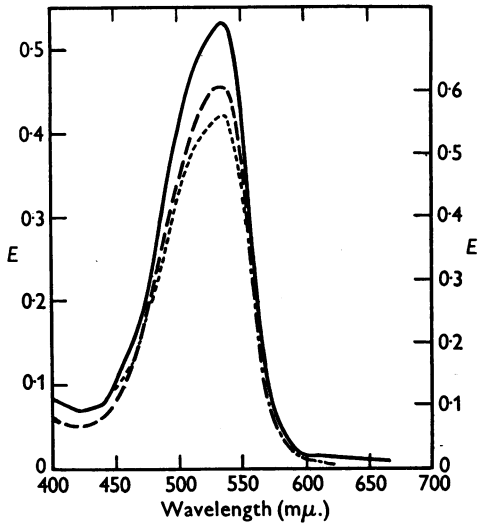

Fig. 3. Absorption spectra of zinc dithizonate solutions in CCl_4. ——, absorption spectrum of zinc dithizonate in CCl_4 (about 0·35 μg. Zn/ml.) — — —, ash solution from choroid (pH 4·75) extracted with dithizone in CCl_4. Absorption curve determined and corrected for excess dithizone (peak at 620 mμ.) - - - -, ash from iris treated similarly. The curve for pure zinc dithizonate corresponds with the ordinates on the left, the other curves with those on the right.

(*d*) Copper solutions: $CuSO_4.5H_2O$ (0·1964 g.) was dissolved in 0·1 N-H_2SO_4 (1 l.). From this a solution containing 1 μg. Cu/ml. was prepared by dilution with 0·1 N-H_2SO_4.

(*e*) Zinc solutions: $ZnSO_4.7H_2O$ (0·2198 g.) was dissolved in 0·1 N-H_2SO_4 (1 l.). From this a solution containing 1 μg. Zn/ml. of 0·1 N-H_2SO_4 was prepared by dilution.

(*f*) Dithizone solutions (0·01 and 0·003 % w/v) were made up in A.R. CCl_4 and filtered. These solutions deteriorate slowly as a result of oxidation. Fresh solutions were made up every 3 months and stored in the dark at 0°.

Calibration. (i) *Copper:* solutions containing 0–10 μg. of metal in 10 ml. 0·1 N-H_2SO_4 were extracted with 0·003 % dithizone solution (6 ml.) in separating funnels. The lower layer was run off into a 10 ml. standard flask and the remaining drops washed through with CCl_4. Readings of the extinction at a wavelength of 510 mμ. were made on the spectrophotometer. A straight-line graph was produced by plotting the extinction against amount of Cu to be extracted. A similar graph was produced for a 0–25 μg. range by using proportionately greater quantities of reagents and final dilution of the CCl_4 extract.

(ii) *Zinc:* solutions containing 0–10 μg. of metal in 10 ml. 0·1 N-H_2SO_4 were placed in separating funnels and 5 ml. buffer (pH 4·75) and 1 ml. sodium thiosulphate solution were added. Each mixture was then shaken with successive 1 ml. portions of 0·01 % dithizone solutions until the lower layer (CCl_4) remained green after shaking for 2 min. Each portion was run off into a 20 ml. standard flask and the last drops were washed through with CCl_4. The solution was made up to volume and readings of extinctions at 535 and 620 mμ. were made.

A straight-line graph was produced by plotting $E_{535\,m\mu.} - 0.25\,E_{620\,m\mu.}$ against the amount of Zn (0–10 μg.) to be extracted. A similar graph was obtained for the range 0–50 μg. Zn, after diluting the final extract to 50 ml.

Blank estimations were carried out with each set of Zn and Cu estimations as a precaution against casual contamination in the apparatus and standard solutions; these estimations also served as a check on the deterioration of the 0·003 % dithizone solution used to extract Cu. If the blank estimations were found to give a lower extinction than when the dithizone solution was fresh, the standard graph was recalibrated. The $E_{620\,m\mu.}/E_{535\,m\mu.}$ ratio of the 0·01 % dithizone solution was checked weekly, and the current value was used in calculating the absorption at 535 mμ. due to dithizone. The average value for the ratio was 4·0 and the greatest drop recorded in 3 months was from 4·2 to 3·8.

