THE DISTRIBUTION OF COPPER AND ZINC IN VERTEBRATE EYES, AND THE ASSOCIATION OF THESE AND OTHER METALS WITH MELANIN PIGMENTATION

--

A THESIS

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By

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FORSWORD

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Chapter I

INTRODUCTION.

The chemical elements of the animal body can be arranged in the following classes:-

1. Organic: C, H, O, N, P, S. The classical biological elements which exist in numerous compounds containing carbon, and whose functions are diverse. These elements together account for 99 per cent. of the body weight if we include the hydrogen and oxygen present as water.

2. Inorganic elements present in macro quantities, e.g. Na, K, Ca, Cl.

3. Trace or minor elements. These are inorganic and present in micro quantities.

   a. Micro-nutrient elements. Those trace elements which have been shown by nutritional or other studies to have physiological function in the animal body.

b. Non-functional trace elements. Those not falling into category a.

This classification is merely a rationalisation of the terminology which is at present employed, and is therefore not entirely logical or watertight; it will be used in the present work because it does not conflict with any of the current conceptions in the literature.

The distinction between groups 2 and 3 is somewhat arbitrary; Green (1941) defines a trace substance as being one which occurs in amounts less than five parts per million dry weight of biological matter; Barcroft (1944) places the demarcation at 50 parts per million wet weight, which is just about the value for the average iron and zinc concentrations in the animal body. The division between the two groups is at present strengthened by an apparent difference in function. Group 2 are mainly concerned with ion balance or structural functions, whilst the trace elements have so far only been found to function (if at all) in connection with enzymes. The distinction becomes less clear, however, when it is considered that inorganic elements present in macro quantities, such as chlorine, magnesium, and calcium, can also function in connection with enzyme systems. In addition, strontium and silicon, which in most

species are trace elements, in Acantharia and Sponges respectively are present in larger quantity, and function as structural elements. There is also an interchange of elements between groups 3,a and 3,b when we consider the whole animal kingdom. An element may be functional in one group of species and not in another.

Vinogradov (1935) has compiled a list of some 60 elements which occur in living matter of one kind or another. Only a small fraction of this total have so far been shown to be physiologically necessary. Though it is possible that many more will be shown in the future to be of importance, it seems likely that there will always remain a number of trace elements to which no function can be assigned.

As mentioned previously, all the specific functions of trace elements which have so far been discovered are connected with enzyme action. Several trace elements have been shown by nutritional studies to be necessary for animal health, but specific functions in connection with enzymes have not been definitely assigned to all of these. Manganese and Magnesium are essential for an animal diet, but their position in connection with enzyme systems is still equivocal.

For the elements which have been assigned a function, deficiency symptoms evident from nutritional studies

are difficult to evaluate, and do not show conclusively whether the known function or functions of an element are the only ones. In fact, the complexity of the deficiency syndrome would seem to indicate that this is not so.

Thus, distribution studies, first on whole tissues, and then on parts of particular tissues rich in the element under consideration, are important as a possible means of shedding further light on the functions of a trace element. Even should the studies fail to bring out specific functions for an element, it is of use to discover whether the concentration of an element in a particular tissue is characteristic of that tissue and different from that in another tissue, for such differences must mean that certain physicochemical or biochemical agencies which are responsible for the accumulation of the element differ from one tissue to another.

Harless (1847) was the first to detect copper in biological matter; he found that it occurred in octopus blood.

Lechartier & Bellamy (1877) first showed the presence of zinc in plants and animals.

Since these years, the two elements have been detected and estimated in very many biological situations,

and their universal occurrence in living matter, first proposed by Gabriel Bertrand soon after the start of the 20th century, is now more or less accepted as fact. In addition, both elements have been shown by nutritional studies to be essential for the maintenance of the health of various animals.

Waddell, Steenbock, Elvehjem & Hart (1928) were the first to show that copper is an essential constituent of animal diets. Stirn, Elvehjem & Hart (1935) were the first to show conclusively that zinc was essential, though several other workers had previously obtained indications that it was so.

Several specific physiological functions of copper have been established. The first to be discovered was evident from the original nutritional studies which showed the necessity for a certain amount of copper in the diet. It was observed that copper, as well as iron, is necessary for the cure of nutritional anaemia in rats. Though it appears that copper is necessary for the formation of haemoglobin, the exact nature of the chemical processes in which the metal is involved are unknown. Redfield, Coolidge & Shotts (1928) showed that the blue compound haemocyanin, to which is combined most of the copper in the blood of marine gastropods, functions

as a respiratory pigment in these animals. It does not occur in vertebrates, however.

Kubowitz (1937) showed that the polyphenol oxidase of the potato contains copper. Several other plant oxidases appear to be copper compounds, but the only enzymes of this class which have so far been shown to occur in animals are tyrosinase and dopa-oxydase. Lerner, Fitzpatrick, Calkins & Summerson (1950) have recently shown that the activity of both these enzymes is dependent on the presence of copper. They believed that both the enzymes were in fact a single copper-containing protein.

The only other organic copper compounds known to occur in vertebrates are haemocuprein and hepatocuprein (Keilin & Mann, 1938). No function was assigned to these materials.

It has never been shown how much of the copper in an animal or an individual tissue is associated with the known functions or compounds of the metal.

Mendel & Bradley (1905) showed that the respiratory protein of the blood of the snail contained zinc, but in vertebrates the only organic compound of zinc which has been shown to occur is carbonic anhydrase (Keilin & Mann, 1939, 1940).

Zinc ions have been reported to activate the enzymes dehydropeptidase (Yudkin & Fruton, 1947) and glycine - L - leucine dipeptidase of animal tissues (Smith, 1949). It is not known how widespread these enzymes are, or whether zinc is the natural activating metal in animal tissues. In the latter case it almost certainly is not.

Zinc has been reported to be a constituent of the enzymes uricase (Holmberg, 1939) and kidney phosphatase (Massart & Vandendrissche, 1940). In neither case did the authors consider that sufficient evidence existed for the belief that zinc was a normal constituent of these enzymes.

As carbonic anhydrase remains the only known organic compound of zinc in vertebrates, it is useful to study the relation between the zinc content and carbonic anhydrase activity of animal tissues. Vallee & Altschule (1949) showed that a good statistical correlation existed between the variations in zinc content and in carbonic anhydrase activity of the red blood corpuscles of man in various pathological conditions. This indicates that all the zinc in the corpuscles is concerned with carbonic anhydrase activity. Such, however, does not seem to be the general rule for all animal cells. In general, the

amount of zinc in animal tissues is much greater than could reasonably be expected to be associated with carbonic anhydrase on the basis of the 0.3 per cent. of zinc which Keilin & Mann (1940) found in the most active of their carbonic anhydrase preparations. Leiner & Leiner (1941, 1943) examined the zinc and carbonic anhydrase contents of a number of fish tissues and found that there was no correlation between the two. Scott & Mendive (1941) also found no correlation between zinc and carbonic anhydrase activity in animal tissues.

It is therefore apparent that though we know of several specific functions of copper and zinc, it is not possible at present to say whether all the copper and zinc in a given animal tissue is concerned with these functions, and whether other functions exist.

There is therefore a clear case for carrying out further work on the distribution of copper and zinc in animal tissues, and fractionations of those tissues found to be rich in these elements.

The present work is concerned almost entirely with vertebrate eyes, whose individual tissues are markedly discrete, and have sharply contrasted functions towards the whole organ.

For the detection and estimation of copper and zinc in foodstuffs and the larger organs of the animal body the classical gravimetric or colorimetric reactions are sufficiently sensitive. For the investigation of their function and distribution in the less abundant tissues of the body, reliable micro-analytical methods are necessary. Such methods have only recently become available and some of the older work in the field is therefore unreliable.

Burdon-Cooper (1928) and Burdon Cooper & Lewis (1929) gave 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 iron, zinc and manganese, which Tauber & Krause (1943) were later able to estimate in cattle eyes by other methods.

Tauber & Krause's (1943) work on cattle eyes contains the only published results for the distribution of copper and zinc in mammalian eye tissues. Their method of estimating copper (Elvehjem & Lindow, 1929) is a standard and satisfactory one, but the method of estimating zinc, as given in their paper, is quite inadequate. They used diphenylthiocarbazone as a reagent for zinc, but they carried out the reaction in a solution whose pH was never controlled and was probably

very acid. The reaction is only specific for zinc in a solution at pH 4.75 and in the presence of $Na_2S_2O_3$ or other complex-forming reagent. In acid solution, without added reagents the reaction with copper is more likely to occur. The Zn/Cu ratio found by them is never much greater than 1, whereas the zinc content of most biological materials is several times as great as that of copper. A re-investigation of the problem therefore seems necessary.