Specificity of the extraction procedures. Sandell (1944) states that several other metals are partially extracted, if present in certain relative concentrations, by the procedures used here for Cu and Zn. To determine whether such metals are present in amounts sufficient to cause significant interference in the present work, absorption curves for extracts from typical ash preparations of eye tissues were obtained by the above procedures.

In the case of Cu, all the dithizone shaken up with a solution of Cu salt in 0·1 N-H_2SO_4 is converted to copper dithizonate merely by the presence of a sufficient excess of Cu in the aqueous phase. Thus Fig. 2 compares the absorption curve of dithizone in CCl_4 shaken up with large excesses of $CuSO_4$ solution, and a solution of mixed eye-tissue ash, respectively.

In the case of Zn, excess dithizone is always present in the extract. However, if it is assumed that the absorption of a zinc dithizonate solution at 620 mμ. is zero (which is very nearly the case), then, if Zn is the only metal extracted, the absorption at 620 mμ. is solely due to dithizone. From the absorption curve for pure dithizone the relations between the $E_{620\,m\mu.}$ value and the E values of a dithizone solution at other wavelengths is known, and thus the amount of light absorption at any wavelength due to dithizone in a mixed solution of zinc dithizonate and dithizone, can be found. By subtraction of the E values calculated to be due to dithizone, from the total E values at various wavelengths of such a mixture of dithizone and zinc dithizonate, the extinction values which should be due to zinc dithizonate, if the extraction procedure is specific, can be found. The absorption curve, plotted from E values obtained in this way for an extract of a typical eye-tissue ash, is given in Fig. 3. There is an almost exact correspondence between this calculated curve and a pure zinc dithizonate curve, obtained by decomposing the excess dithizone with Na_2S solution, in an

extract from a solution of $ZnSO_4$ under the standard conditions described above. Such a correspondence would hardly occur if another metal dithizonate were present, even though the original assumption, that the 620 mμ. absorption was wholly due to dithizone, was unjustified.

The correspondence between the Cu curves demonstrates that here, also, the extraction procedure is specific for Cu.

The curves, both for Zn and Cu, agree with those given by Fischer & Weyl (1935) and Fischer (1937) for the pure metal dithizonates.

Estimation procedure. The solution of ash in 0·1 N-H_2SO_4, obtained as described in the section on preparation of tissues for analysis, was made up to a standard volume with 0·1 N-H_2SO_4 (the exact volume depending on the weight and kind of tissue analysed) and a sample taken for Cu extraction. The sample was made up to approximately 10 ml. with 0·1 N-H_2SO_4 and shaken with 6 ml. of 0·003% dithizone solution in a separating funnel. If the CCl_4 layer was then red, a further 9 ml. of dithizone solution were added and the whole again shaken. The extract, either 6 or 15 ml. in volume, was then run off and made up to volume as described before. Comparison of the value of the extinction at 510 mμ. with one of the standard graphs then indicated the amount of Cu present.

A smaller sample was taken for Zn estimation. This was first made up to approximately 10 ml. with 0·1 N-H_2SO_4 in a separating funnel and extracted with excess dithizone solution to remove Cu. Buffer (pH 4·75) and sodium thiosulphate solutions were then added and Zn extracted as described in the section on calibration. The extract was washed through with CCl_4 and made up to a volume putting the extinction values within the effective range of the spectrophotometer. The amount of Zn present was obtained by referring to the standard graphs.

Accuracy of the method. The reproducibility of the results was tested by homogenizing a mixture of cattle irises, choroids and lenses in a Waring Blendor. Portions of the homogenate were dried, reduced to ash and the Zn and Cu estimated by the standard procedure given above. Dry weights of the homogenate portions were of the order of 0·5 g., which was a rough average figure for the dry weights of the whole tissues taken for analysis in the following work.