Leiner & Leiner (1942, 1944) give the results of zinc estimations on fish eye tissues. Their method of estimation appears to be satisfactory and sufficiently standardised. Their results for the zinc content of choroids and pigmented tissues of some fresh-water fish are so remarkably high in comparison with the zinc content of most biological material, that they deserve independent confirmation.

Shakir (1948) began an investigation of the whole problem of trace elements in eye tissues.

He carried out semi-quantitative emission spectroscopic estimations of trace elements in dialysed and undialysed fish eye tissues. The presence of comparatively large quantities of dialysable inorganic material results in a cloud of vapourised salts round the electrode, 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 of dialysed and undialysed preparations. A five-fold difference is certainly demonstrable but it is difficult to be sure of small differences. In spite of such limitations it seemed clear that tissues from fish eyes contained non-dialysable zinc, and possibly copper, in excess of the retained amounts of other trace metals.

Shakir also carried out estimations of the zinc and copper content and carbonic anhydrase activity of all the sheep eye tissues, and of the zinc and copper content of retinae and choroids of cattle and whales. He found no correlation between the zinc content and carbonic anhydrase activity of the tissues. Leiner & Leiner (1941, 1943) also estimated the zinc content and carbonic anhydrase activity of some fish eye tissues, and found no correlation between the two. It therefore seems established that all the zinc in eye tissues is not associated with carbonic anhydrase. The present work was initiated with the intention of confirming and extending the other results obtained by Shakir.

In the course of the distribution studies, described in the first part of the thesis, it became evident that zinc, and to a lesser extent copper, were associated with the melanin pigment of vertebrate eyes. The second part of the thesis contains the results of a study of the association between various metals and the ocular pigment material.

The thesis is divided into chapters in such a manner that the material and argument of each chapter as nearly as possible follows on from, and is a development of, the previous chapters.

ORIGINAL WORK

Part I

Chapter II

THE TECHNIQUE OF ZINC AND COPPER ESTIMATION

Section 1. Introduction

Vallee & Gibson (1948) state that the only two reliable micro-methods for estimating zinc in biological material are polarography, and the reaction with diphenylthiocarbazone. The latter employs a spectrophotometric technique, apparatus for which was readily available, and it was therefore chosen as the most convenient method.

There are several semi-micro and micro-methods available for the estimation of copper. Tauber & Krause (1943) used the Elvehjem & Lindow (1927) method. This has been shown to be specific in cases where the iron content is not more than ten times that of copper. Tauber & Krause obtained a good recovery of copper added to lens tissue (but they give no figures)

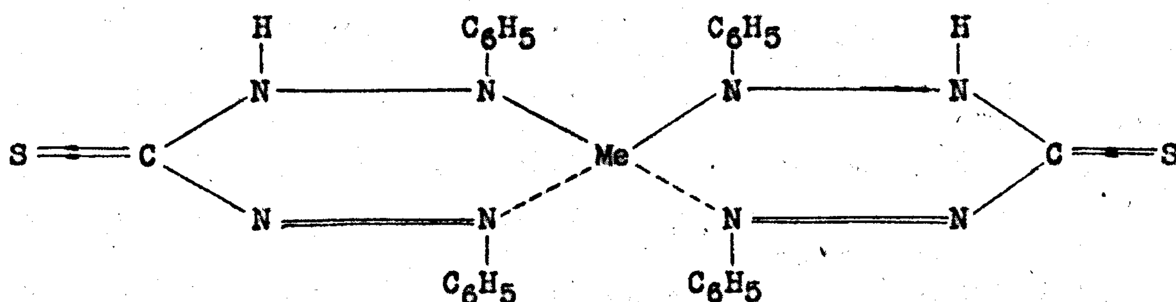
The minimum weight of copper which can be estimated by this procedure is 20 ugs. and it is therefore unsatisfactory for work with small quantities of material.

The estimation of copper by diphenylthiocarbazone is quoted by Sandell (1944) as being one of the most sensitive methods. By adopting this procedure, and removing copper before proceeding to estimate zinc, it is possible to use the same solution of biological ash for the determination of both metals. It was therefore decided that this method was most convenient for estimating copper.

Diphenylthiocarbazone ($C_6H_5.N = N.CH.S.NH.NH.C_6H_5$) has a long history as a reagent for the estimation of metals. It was discovered by Emil Fischer in 1878, who found that many of its metallic salts were highly coloured. Helmut Fischer in 1934 first used dithizonates for the estimation of various metals, and due mainly to his work it has become one of the most valuable organic reagents.

Though dithizone will react with many metals, a specific extraction of several of these can be achieved by performing the reaction at a controlled pH and in the presence of other reagents which form complexes with the unwanted metals.

Copper dithizonates can exist in either keto or enol forms. However, a solution of a cupric salt in dilute (0.1 N) acid solution forms only the keto complex. Zinc forms a keto complex on every occasion. The formula of both complexes can be represented as:-



where 'Me' represents the metal atom.

Dithizone and the metal dithizonates are insoluble in aqueous solutions, but readily soluble in many organic solvents. In the present case, where two metals are being estimated, it is an advantage to use an immiscible solvent. The first metal, copper, to be extracted is then taken up into a non-aqueous phase, and can be easily separated in this way. The two solvents usually used for solutions of dithizone are chloroform and carbon tetrachloride. Analytical reagent carbon tetrachloride can be used without further purification, and this solvent was therefore adopted in the present work.

A 0.1 N acid solution decomposes all the metal dithizonates except those of palladium, silver, mercury, gold, bismuth and copper. None of the first five occur in biological tissues in significant amounts. Extraction of a solution of the ash of biological material in 0.1 N acid with a solution of dithizone in carbon tetrachloride should therefore produce only copper dithizonate. That none of the first five metals occurs in significant amounts in eye tissues is demonstrated in Section 2 of this chapter.

If the pH of the solution is adjusted to pH 4.75, and sodium thiosulphate added, the only metals which react with dithizone are palladium, stannic tin, cadmium and zinc. The first three are not normal biological elements, and it is demonstrated in Section 2 that none of them occur in detectable amounts in eye tissue ashes.

Relatively large amounts of ferric iron oxidise dithizone to a brown substance whose absorption spectrum is quite different from the original material. The presence of any quantity of this brown material therefore upsets any colorimetric estimations involving standard dithizone solutions. The amounts of iron in eye tissues were not considered sufficient to warrant the paying of particular attention to this matter. Sandell (1944) considers the effect unimportant in connection with biological ashes generally.

Shakir (1948) used diphenylthiocarbazone for the estimation of both zinc and copper. His method was based on that of Fischer & Leopoldi (1934). For both metals this involves treatment of the metal dithizone complex, dissolved in carbon tetrachloride, with excess sodium sulphide solution, which decomposes the excess dithizone reagent. It is an axiom of quantitative analysis that the fewer the chemical processes involved in the estimation, the better the method. By purely photometric and spectrophotometric methods it is possible to estimate the excess of dithizone reagent in the mixed solution of the metal complex and unchanged dithizone reagent. The treatment with sodium sulphide, which has been criticised in the case of copper by Sandell (1944), and in the case of zinc by Vallee & Altschule (1949), on the grounds that too much sodium sulphide decomposes the metal-dithozone complex, is then eliminated.

The steps involved in the estimations are:-

a. Reduction of the tissue to ash.

b. Solution of the ash.

c. Extraction of copper, and then zinc, by means of dithizone in carbon tetrachloride.

d. Spectrophotometric determination of the amounts of copper-dithizonate and zinc dithizonate in the CCl_4 solutions.

In the present work the processes involved in (a) were taken from the literature (apart from the distilled water treatment for breaking up carbon residues). The processes involved in (b) were mainly constructed on the example of Dr.H.Fore's manganese determinations. In (c) a modification of the method of Sandell (1944) was used for copper, and a modification of that of Vallee & Gibson (1948) for zinc. Vallee & Gibson used potassium cyanide and sodium potassium tartrate as additional complex-forming reagents. They were found to be unnecessary in the present work.

In (d) it was decided to use the Beckmann & Unicam photoelectric spectrophotometers. These instruments can measure the absorption by a solution of light radiation of selected wavelength bands of less than 1 millimicron in width. Their use involved preliminary work in determining the strengths of the various solutions which would give optimal readings on the instruments.

At each stage it was necessary to eliminate as far as possible the risk of casual contamination from reagents, solvents, vessels, and dust.

The following is a description of the final procedure adopted, a proof of the specificity, and an estimation of the accuracy of the method.

Section 2. Experimental Procedure.

Reduction of the tissue to ash.

The tissues were dried to constant weight in a stainless steel 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 will froth over.

Solution of ash.

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 minutes. The contents were then washed into the beaker used to support the crucible in the extraction procedure, and the solution

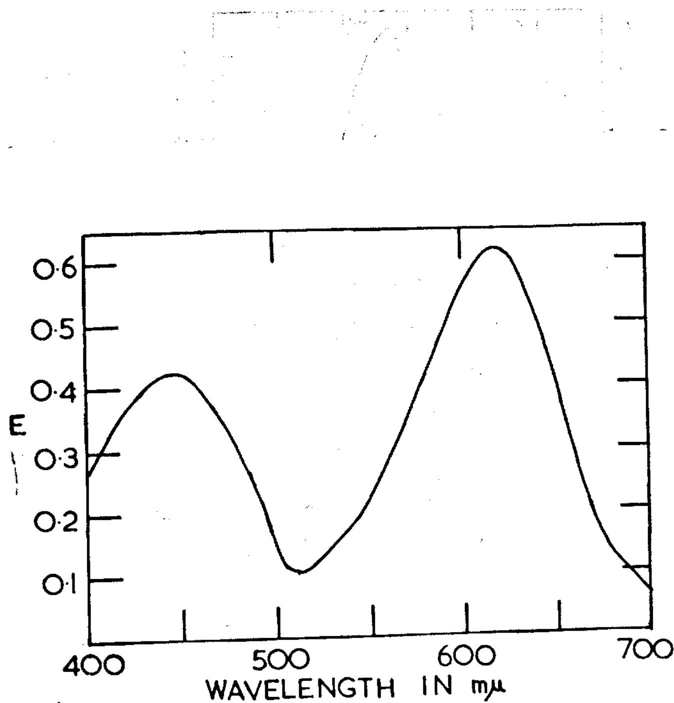
made up to a standard volume ready for the estimation of copper.

Spectrophotometric Determination.

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

Figure 1

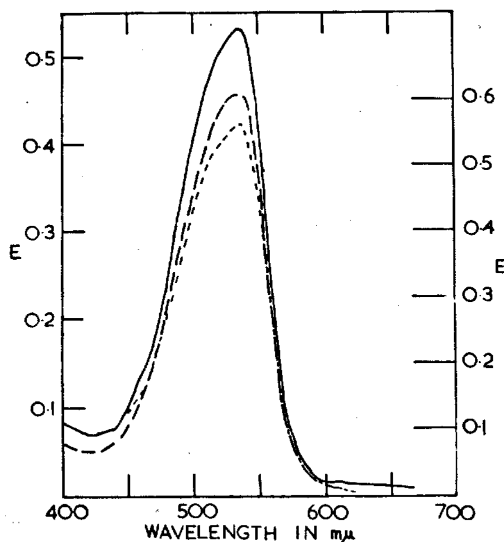
Absorption spectrum of diphenylthiocarbazone in carbon tetrachloride.



$E^{1\%}_{1cm.}$ at 620 mμ = 447

Figure 2

Absorption spectra of zinc dithizonate solutions in carbon tetrachloride.



——————Absorption spectrum of zinc dithizonate in carbon tetrachloride (0.5 ug. Zn/ml.)

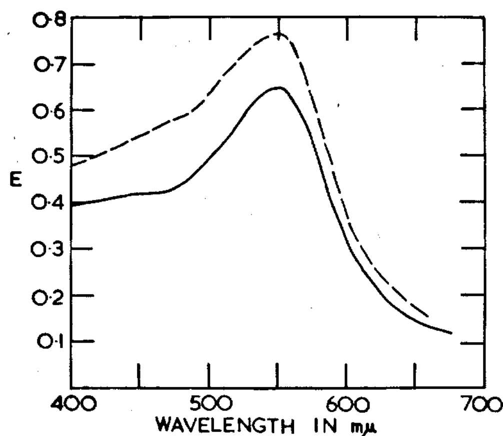
— — — — —Ash solution from choroid (pH 4.75) extracted with dithizone in CCl_4. Absorption curve determined, and corrected for excess dithizone (peak at 620 mu.)

---------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.

Figure 3

Absorption spectra of copper dithizonate solutions in carbon tetrachloride.



- - - - - - 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.

Extraction Procedure.

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 (B.D.H.) 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 gms. $Na_2S_2O_3 5H_2O$ 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 zinc 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.

(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 ug. (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 ug. Zn/ml. of 0.1 N H_2SO_4 was prepared by dilution.

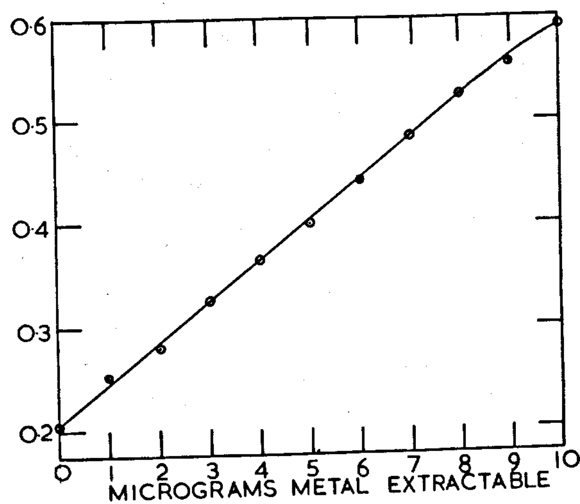
(f). Dithizone solutions (0.01% and 0.003% w./v.) were made up in A.R. carbon tetrachloride. These solutions deteriorate slowly as a result of oxidation. Fresh solutions were made up every 3 months and stored in the dark at 0°.

Calibration.

(i). Copper: Solutions containing 0-10 ug. (inclusive) of metal in 10 mls. 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 carbon tetrachloride. Readings of the extinction at a wavelength of 510 mu. were made on the spectrophotometer. A straight line graph (e.g. Fig. 4) was produced by plotting the extinction against amount of copper to be extracted. A similar graph was produced for a 0-25 ug. range by using proportionately greater quantities of reagents and final dilution of the CCl_4 extract.

Figure 4

A typical standard graph for the extraction of copper from 0.1 N H_2SO_4 solution by dithizone in carbon tetrachloride (6 mls. 0.003% dithizone; final volume of the extract 10 mls.).



(ii). Zinc: Solutions containing 0-10 ug. (inclusive) 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 carbon tetrachloride. The solution was made up to a definite volume and readings of extinctions at 535 and 620 mu. were made.

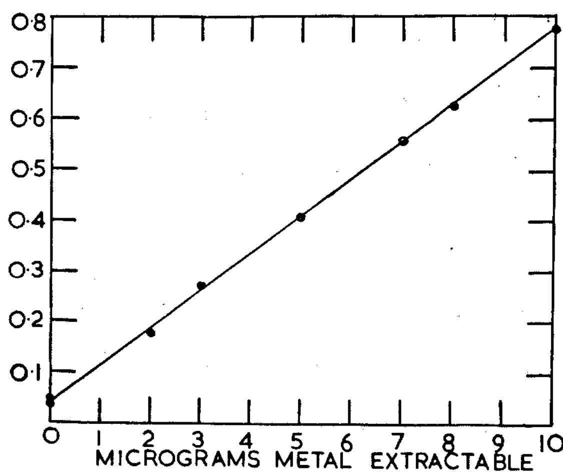
A straight line graph (e.g. Fig. 5) was produced by plotting $E_{535\ mu.} - 0.25\ E_{620\ mu.}$ against the amount of zinc to be extracted.

A similar graph was obtained for the range 0-50 ug. zinc, after diluting the final extract to 50 ml.

Blank estimations were carried out with each set of zinc and copper 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 copper. If the blank estimations were found to give a lower extinction than when the dithizone solution was fresh, the standard

Figure 5

A typical standard graph for the extraction of zinc from an aqueous solution (pH 4.75) containing sodium thiosulphate, by dithizone in carbon tetrachloride (final volume of extract 20 mls.).



graph was recalibrated. The $E_{620\ mu.}/E_{535\ mu.}$ ratio of the 0.01% dithizone solution was checked weekly, and the new value used in calculating the absorption at 535 mu. 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.

Accuracy of Extraction Procedure from Solution.

This was tested as follows.

A solution containing approximately 0.5 ugms. of Copper and Zinc per ml. in N/10 H_2SO_4 was prepared.

Five estimations of Zinc and Copper in 10 mls. solution were carried out. The results are tabulated below.

Table 1.