Results

The results are summarized in Tables 1–4. In Table 1, samples 1–6, inclusive, were ashed in silica crucibles, and samples 7 and 8 in platinum crucibles. When tissues containing little organic matter, such as the aqueous and vitreous humours, were ashed, the deviation from the mean for samples in silica crucibles was somewhat greater than that shown above, and the mean of the results for these samples was consistently less than that for samples ashed in platinum crucibles. This effect is presumably due to

Table 1. *Reproducibility of zinc and copper estimations on eight equal portions of a homogenate of cattle lenses, irises and choroids*

Sample	Zinc (μg./g. dry material)	Copper (μg./g. dry material)
1	168	87
2	161	90
3	159	85
4	160	81
5	154	86
6	148	80
7*	170	92
8*	160	85
Mean	**160**	**86**
Standard deviation	7·03	3·80

* Ashed in Pt crucibles; all others in silica crucibles.

Table 2. *Concentration of zinc in the eye tissues of some mammals*

(A dash indicates that no analysis was made. Numbers in brackets denote μg./g. wet tissue.)

Tissue	Cattle (μg./g. dry tissue)	Sheep (μg./g. dry tissue)	Sperm whale (μg./g. dry tissue)	Rabbit	
				Coloured (μg./g. dry tissue)	Albino
Iris plus ciliary body	246 (41·0)	436 (65·1)	99·5	127	54·4
Choroid plus pigment epithelium	139 (26·5)	277 (69·2)	37·2	466	86·2
Retina minus pigment epithelium	71·0 (7·2)	80·0 (7·3)	54·1	—	—
Lens	37·3 (15·0)	117 (47·2)	35·2	15·8	12·5
Aqueous humour	30·0 (0·29)	—	—	—	—
Vitreous humour	26·4 (0·35)	23·2 (0·29)	10·5	—	—
Sclera	14·6 (4·1)	56·0 (16·1)	0·33	—	—
Cornea	13·5 (2·3)	25·0 (3·6)	35·3	6·6	12·1
Optic nerve	6·8 (2·2)	—	—	—	—

Table 3. *Concentrations of copper in the eye tissues of some mammals*

(A dash indicates that no analysis was made. Numbers in brackets denote μg./g. wet tissue.)

Tissue	Cattle (μg./g. dry tissue)	Sheep (μg./g. dry tissue)	Sperm whale (μg./g. dry tissue)	Rabbit (μg./g. dry tissue)	
				Coloured	Albino
Iris plus ciliary body	27·5 (4·6)	50·1 (7·4)	5·9	11·6	14·7
Choroid plus pigment epithelium	9·8 (1·7)	13·5 (3·4)	2·2	16·8	21·0
Retina minus pigment epithelium	6·8 (0·67)	11·5 (1·3)	10·6	—	—
Lens	1·2 (0·46)	2·1 (0·8)	4·2	0·62	0·49
Aqueous humour	10·4 (0·10)	—	—	—	—
Vitreous humour	17·7 (0·24)	24·0 (0·3)	5·9	—	—
Sclera	4·8 (0·13)	5·1 (1·4)	0·074	—	—
Cornea	3·2 (0·57)	1·9 (0·26)	3·4	0·30	1·5
Optic nerve	5·6 (1·8)	7·9 (2·6)	—	—	—

the formation of stable silicates. (Only two platinum crucibles were available, so that silica crucibles were used for all analyses except where otherwise stated.)

Table 4. *Zinc and copper concentrations in the eye tissues of three individual sheep*

(Results are in μg./g. dry tissue.)

Animal	Lens		Iris		Sclera	
	Cu	Zn	Cu	Zn	Cu	Zn
1	2·8	124	40	458	4·6	41
2	1·7	129	41	401	6·6	47
3	2·1	92	69	450	4·0	67

The results obtained for Cu in Table 1 are not indicative of the physiological Cu concentrations of the tissues used, for it has been observed that any aqueous solution placed in the Waring Blendor accumulates Cu, presumably from the alloys of the stirring mechanism.