	Copper / 10 mls. soln.	Zinc / 10 mls. soln.
	4.82	5.21
	4.76	5.35
	5.10	5.20
	5.15	5.33
	5.05	5.08
Mean	4.98	5.23
Max. Deviation	0.22	0.15
Thus Error	± 4.4%	± 2.9%

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 copper and zinc. 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 copper, all the dithizone shaken up with a solution of copper salt in 0.1 N H_2SO_4 is converted to copper dithizonate merely by the presence of a sufficient excess of copper in the aqueous phase. Thus Fig. 3 compares the absorption curve of dithizone in CCl_4 shaken up with large excesses of copper sulphate solution and a solution of mixed eye tissue ash respectively.

In the case of zinc, excess dithizone is always present in the extract. However, if it is assumed that the abrosption of a zinc dithizonate solution at 620 mu. is zero, (which is very nearly the case) then, if zinc is the only metal extracted, the absorption at 620 mu. is solely due to dithizone. From the absorption curve for pure

dithizone the relations between the $E_{620\ mu.}$ 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. 2. There is an almost exact correspondence between this calculated curve and a pure zinc dithizone curve, obtained by decomposing the excess dithizone in an extract from a solution of zinc sulphate 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 absorption was wholly due to dithizone, was unjustified.

The correspondence between the copper curves in Fig. 2 demonstrates that here also the extraction procedure is specific for copper.

The curves, both for zinc and copper appear to 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 an aliquot taken for copper extraction. The aliquot 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 colour of the carbon tetrachloride 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 mu. with one of the standard graphs then indicated the amount of copper present.

A smaller aliquot was taken for zinc 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 copper. Buffer (pH 4.75)

and sodium thiosulphate solutions were then added and zinc 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 zinc present was obtained by referring to the standard graphs.

Section 3. - Accuracy of the Method.

The reproducibility of the results was tested by homogenizing a number of cattle irises, choroids and lenses in a Waring Blender. Portions of the homogenate were dried, reduced to ash and the zinc and copper 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.

Table 2.

Results of zinc and copper estimations on eight equal portions of a homogenate of cattle lenses, irises and choroids.

Sample	Zinc (ug./g. dry material)	Copper (ug./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

In Table 2, 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 for samples ashed in platinum crucibles. This effect is presumably

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

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

Section 4. - Recovery Experiment.

This was designed to test whether any constant proportion of the zinc and copper of the ashed tissues is lost during the procedure. The accuracy of the determinations has already been assessed; this experiment showed that if there is a loss of these metals in the estimation procedure, it must be a fairly constant one.

Dried cattle lenses were ground to a fine powder in a pestle and mortar. Four approximately equal portions of the powder were placed in four weighed silica crucibles, and the crucibles again weighed. To each of two crucibles was added 50 micrograms of copper and 50 ugms. of zinc in the form of their sulphates dissolved in 10 mls. of twice

distilled water. This solution was prepared by dilution from one containing .05% Zn. and .05% Cu. in the form of sulphates. To the remaining two crucibles was added 10 mls. twice distilled water. All four crucibles were dried, ashed, and their zinc and copper contents determined.

Results:

Table 2a. (Controls).

Dry weight in gs.	Cu content (ugs.)	Zn content (ugs.)	Metal content in p.p.m.	
			Cu	Zn
0.9243	2.22	31.1	2.40	33.7
0.9921	2.58	31.6	2.60	31.9
		Average	2.5	32.8

In the following Table the recoveries are calculated by subtracting the metal contents obtained by multiplying the dry weights of lens powder by the average metal content given in Table 2a.

Table 2b. (Tests).

Dry weight in gs.	Cu content (ugs.)	Zn content (ugs.)	Metal recovered in ugs.	
			Cu	Zn
0.8960	50.4	78.5	48.2	49.1
0.9535	51.8	84.6	49.4	53.3

It was considered unnecessary to substantiate this experiment with further recovery experiments on other tissues, for the same conditions of ashing and extraction were used for each tissue, and it is the satisfactoriness of these conditions which is tested by the experiment.

Previously, recovery experiments were carried out on sclerotic tissue. The results were inconclusive because it was not possible to obtain a homogenate of the tissue; as cut strips of tissue had therefore to be used, doubt occurred upon the question of whether the sclera is homogeneous in respect of copper and zinc concentrations. In addition, the quantities of metals added for recovery were too small; the error inherent in the method therefore made the results difficult to interpret.

Section 5 - Discussion

The technique described for the estimation of copper and zinc was considered satisfactory for carrying out a survey of the distribution of these metals in eye tissues, the following points being noted.

Firstly; the method has been shown to extract copper and zinc specifically from eye tissue ash; other

metals which might have interfered do not seem to be present in sufficient quantity to cause any noticeable effect on the absorption curves of the copper and zinc dithizonates.

Secondly; the variation in the results obtained on a number of samples of a homogenate of eye tissues is less than the probable biological variation.

Thirdly; a good recovery of zinc and copper added to lens tissue was obtained with the method.

CHAPTER III

COPPER AND ZINC IN MAMMALIAN EYE TISSUES.

Section 1 - Materials and their Sources.

Cattle and sheep eyes were obtained from animals killed at the Liverpool Abattoir. The animals were of various breeds, mainly middle-aged, and of both sexes. The eyes were removed from the heads, usually within a few minutes of the death of the animals; they were then separated into their constituent tissues within 12 hours of the death of the animals. If some were undissected more than two hours after death, they were kept in a refrigerator.

Whale eyes were obtained from sperm whales caught in the Antarctic region by boats of the whaling ship "Balaena", and had been stored in tins at a temperature below 0° C. for several months before dissection.

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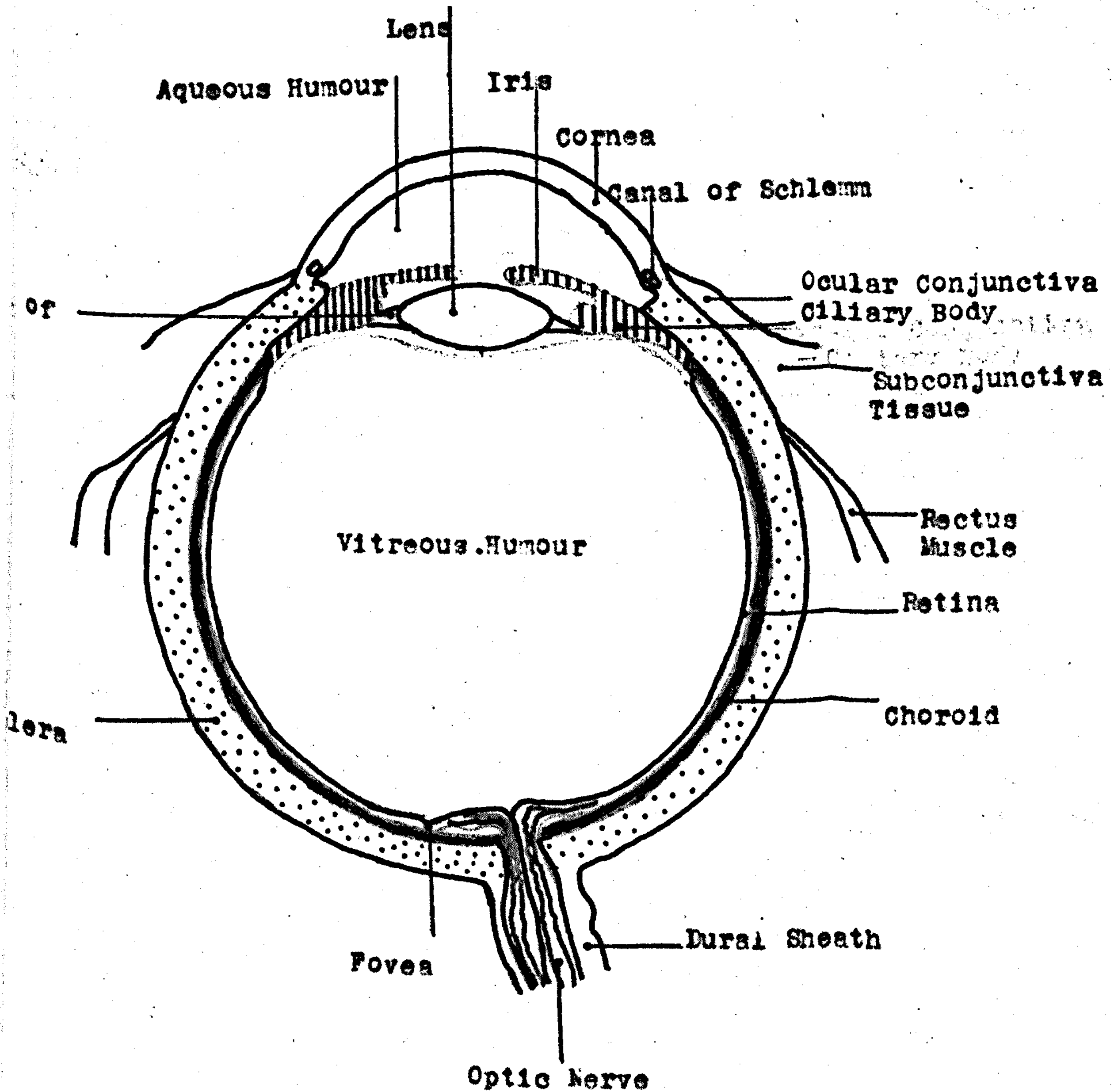
Fig.6: Diagrammatic Section through a Generalised Vertebrate Eye.



the outermost layer, next is the uveal tract, comprising the choroid, and ciliary body, and the innermost layer is the retina, in which are embedded the sensory elements receiving the light stimuli.

Sclera. This is composed of tough, inelastic, tendinous material, organised in ribbon-like bundles of microscopic fibres which are fitted together in such a way that the whole tissue is equally strong in all directions. It is the main supporting tissue of the eye. The tissue of the sclera contains relatively few cells, and consists chiefly of relatively inert fibres, whose metabolism is so low that it requires no direct blood supply.

Choroid. This is a deeply pigmented layer, next to the sclera, consisting mainly of blood vessels, with connective tissue binding them into a membrane. The rich vascularity of the tissue is concerned with the nutrition of the retina.

Pigment epithelium. This is firmly joined to the inner surface of the choroid coat, though embryologically it is classed as part of the retina. The cells of the epithelium contain granules of melanin; on the side next to the choroid they secrete a cement substance which binds them to the choroid, so that the layer usually adheres to

this tissue when the retina and choroid are separated; on the side next to the retina the surface of the layer usually bears a number of processes put out by the cells, from these cells pigment migration into the retina takes place in some species.

Retina. The receptive layer of rods and cones stands on the outer surface of the retina; the inner part of the retina is mainly connective and nervous tissue.

Lens. This is a crystalline, glassy, cushion-shaped body which lies behind the iris. It is supported from behind by the vitreous body, and from the front to some extent by the iris. The body of the lens, released from its capsule in a fresh condition, is a glutinous, almost inelastic mass, which is composed of innumerable layers.

The humours. The aqueous humour is continuously produced at a low rate and drains out of the eye-ball into the blood stream by a complex arrangement, so regulated that the intra-ocular pressure remains roughly constant. The vitreous humour is rendered gelatinous by the addition to it of protein secreted at an early stage by the retina. It is relatively permanent, and in the adult eye it is fixed in amount, so that any portion of it which is lost through

a wound is only replaced by watery aqueous humour.

Cornea. The layers of fibres are not so much felted as in the sclera, but run more nearly parallel, with less interchange of fibres between layers. The cells between them are consequently more definitely organised into layers. The substance of the healthy cornea is quite devoid of blood vessels.

Iris. This is attached to the anterior surface of the ciliary body and forms a delicate diaphragm across the anterior part of the eye. It possesses an opening, the pupil, which is usually somewhat eccentrically placed towards the nasal side. The pupillary border rests upon the lens. The iris houses two muscles, which are both derived from the pigment epithelial layer of the retina. The colour of the tissue varies in different individuals; the posterior border (retinal portion) is heavily pigmented.

Ciliary Body. The front of the uveal tract is thickened and modified to form the ciliary body which runs around the eye as an asymmetrical girdle. It forms the attachment for the suspensory ligament of the lens and gives origin also to the iris. The inner (vitreal) surface of the ciliary body presents two zones. The posterior two-thirds

is deeply pigmented and relatively smooth; this is a continuation of the pigment epithelium. The anterior third bears the ciliary processes. The two layers constitute the so-called pars ciliaris retinae. They continue over the posterior surface of the iris as the pars iridica retinae. Within the ciliary body is the ciliary muscle; this is concerned with accommodation in the eye.

For more detailed accounts of the anatomy and functions of the eye tissues, reference should be made to Duke-Elder (1938) and Detwiler (1943). Fig. 6 is taken from the former book.

c Dissection was carried out with stainless steel instruments. For the cattle, sheep, and rabbit eyes, an incision was made with a scalpel in the sclera and the front portion of the eye parted from the back by cutting as nearly as possible round the outer margin of the iris and ciliary body. For the whale eyes a cut was made round the outer margin of the cornea with a scalpel. The sclerotic coat was too thick to allow the initial incision to be made there. The tissues of the front and back portions in all cases

were then separated. The iris, vitreous humour, retina and choroid were then fairly easily removed, mainly by the use of tweezers. Before the removal of the iris it was usually necessary to free the lens from the front part of the eye by cutting through the zonula of Zinn with a pair of scissors. In the species where the cornea was not previously separated, it was cut away from the front part of the sclera. The two parts of the sclera were cleared to remove adhering muscle and adipose tissue, and combined. Each individual tissue was then washed with twice distilled water. All manipulations after washing were carried out with glass rods.

The state of the vitreous humour, and the attachment of the lens to the ciliary body via the zonula of Zinn vary according to the species of the animal, and the length of time the eyes are left after the death of the animal. In the whale specimens, after warming the eyes to room temperature, the vitreous humour was found to be almost entirely liquid, with the lens floating in the fluid, and the aqueous humour from the anterior chamber of the eye admixed. In cattle and sheep eyes the vitreous humour was found to adhere, in a gelatinous mass, to the front portion of the eye,

mainly round the margin of the ciliary body. The aqueous humour was obtained from cattle and sheep eyes by slitting the cornea with a scalpel before any incision was made in the sclera. The pressure of the fingers is sufficient to empty the fluid from the chamber of the eye anterior to the lens, without disturbing the posterior chamber containing vitreous humour.

Section 3. - Discussion of methods of presenting Results.

Many different units for the weight of element and of tissue have been used in the presentation of results of estimations of trace element concentrations in living matter. The size of the units used is determined formally by the sensitivity of the analytical method. Thus in the older literature results are expressed in mgs./kg., mgs./100 gr., or gs.%. Since the development of reliable micro-analytical techniques concentrations have usually been expressed in ugs./100 mgs. or parts per million (p.p.m. or ugs./g.). These units possess an advantage over the older ones in that they represent weights which correspond better with the natural size of the eye tissues. Concentrations

in the present work are expressed in p.p.m. except in some discussions, where high concentrations are emphasised by reference to percentage weights.

There are four ways in which the trace element contents of tissues have been compared:-

i. Weight per whole organ or tissue.

This method gives no impression of the element in relation to the amount of other tissue constituents and is of interest in physiology rather than biochemistry.

ii. On a wet weight basis.

If the element is evenly distributed throughout the tissue, then expression of results on a wet weight basis will show its natural functional concentration. If, however, the element be bound in certain specialised cells which constitute only a small volume of the total tissue, the rest of the volume containing less solid matter, then wet weight concentrations will not give such a true picture as dry weight concentrations. There is an important practical difficulty in expressing results in this way; it is often necessary to wash the tissue to remove blood and other extraneous matter after dissection, and this introduces doubt as to how much superfluous water is present.

iii. On a dry weight basis.

In this procedure one of the functional constituents of the tissue is deliberately removed; if the amounts of water in the various tissues differ greatly, then comparison of the tissues on this basis will be misleading. In the very watery tissues such as the aqueous and vitreous humours there is always a high relative concentration of all the mineral elements on a dry weight basis, and this probably does not correspond with any special organic association or function in these tissues.

From an analytical point of view this procedure is the easiest to standardise, but it should be used in the comparison of functional concentrations only if the tissues compared have roughly the same water content, or if the concentrations on a wet weight basis correspond with those on a dry weight basis.

iv. On an ash weight basis.

As a basis for comparing the concentrations of trace elements in the various tissues for the purpose of discovering functional accumulations or organic associations of particular elements, this method is of little use. The functions of the trace elements are concerned with the

organic matter of the tissue and normally with the organic matter in aqueous solution or suspension. To demonstrate a functional accumulation of a trace element in a tissue, the amount of that element must therefore be related either to the weight of solid organic matter, or to the weight of the aqueous mixture. If such an accumulation is demonstrated, it is then desirable to estimate the total ash content of the tissue to discover whether there is a general accumulation of inorganic elements, or whether a specific element is accumulated.