Discussion

Table 2 shows that the zinc concentrations in the various types of tissue in mammalian eyes differ to a considerable extent. The differences are, in most cases, very much greater than could be accounted for by the experimental error (Table 1). The differences between the concentrations of copper in the various tissues, shown by the results of Table 3, are not so great, but in most cases are larger than the latitude, indicated in Table 1, which could arise from the experimental procedure. Table 4 shows that the differences in zinc and copper concentrations between the eyes of three individual sheep were small compared with the differences between the various types of tissue of the same sheep. Variation between individuals of the same species was not studied further because the eyes were obtained from a large abattoir and it was not easy to ascertain the breed, or the history, of the animals.

That the differences between the various eye tissues go deeper than peculiarities either of individuals or species, is indicated by the fact that the tissues can be placed in an order (in respect of their zinc and copper concentrations) which is roughly the same in each of the species examined. This order is very similar to that obtained from the results of Leiner & Leiner (1944) on the zinc content of fish-eye tissues, though the absolute concentrations were often much higher than those in the mammalian species examined here.

The results given in Table 2 for zinc concentrations in cattle-eye tissues are very much higher than any of the values found by Tauber & Krause (1943) for bullocks. However, the specificity of their method of estimation is questionable. They extracted the dithizone complex from an aqueous solution, the pH of which was probably acid, and in any case was never rigorously controlled. The reaction of dithizone with zinc can be made specific only in a solution at about pH 4·75, and in the presence of sodium thiosulphate or some other suitable complex-forming reagent. In acid solution, without any added reagents, only copper is likely to be extracted. The zinc/copper ratio found by them is actually never much greater than 1, whereas the zinc content of most biological materials is several times as great as that of copper.

The distribution of zinc and copper between the various types of sheep-eye tissues coincides with that found by Shakir (1948), and the concentrations of zinc found in each case are very similar. The concentrations of copper shown in Table 3 tend to be somewhat lower than the results for corresponding tissues given by Tauber & Krause for cattle, and by Shakir for sheep; this difference may be due to individual variation of the animals and to variation in pasture food.

It is significant that, both in the present work on zinc and copper in mammalian eyes and in the work on zinc in fish eyes, the highest concentrations of the metals were found in the pigmented parts of the eye. It is relevant that the only species (of those examined here) in which the retina, or any other tissue, contains a higher concentration of either metal than the iris, or choroid plus pigment epithelium, is the whale, whose choroid is thick, spongy and low in pigment. In addition, the whale eyes had been preserved and, on dissection, it was found impossible to separate spots of adhering pigment epithelium from the almost liquid retina.

The results for coloured and albino rabbit eyes show without doubt that the high concentration of zinc in the iris and choroid tissues is dependent, at least partly, on the presence of melanin pigment. No large difference was observed between the copper concentrations in the two types of eye, but the total amount of copper estimated was so small in each case (0·8–2·0 μg.) that the error introduced by ashing makes the results probably not significant.

Flesch (1949) reported a connexion between the concentration of copper in rabbit hair, and pigmentation. He observed a small difference between the copper concentrations in white and black hair from the same rabbit, though white hair from one rabbit might contain more copper than the black hair from another. Fore (1950) found no difference in manganese contents of the two types of hair, but a big drop in ash weight in white hair. We have found a small lowering of zinc concentration in white hair, no difference in copper concentration, and again a big drop in ash weight. Thus, in the rabbit, it appears that though zinc and copper may be concerned in the pigment problem, some other mineral element or elements are present in enhanced quantity in pigmented hair.

2. FRACTIONATION OF PIGMENTED TISSUES

Flesch (1949) has shown that pigment separated from a mouse melanoma contains much more copper than the melanoma tissue itself. Leiner & Leiner (1944) showed that grey or black powders, separated by differential centrifugation from powdered dry fish eyes, contained rather more zinc than colourless fractions, though a yellow fraction contained more still. They did not postulate a connexion between zinc concentration and pigmentation.