To summarise the usefulness of the various methods: Comparison of the concentrations of an element in various tissues on a wet weight or a dry weight basis will show up a special organic association of the element in a particular tissue, if enough of the organic complex concerned is present. Estimation of the ash content of the tissue and the ash content of the organic complex will then show whether the association is specific.

In the present work most of the results were obtained on a dry weight basis, but the general conclusions to be drawn from these results were checked by reference to results on a wet weight basis for some species. Results

for the metal contents of single tissues are also given for all the species except rabbits. The smaller the eye, the less easy it is to tell whether all of a particular tissue has been obtained by the dissection process. If much of the tissue remains behind, the results expressed per whole tissue will not have much meaning. Similarly, because the amounts of tissue obtained from each eye were so variable, results are not given for the total copper and zinc contents of aqueous humour and optic nerve from cattle and sheep.

Section 4. Purpose and design of the distribution studies.

The main purpose of the initial investigation was to discover whether the tissues of the mammalian eye differed in their accumulation of copper and zinc, and, if they did, which tissues contained the highest concentrations of these metals. Should differences in the zinc and copper concentrations of the tissues be detected, it is also necessary to show whether such differences are random variations, or are due to general properties of the eye tissues of all the individuals of a species, or all the species of the Mammalia. To establish these last two principles it is necessary to show that variations in the concentration of the metals from

one individual of a species to another could not account for the differences between the various eye tissues. The study of variations among the individuals of a species is difficult, if, as in the present case, the breed and dietary history of the animals is not readily available. It will appear later (in Section 6), that it is unnecessary to undertake a detailed survey of individual variations to achieve the initial purpose of the present study.

A secondary purpose of the investigation was to estimate the probable differences between the zinc and copper concentrations of the same tissue from left and right eyes of the same animal. If the differences were not great, then experiments could be carried out on one eye, whilst using the other as a control. Cattle and sheep eyes from the abbatoir were therefore divided into two batches, one eye from each animal being placed in batch A, and the other in batch B. This process was not practicable for whales, where pairs of eyes from several animals had been mixed in one tin. In rabbits the possible variation in the amount of a single tissue removed by the dissection technique was too great for such a comparison to be significant.

The limiting factors in the amount of tissue used for analysis are:-

i. Lower limit. The weight of tissue which contains an estimable amount of copper and zinc.

ii. Upper limit. The volume of tissue which will fit into a crucible of a size suited to a muffle furnace, and which will not overflow the crucible during the ashing process.

For some of the eye tissues the first factor was more important, for others, the second. For cattle and sheep, the eyes were dissected in batches of ten; all the tissues were thus obtained in amounts above the first limit. For those tissues, such as the sclera, which then had a total volume outside the second limit, part only of the tissue from each eye was taken for analysis. This sampling procedure makes the estimated concentrations of metals in each tissue directly comparable. Six whale eyes were analysed and the same procedure adopted.. In the first experiment with rabbits, carried out with batches of six eyes, the amounts of the eye tissues from the albino eyes were below the lower limit. In the second experiment (results of which are given) batches of greater numbers were used, but

in no case was it found necessary to sample the tissues to reduce the total volume obtained in dissection.

Preliminary work using the analytical techniques given by Shakir (1948) showed that the iris, lens and sclera contained enough copper and zinc to give significant figures for the contents of a single eye tissue. It was therefore decided to test the order of the variation in zinc and copper concentrations in these tissues from one individual animal to another.

Section 5. - Results.

The copper and zinc results for separate species are given in Tables 4 - 9. Results for copper in the various species are compared in Table 10, and for zinc in Table 11.

In all the tables a dash indicates that no result was obtained.

TABLE 4. Copper and Zinc in the Eye Tissues of Cattle.

Tissue	Batch	COPPER ug./whole tissue	COPPER ug./g.dry tissue	COPPER ug./g.wet tissue	ZINC ug./whole tissue	ZINC ug./g.dry tissue	ZINC ug./g.wet tissue
Aqueous humour		-	10	0.10	-	30	0.29
Vitreous humour	A	2.5	20	0.25	3.5	28	0.25
	B	2.4	18	0.23	3.6	25	0.24
Cornea	A	0.5	3.6	0.66	1.6	11	2.2
	B	0.4	2.9	0.51	1.9	14	2.4
Sclera	A	-	4.1	1.2		17	4.7
	B	-	5.4	1.5	-	13	3.5
Retina minus pigment epithelium	A	0.46	6.4	0.66	4.8	68	6.9
	B	0.49	7.1	0.68	5.2	75	7.4
Choroid plus pigment epithelium	A	0.46	4.9	0.89	11.9	126	24.5
	B	0.50	5.4	0.99	14.4	152	28.6
Optic nerve		-	5.6	1.8	-	6.8	2.2

TABLE 5. Copper and Zinc in Individual Cattle Irises and Lenses.

Tissue		COPPER			ZINC		
		ug./whole tissue	ug./g.dry tissue	ug./g.wet tissue	ug./whole tissue	ug./g.dry tissue	ug./g.wet tissue
Iris plus ciliary body.							
1st Animal	Eye A	4.6	28	4.7	42.5	252	42.8
	Eye B	3.7	23	3.7	41.8	259	42.4
2nd Animal	Eye A	4.9	30	4.7	38.2	229	36.5
	Eye B	4.6	29	5.1	39.2	245	42.3
Lens							
1st Animal	Eye A	1.0	1.1	0.39	40.6	42.4	16
	Eye B	1.1	1.1	0.43	39.0	40.6	15
2nd Animal	Eye A	1.6	1.7	0.67	33.6	35.0	14
	Eye B	1.0	1.0	0.42	35.0	35.2	15

TABLE 6. **Copper and Zinc in the Eye Tissues of Sheep.**

Tissue	Batch	COPPER			ZINC		
		ug./whole tissue	ug./g.dry tissue	ug./g.wet tissue	ug./whole tissue	ug./g.dry tissue	ug./g.wet tissue
Vitreous humour	A	2.4	22	0.29	2.7	24	0.32
	B	2.8	26	0.34	2 3	22	0.26
Cornea	A	0.16	2.2	0.36	1.7	23	3.8
	B	0.11	1.7	0.24	1.5	27	3.4
Retina minus pigment epithelium	A	0.53	13	1.4	3.3	77	9.0
	B	0.42	10	1.2	3.4	83	9.5
Choroid plus pigment epithelium	A	0.59	12	2.7	13.7	275	62
	B	0.95	16	4.0	16.4	278	70
Optic nerve		-	7.9	-	-	55	-

TABLE 7. Copper and Zinc in Individual Sheep Irises, Scleres and Lenses.

Tissue		COPPER			ZINC		
		ug./whole tissue	ug./g.dry tissue	ug./g.wet tissue	ug./whole tissue	ug./g.dry tissue	ug./g.wet tissue
Lens	1	1.6	2.8	-	67	123	-
	2	0.95	1.7	-	70	129	-
	3	1.2	2.1	-	54	92	-
Sclera	1	3.2	4.6	1.3	28	41	11.4
	2	4.9	6.6	2.2	34	47	15.4
	3	3.6	4.1	1.4	57	67	22.0
Iris	1	3.2	40	5.8	36	458	66
	2	3.1	41	6.2	31	401	60
	3	4.7	69	10.2	30	450	65

TABLE 8. Copper and Zinc in the Eye Tissues of the Whale.

Tissue	COPPER		ZINC	
	ug/whole tissue	ug./g. dry tissue	ug./whole tissue	ug./g. dry tissue
Vitreous humour	0.44	5.9	0.79	10.5
Cornea	0.26	3.4	2.7	35
Sclera	0.36	0.074	1.65	0.33
Lens	0.25	4.2	2.1	35
Retina	0.49	10.6	2.3	54
Choroid	0.40	2.2	7.1	37
Iris	0.40	5.9	6.9	99.5

TABLE 9. Copper and Zinc in the Eye Tissues of Rabbits.

Tissue	PIGMENTED ANIMALS				ALBINO ANIMALS			
	Total wt. of metal found (ugs.)		ugs. metal per g. dry tissue		Total wt. of metal found (ugs.)		ugs. metal per g. dry tissue	
	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn
Lens	1.2	30	0.62	16	0.78	20	0.49	12
Cornea	0.1	2.2	0.3	6.6	0.4	3.2	0.15	12
Iris	2.0	23	12	127	1.7	6.3	15	54
Choroid	0.8	22	17	466	1.1	4.5	21	86

TABLE 10. ***Concentrations of Copper in the Eye Tissues of some Mammals.***

Tissue	CATTLE		SHEEP		SPERM WHALE	RABBIT	
	ug./g.dry tissue	ug./g.wet tissue	ug./g.dry tissue	ug./g.wet tissue	ug./g. dry tissue	coloured ug./g.dry tissue	albino ug./g.dry tissue
Iris plus ciliary body	27	4.6	50	7.4	5.9	12	15
Choroid plus pigment epithelium	9.8	1.7	13	3.4	2.2	17	21
Retina minus pigment epithelium	6.8	0.67	11	1.3	11	-	-
Lens	1.2	0.46	2.1	0.8	4.2	0.62	0.49
Aqueous humour	10	0.10	-	-	-	-	-
Vitreous humour	18	0.24	24	0.3	5.9		
Sclera	4.8	1.3	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	-	-	-

TABLE 11. **Concentration of Zinc in the Eye Tissues of some Mammals.**

Tissue	CATTLE		SHEEP		SPERM WHALE	RABBIT	
	ug./g.dry tissue	ug./g.wet tissue	ug./g.dry tissue	ug./g.wet tissue	ug./g.dry tissue	coloured ug./g. dry tissue	albino ug./g. dry tissue
Iris plus ciliary body	246	41	436	65	99	127	54
Choroid plus pigment epithelium	139	26	277	69	37	466	86
Retina minus pigment epithelium	71	7.2	80	7.3	54.1		
Lens	37	15	117	47	35	16	12
Aqueous humour	30	0.29	-	-	-	-	-
Vitreous humour	26	0.35	23	0.29	10	-	-
Sclera	15	4.1	56	16	0.33	-	-
Cornea	13	2.3	25	3.6	35	6.6	12
Optic nerve	6.8	2.2	-	-	-	-	-

Section 6. Discussion.

For all the species examined, the results presented show that there are differences between the copper or zinc concentrations in the various tissues of the eye which are greater than can be accounted for by the experimental error (about ± 5%), which was illustrated by Table 2 in Chapter 2.

That the observed differences are not due to the idiosyncrasies of individual animals is indicated by the facts:-

(a). that large differences occur even when the results concerned are those for batches of tissue from 10 eyes. The individual variation would have to be very high which could give such differences between the averages of groups of ten individual tissues.

(b). that the differences between the zinc or copper concentrations in the same tissue from different animals of the same species (cf. Tables 5 and 7) are nowhere near as great as most of the differences between the eye tissues of one animal.

With the single exception of the vitreous humour of the sheep the concentration of zinc in the tissues is always greater, and may be up to 20 times greater, than that of copper in the same tissue.

That the differences between the zinc concentrations in various tissues is a common and necessary property of mammalian eyes is indicated by the similarity, in the pattern of the distribution of zinc concentrations in the different tissues, between all the species examined. In every case, one of the pigmented tissues contains the highest zinc concentration either on a wet or dry weight basis. In the rabbit the zinc concentration is highest in the choroid, whereas in the other three species it is highest in the iris. It is notable that the only species where any tissue contained more zinc than either of the pigmented tissues was the whale, whose choroid is thick, spongy, and contains little pigment. In addition the whale eyes had been preserved at 0° C. for some time and, on dissection, it was found impossible to separate spots of adhering pigment from the almost liquid retina. The fact that the whale retina contains more zinc than the choroid may thus be fitted into the general scheme without affecting the conclusions to be drawn.

Apart from the whale, the distribution of copper concentrations follows a pattern similar to, but less distinct, than, that for zinc. In rabbits, sheep and cattle the highest copper concentrations in fresh tissues were found in

the iris and choroid. On dry weights of tissues, the vitreous and aqueous humours were higher than the choroid.

Comparison of the/results with others in the literature.

Baroni (1937) by a cytochemical method identified copper as a constituent of cattle retinas, and Nitzescu & Georgescu (1935) estimated this element in beef vitreous humour, but the only surveys of the quantitative distribution of copper and zinc in the eye tissues of mammals are those of Tauber & Krause (1943) for cattle, and Shakir (1948) for sheep, and the retinas and choroids of cattle. These results are compared with the present work in Tables 12 and 13.

The zinc concentrations given by Tauber & Krause for cattle are of a much lower order than those obtained either by Shakir, or in the present work. It has been mentioned in Chapter I that the analytical method they used was probably entirely inadequate; their results were therefore not considered to be significant. In comparing the work of Tauber & Krause with that of Shakir, and with the present work, it should also be noted that the Americans worked on bullocks, whilst we worked on cows and bullocks. There are thus at least two biological factors which may account for

TABLE 12. The Concentrations of Copper and Zinc (in ugs./g. dry tissue) found by various workers in the eye tissues of cattle.

	Iris	Choroid	Retina	Vitreous Humour	Lens	Sclera	Cornea	Optic Nerve
COPPER								
Tauber & Krause	7-16	11-17	10-34	10-18	3-6.8	5.7-10	5-8.7	9-15
Shakir		14-44	28-30					
Present work	27	9.8	6.8	18	1.2	4.8	3.2	5.6
ZINC								
Tauber & Krause	12-15	7-9	21-26	12-20	5-9.5	5-7	7-8	8-12
Shakir		106-240	114-138					
Present work	246	139	71	26	37	15	13	6.8

TABLE 13. The Concentrations of Copper and Zinc (in ugs./g. dry tissue) found by various authors in the eye tissues of Sheep.

	Iris	Choroid	Retina	Vitreous Humour	Lens	Sclera	Cornea
COPPER							
Shakir	14	12-74	25	15	5.2	7.1	4.8
Present work	50	13	11	24	2.1	5.1	1.9
ZINC							
Shakir	249	200-343	340	-	86	43	63
Present work	436	277	80	23	117	56	25

some differences between the results; age, and the difference in diet of the two sets of animals.

Shakir apperently attempted to separate the pigment epithelium from the choroid and analyse it with the retina; whether or not this process was successful, it would be inevitable that much melanin pigment would be added to the retina during the procedure; this could account for the fact that the results given by Shakir for the retinal copper and zinc are much higher than those of the present work, in which the pigment epithelium was allowed to remain attached to the choroid, and care was taken to have as little pigment attached to the retina as possible. Tauber & Krause did not give details of their dissection procedure but it is possible that the same factor is operative in their results.

The figures given for retinas and choroids of sheep in the present work are confirmed by the work on dark adaptation (given in Chapter IV) which also shows the great increase in the zinc concentration in the retina which can result from attachment of pigment to this tissue. With the addition of pigment from the pigment epithelium the zinc concentration in the retina was found to become equal to that in the choroid.

The discrepancies between the figures of Shakir and of Tauber & Krause (disregarding the results for zinc given by the latter), and those of the present work, for most of the tissues can be explained by variation due to breed, age, race, and inaccuracies in estimation procedure. There remains, however, the fact that the concentrations of zinc and copper previously reported to occur in the iris in no case were significantly greater than those in the choroid and retina. Work on the fractionation of irises given in Chapter IV confirmed the high figures for the copper and zinc content of the iris; in the four batches of irises examined the copper concentrations ranged from 17 to 20.5 ugs./g. dry tissue, and the zinc from 160 to 200 ugs./g. dry tissue. These are a little less than the figures given above, but are still much higher than the results for any of the other tissues. Neither Tauber & Krause nor Shakir mention the ciliary body in their work, but it is possible that they removed it from the iris before analysis. This could then account for the discrepancy in the results.

Despite the partial (and perhaps only apparent) disagreement with some of the previous work over the figures for retina and iris, it was considered that the evidence from

the present work was sufficient to justify the general conclusion that zinc, and to a lesser extent, and not in every case, copper, are concentrated in the parts of the eye containing melanin pigment, namely the iris, the choroid and the other, minor, parts of the uveal tract.

The results, and the general conclusions of the present work concerning zinc, are confirmed by the work of Leiner & Leiner. Their extensive studies (1942; 1944) on the zinc contents of salt and fresh-water bony fish (see Chapter V) support by analogy the present work. In addition they analysed specimens of the eyes of man, fowls and dogs. The human choroid contained more zinc than the iris, which in turn contained more than any of the other eye tissues. Figures were not given for the irises of the dog or fowl, but of the remaining eye tissues, the choroid had the highest concentration of zinc in both species. In all three species the choroid and the retina (and in man the iris) were found to have higher concentrations of zinc than blood, milk, lungs, skeletal muscle, ovaries, testicles, kidney, liver, or brain from the same species.

Difference between the results for albino and pigmented rabbits.