In view of these facts and the evidence recorded in the first part of this paper, it was decided to determine whether zinc and copper are concentrated in the pigment fraction of the iris and choroid tissues of mammals.

Experimental methods and results

It was established by analysis that cattle irises and choroids from left and right eyes of the same animal differ in their zinc concentrations by less than 15% of the mean between the two, and in their copper concentrations by less than 20% of the mean.

A number of pairs of cattle eyes were obtained from the abattoir; one of each pair of irises dissected out was analysed whole, and the other of the pair fractionated to obtain pigmented material.

Fractionation

Whole irises (3–6) were digested with 5 ml. of an approximately 2% (w/v) trypsin suspension and about 25 ml. of twice-distilled water for 24–36 hr. at 37°. The melanin pigment can then almost all be washed away from the bulky residue of undigested fibrous, connective and muscular tissue, which is allowed to remain in the digestion flask.

Table 5. *Zinc and copper present in various fractions of cattle irises obtained by digestion with trypsin*

Fraction	Dry weight (g.)	Zinc (μg.)	Copper (μg.)
Exp. 1			
Undigested residue	0·0430	9·6	0·9
Combined washings	0·2679	24·6	8·0
Pigment fraction	0·1383	101·0	9·0
Total	0·4462	135·2	17·9
Trypsin blank	0·0508	23·6	1·9
Whole irises ashed	0·5050	99·0	9·8
Total	0·5558	122·6	11·7
Exp. 2			
Undigested residue	0·2175	22·8	2·4
Combined washings	0·1945	11·1	4·6
Pigment fraction	0·0997	74·5	8·0
Total	0·5127	108·4	15·0
Trypsin blank	0·0534	23·4	2·4
Whole irises ashed	0·4992	79·6	9·8
Total	0·5526	103·0	12·2
Exp. 3 (calves)			
Undigested residue	0·1766	9·0	4·0
Combined washings	0·2048	17·8	4·2
Pigment fraction	0·1207	62·8	5·3
Total	0·5021	89·6	13·5
Trypsin blank	0·0431	12·0	2·8
Whole irises ashed	0·4647	80·0	10·5
Total	0·5078	92·0	13·3

The washings were centrifuged until the supernatant liquid was yellow, and this solution was then poured off. The residue was stirred up with twice-distilled water and

recentrifuged, the operation being repeated five or six times, until no opacity due to protein was observable in the washings. The washings were all added to the original supernatant liquid.

The undigested residue, the washings, the pigment fraction and a blank containing 5 ml. of the trypsin suspension were all dried, ashed and analysed for Zn and Cu by the methods already described. The results are compared in Table 5 with the analysis for the 'control' irises, which had been dried and ashed whole. These results, plus the trypsin blank figures, should equal the total for the various fractions if no contamination or loss has occurred.

Pairs of whale eyes were not available, and digestion of the irises, possibly due to the presence of fat, only partially separated the pigment from the rest of the tissue. Thus it was not possible to construct a balance sheet showing the amounts of Zn and Cu to be found in the pigment fraction and in the other parts of the tissue. However, in the pigment which was separated, Zn and Cu were present in much greater concentration than in the original tissue.

It was likewise found impossible, even after 3 or 4 days digestion, to separate more than a small amount of the pigment from cattle choroids, in the manner used for irises. It was again found, however, that the pigment fraction which was isolated was much richer in Cu and Zn than the original tissue. In Table 6 are collected the results of analyses of dialysed pigment fractions from the various sources mentioned above.

Table 6. *Zinc and copper concentrations in various pigment samples, prepared from eye tissues by the trypsin digestion procedure followed by dialysis*

Source of pigment	Zinc (μg./g. dry material)	Copper (μg./g. dry material)
Adult cattle irises:		
Batch 1	1000	78
2	730	65
3	719	87
4	748	80
Calf irises	522	44
Humpback whale irises	968	56
Adult cattle choroids	737	48

Further examination of the pigment fraction

Renewed digestion. Part of the pigment fraction obtained from six cattle irises by the above procedure was analysed as such for Zn and Cu, and part was digested with 2% (w/v) trypsin suspension for a further 3 days at 37° before analysis. Results for the two analyses are shown in Table 7.

Table 7. *Zinc and copper concentrations in two pigment preparations before and after a second trypsin digestion*

Pigment preparation	Zinc (μg./g. dry material) Before	Zinc (μg./g. dry material) After	Copper (μg./g. dry material) Before	Copper (μg./g. dry material) After
1	871	1073	107	84·9
2	874	1028	113	82·1

Hydrolysis with acid. Part of a pigment fraction was analysed intact, and part weighed and then treated with 0·1 N-HCl for 1 hr. on a steam bath. The insoluble residue was centrifuged down, washed, dried, weighed and analysed. Results are shown in Table 8.

Table 8. *Effect of acid hydrolysis upon the zinc and copper concentrations and the dry weight of insoluble material, of an iris pigment fraction*

	Dry weight (g.)	Zinc content (μg.)	Copper content (μg.)
Original pigment material (*A*)	0·1225	100	9·1
Insoluble residue after hydrolysis of (*A*)	0·0741	0·8	8·1

Part of the insoluble residue was retained as a wet suspension. The absorption curves of this suspension in twice-distilled water and of a suspension of the original pigment fraction are given in Fig. 4.

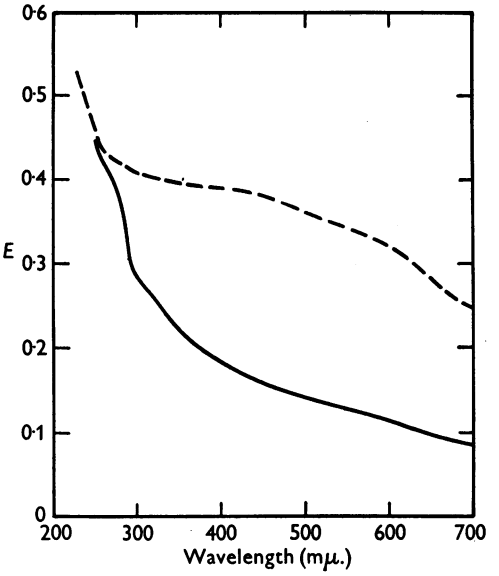

Fig. 4. Absorption spectra of suspensions of melanin material from cattle irises, before and after acid hydrolysis. ——, absorption spectrum of the original melanin material suspended in twice-distilled water (pH 5·0). — — —, absorption spectrum of a suspension of the insoluble residue after hydrolysing melanin material.

The amount and nature of the ash of the pigment fraction. The percentage of the dry weight of the pigment fraction recoverable as ash was determined for a sample repeatedly washed and centrifuged down from twice-distilled water, and on a sample dialysed for 3 days against twice-distilled water. Ashing was carried out in a platinum crucible and the respective percentages for the two samples were found to be 2·594 and 2·590.

It is clear that no more than a small part of the ash can be accounted for by Zn and Cu salts or oxides, for these make up only 0·077 and 0·0063%, respectively, of the dry weight of the pigment sample.

It was therefore decided to investigate the other metallic constituents of the ash by a qualitative emission spectroscopic technique.

The Hilger E 1·301 version of the Littrow emission spectrograph was used, and an arc source was used for excitation of the electrode and ash.

By means of the Hartmann diaphragm three spectrograms were taken without moving the photographic plate: (*a*) a control consisting of pure graphite rods; (*b*) the test material placed on a hollowed graphite cathode opposite a graphite anode and (*c*) a reference standard consisting of two pure iron electrodes.

Spectrograms were photographed for two wavelength ranges: 2250–2880 and 2880–5050 A.

The two spectrograms for these ranges, each comprising three spectra in exact juxtaposition, were examined on a Judd Lewis comparator. Lines in the test spectrogram not present in the control were identified by reference to the iron lines in the third spectrogram. The iron spectrogram was charted, and the unknown lines in the test spectrogram identified by reference to labelled photographs in Brode (1939) and the wavelength and element tables in the Massachusetts Institute of Technology *Wavelength Tables* (1939). Unequivocal 'raies ultimes' were identified for Ca, Ba, Mg, Fe, Cu and Zn. The number of iron lines in the test spectrogram was very large and it appears that this element was the major metallic constituent of the ash. These metals, together with Na, were identified in a similar manner in a batch of irises which had been repeatedly washed with twice-distilled water. This demonstrates that they have not been introduced into the material during the digestion or dialysis procedures.

Discussion

The results presented in Table 5 show, without further inquiry, that greater amounts of zinc and copper are associated with the pigment fraction of cattle irises than with any other fraction. However, on close inspection of the results, several matters are seen to require comment.

It was to be expected that the sum of amounts found in the iris fractions would be less than the amount in whole iris since losses due to decomposition of organic compounds are inevitable during digestion and in evaporation at 100° of the large quantities of water present in the washings. Differences between the dry weights and zinc and copper contents of the two irises from the eyes of the same animals are also bound to occur as a result of biological variation and imperfect separation in dissection. Taking these factors into account, there appears to be fairly good agreement between the results for corresponding groups of irises, and it may be concluded that little or no contamination or loss of zinc and copper took place.

The proportion of the total dry weight, zinc content, and copper content, found in each fraction varied somewhat from one experiment to another; this was probably due to variations in the extent of digestion of the tissue and to small variations in the separation technique.

In each experiment, the concentrations of zinc and copper in the dry pigment were much higher than in the other fractions of the original tissue. The accumulation of zinc was much greater than that of copper, and the concentration of zinc was increased by further trypsin digestion of the pigment fraction, whereas that of copper was decreased. Dialysed samples of pigment retained the high concentrations of both metals. Pigment fractions isolated from cattle choroids and from humpback whale irises contained concentrations of zinc and copper similar to those in cattle iris pigment fractions.

Table 5 shows that the high concentrations of zinc and copper in cattle irises can be ascribed to accumulation in the pigment fraction. Table 6 shows that similar accumulations occur in cattle choroids and whale irises. In conjunction with the results in the first part of this paper, it thus appears that the pigment material in mammalian eyes is closely associated with much more zinc and copper than is present in most mammalian tissue constituents.

That the pigment from cattle irises is of the melanin type, is indicated by the retention of colour in the portion of the pigment fraction which is insoluble and resistant to acid hydrolysis. Natural melanins have usually been isolated as the insoluble residues of prolonged acid or alkaline hydrolysis of pigmented tissues. The term 'melanin' is ill-defined; different workers have used different combinations of properties to characterize the material, and it is by no means certain that all the substances which have been given this name have the same chemical structure. The tests for melanin identity employed in the present work are quoted in the classification by Mason (1948).

Physical evidence for the identity of the pigment from cattle irises is given by the absorption curves (shown in Fig. 4) for suspensions of the original pigment fraction, and of the residue, after acid hydrolysis of this fraction. These are similar to those for melanins from other sources (Edwards & Duntley, 1939; Serra, 1945; Zwicky & Almasy, 1935). Shakir (1948) obtained similar curves for pigment in a supernatant suspension made by grinding choroids; this material had a higher concentration of zinc and copper than the whole tissue. A chemical test used by many workers (e.g. Sachs, 1944) for melanin is the bleaching of the colour by strong oxidizing agents. Concentrated nitric acid rapidly destroyed the colour of the material obtained by us.

A material analogous to the pigment fraction of cattle irises was obtained by Greenstein, Turner & Jenrette (1940), who showed that the black insoluble fraction from mouse melanomas hydrolysed with trypsin contained much protein. This protein

dissolved on acid hydrolysis, leaving a highly coloured residue having a nitrogen content which coincided with that of melanins synthesized from tyrosine.

It is considered that the evidence presented gives sufficient justification for placing the chromogenic material of the pigment fraction from cattle irises in the melanin category.

It has now been fairly well established that mouse-melanoma tissue contains a tyrosinase and that copper is an essential part of this enzyme (Lerner, Fitzpatrick, Summerson & Calkins, 1950). Flesch (1949) suggests that copper becomes bound to the pigment in the neighbourhood of the sulphur atoms of sulphur-containing amino-acids of the attached protein. The relatively large amount of copper in the pigment fraction certainly cannot be due to tyrosinase in the free state, for the enzyme is water-soluble, and would have been removed in the fractionation process.

The results given in Tables 4 and 5 show a difference between the states of zinc and of copper in the pigment fraction. The concentration of copper was slightly reduced by both trypsin digestion and acid hydrolysis. The concentration of zinc was slightly increased by trypsin digestion and reduced almost to zero by acid hydrolysis. This indicates a probable difference between the binding of the two metals in the pigment fraction.

The results of the emission spectroscopic analysis show that the accumulation of metals is not limited to zinc and copper. These two metals together constitute only 0·083 % of the dry weight and 3·21 % of the ash of the pigment fraction. The iron, calcium, magnesium and barium, which were the only other elements identified, together must therefore account for much more of the ash weight than do zinc and copper. The great number and strength of the iron lines in the spectrum indicate that this is a major constituent of the ash.

The demonstration of the presence of barium confirms the report of Ramage & Sheldon (1931) that this metal occurs in irises and choroids of cattle, and in the pigment separated from these tissues by rubbing. They did not find the metal in any other cattle tissues, nor in the choroids of a number of other animals. Waelsch (1932) found that melanin from the choroid gave an ash which was 1·9 % by weight of the dry material; this ash contained a demonstrable concentration of iron.

It thus appears that a number of metals are accumulated in the pigment fraction of mammalian eyes in unusually high concentration. This circumstance may perhaps be connected with a number of other observations. As mentioned in the first part of this paper, white rabbit hair contains much less ash material than black. Gortner (1911) showed that black pigment from rabbit hair, horse hair and from black feathers contained 2–3 % ash, which was chiefly iron oxide. Rothman & Flesch (1943) isolated a red pigment, containing iron in the ferric form, from bright-red human hair. They stated, however, that such a pigment had not been obtained from any human hair except red, nor from the hair of any other animals. Giuliani (1938) found that dried ink from the squid (*Sepia officinalis*) gave a total ash of 1·86 % of the dry weight, and a copper analysis of 1·17 % of the dry weight.

There exists no direct evidence yet which might settle the problem of whether the high concentrations of these metals in pigmented tissues, and in pigment fractions of these tissues, have any function in developing or maintaining the natural coloration.

SUMMARY

1. Details of modified standard microprocedures for estimating copper and zinc are presented. The methods, as applied to eye tissue, are shown to be specific for these metals.

2. Results are presented for copper and zinc concentrations in eye tissues of sheep, cattle, whales, and rabbits. It is shown that differences in the concentrations of zinc and copper between the various tissues exceed the experimental error.

3. Ranged in respect of zinc and copper concentrations, the tissues fall in roughly the same order in each of the species examined. The highest concentrations, in general, are in the pigmented tissues.

4. The zinc content of the iris or choroid of albino-rabbit eyes is lower than that of the same tissues of pigmented rabbits. There is no corresponding difference in zinc content for the lens or cornea.

5. The zinc and copper of irises occurs mainly in pigment material found in the supernatant liquor from a trypsin digest of the tissue. The concentrations of zinc and copper are higher in the pigment material than the original tissue, and are not reduced by dialysis.

6. The zinc concentration in the insoluble pigment material is slightly increased by prolonged trypsin digestion, but reduced almost to zero by acid hydrolysis. The copper concentration is slightly reduced by trypsin digestion and little affected by acid hydrolysis.

7. The ash of dialysed pigment fractions contains calcium, magnesium, barium and iron, in addition to zinc and copper. Iron appears to be the major constituent.

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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