The distribution of zinc and copper in the eye tissues of the four mammals examined indicate that zinc, and perhaps copper, is associated with melanin pigmentation in the eye. The results obtained with rabbits show definitely that the high concentration of zinc in the iris and choroids of coloured rabbits is at least partially associated with the presence of melanin pigment. The concentrations of zinc in the iris and choroid of albino rabbits were considerably less than in pigmented rabbits, though the concentrations in the lens and cornea were the same in both types.

There appeared to be no significant difference between the copper concentrations in the iris or choroid of the two types, but the total amounts of copper found were so small that it is doubtful whether this can be regarded as proved.

The work of Leiner & Leiner (1941) showed that, if anything, the carbonic anhydrase content of albino rabbit choroids was higher than that of the choroids of coloured species. The difference in zinc content is therefore not at all concerned with carbonic anhydrase activity.

CHAPTER IV

COPPER AND ZINC IN FRACTIONS OF SOME MAMMALIAN EYE TISSUES

In the previous chapter a survey was made of the distribution of copper and zinc in the various tissues of some mammalian eyes. The present chapter deals with the more precise location of copper and zinc in some of these tissues. Several procedures, some successful, and others not, were used in attempting to fractionate the tissues. The technique of each will be described in its appropriate section.

Section 1. Copper and Zinc in Water Soluble and Insoluble Fractions of Eye Tissues.

Shakir (1948) carried out a preliminary separation of sheep choroids and retinas into water soluble and insoluble fractions. He found 70% of the copper and 50% of the zinc

in an aqueous extract from choroids; 99% of the copper and 74% of the zinc in retinas were extracted by water. He did not attempt to remove substances in temporary suspension from the supernatant extract, and it seems likely that the residue did not represent the true total of insoluble material.

It appeared from the distribution studies of Chapter III that zinc and copper concentrations of left and right eyes from one animal did not differ from each other by more than 30% of the mean between the two figures. In the case of the cattle iris the difference between the dry weight concentrations was less than 20% of the mean for copper and less than 10% for zinc.

As a means of testing the efficacy of the fractionation technique and of obtaining preliminary results, it was therefore decided to use one batch of eyes for fractionation, and the opposite eyes as a control for analysis whole.

Technique.

The tissue (from cattle eyes) was finely ground with acid-washed silver sand, or purified crushed quartz, and twice distilled water. The mixture was centrifuged, the supernatant poured off, the residue washed, and the washings added

to the supernatant. The residue was stirred up with water and separated as far as possible from sand or quartz. It was not possible to remove more than a little of the quartz from the residue in this way. The supernatants, and the residue mixed with sand or quartz, were dried, ashed, and analysed for copper and zinc by the method given in Chapter II.

Results.

These are shown in Table 14.

It was evident from these results that the technique as it stood would not give a quantitative picture of the relative amounts of copper and zinc in the water soluble and insoluble parts of tissues. It is possible that it might have been made to do so by using larger quantities of material and a better system of controls, but the presence of much sand or quartz would always introduce error due to contamination or loss in the ashing procedure, and it was decided not to pursue the method further.

Qualitatively, the results show:-

(a). That centrifuging the extracts of choroids removes much of the copper and zinc which Shakir found there. The difference could be due to the spinning down of small pigment particles.

TABLE 14. Copper and Zinc in Fractions of Eye Tissues.

Tissue	ugs. metal in whole tissues		ugs. metal in aqueous extract		ugs. metal in residue		ugs. total recovered in fractions	
	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn
Irises (3 pairs)	-	84	-	10	-	70	-	80
Choroids (6 pairs)	-	99	-	13	-	34	-	47
do. (6 pairs)	4.5	101	4.7	11	1.2	68	5.9	79
Retinas (6 pairs)	3.9	32	3.5	29	4.5	21	8	50
Lenses (3 pairs)	1.5	95	1.3	82	0.9	15	2.2	97

(b). That in all the tissues some of the copper and zinc is associated with fractions which precipitate on centrifuging the homogenate. The proportion of the metals associated with the precipitate is much greater for the iris and choroid than the retina, and least for the lens.

Section 2. Copper and Zinc in the Corneal Epithelium.

Tauber & Krause (1943) reported that the corneal epithelium contained higher concentrations of copper and zinc than the whole cornea; indeed they were higher than those in most of the other tissues of cattle eyes. In view of the inadequacy of the technique for estimating zinc used by them it was thought that confirmation of their figures was desirable.

Experiment 1.

The outer portion of the cornea was separated by making an incision in the cornea with scissors to a depth of 1 mm. and cutting round the periphery at this depth. It was found that the outer layer of the cornea could be peeled off the main body of the tissue within this circular cut. The tissues were washed with twice distilled water, dried, ashed, and analysed for copper and zinc.

TABLE 15. ***Copper and Zinc in Cattle Corneas.***

Fraction	No. of eyes dissected	Dry wt. of material(g.)	Ash wt.(g.)	ug.Zn/g.dry tissue	ug.Cu/g.dry tissue
Outer layer	8	0.299	-	35	9.5
Outer layer)	24	0.894	0.036	34	5.4
Inner layer)	22	1.288	0.041	17	6.1

These results indicate that the outer layer of the cornea is richer than the inner layer. The results for copper are not as high as those given by Tauber & Krause (18-24 ugs.Cu per g. dry corneal epithelium). The relative weights of the two parts of the cornea from the same eyes indicate that the outer layer includes much more of the tissue than the epithelium. A second technique was therefore tried.

Experiment 2.

The surface of the cornea was scraped with a sharp scalpel before the tissue had been dissected away from the eye. Only the outermost layer, which is fragile and easily removed, was taken. This layer was much thinner than that obtained in the first experiment. The nature of the layer was not studied histologically, but as it could not by any means be split into further layers it was assumed to be the corneal epithelium.

TABLE 16. Copper and Zinc in the Layers of Cattle Corneas.

Layer	No. of eyes dissected	Dry wt. of material (g.)	Ash wt. (g.)	ug.Zn/g.dry tissue	ug.Cu/g.dry tissue
'Epithelium'	18	0.272	-	71	8.3
'Epithelium')	12	0.157	-	76	7.8
)	Parts of same				
Inner layer)	12	0.644	-	13	2.9
'Epithelium')	24	0.386	0.018	68	6.5
)	Parts of same				
Inner layer)	24	0.980	0.031	7.1	1.7

These results show that the epithelium of the cornea is much richer in both zinc and copper than the main body (stroma) of the tissue. It appears, both in the first and second experiments, that the outer part of the cornea has a higher ash content than the inner.

The figures for copper in the epithelium are much lower than those given by Tauber & Krause for bullocks, but the general conclusion to be drawn from their work is confirmed by the present results.

It is interesting to note that the stroma of the cornea contains very few cells, whereas the epithelium consists almost entirely of cells.

Section 3. Pigment Epithelium and Pigment Migration.

The position and form of the pigment epithelium has been discussed in Section 2 of Chapter III. Normally the retina from mammalian eyes is obtained, on dissection, almost free from melanin pigment, whilst the pigment epithelium adheres firmly to the choroid. In some animals, however, the cells of the pigment epithelium, in response to the stimulus of light, put forth processes which penetrate the receptor cell layers of the retina. When this happens

the retina on dissection contains much more melanin pigment than would normally be the case. The phenomenon of pigment migration thus provides a testing ground for the hypothesis that copper and zinc are associated with the melanin pigment of the choroid. If the hypothesis is correct, then migration of these metals into the retina on exposure of the eyes to light should take place in those animals in which pigment migration is known to occur.

The most extensive migration of pigment into the retina takes place in fishes and anuran amphibians. Though photomechanical responses such as the elongation of the rods take place, the evidence for pigment migration in mammals is very insecure (Detwiler, 1943).

Shakir (1948) reported a decrease in the copper and zinc contents of the choroids of sheep on dark adaptation and an increase in the copper and zinc concentrations in the retina. This is an effect which appears to be the reverse of light adaptation.

The present work is concerned with investigating a possible increase in the copper and zinc concentrations of sheep retinas on exposure to light, and determining whether this increase, if it occurs, is correlated with an increase in the melanin pigment attached to the retina.

Technique.

Advantage was again taken of the observation (cf. Chapter III) that zinc and copper concentrations of left and right eyes of the same animal do not differ by more than 30% of the mean between the two.

One of each pair of eyes from six sheep were light adapted by placing them under an electric light for four hours. At the same time the opposite eyes of the pairs were dark adapted by keeping them in a closed tin in a dark room.

Both sets of eyes were dissected by a standard procedure and the choroids and retinas separated. The tissues were then weighed, dried, weighed, and ashed. The ash of each was analysed for zinc and copper. In the first experiment the retinas were analysed entire. In the second and third experiments the retinas from both sets of eyes were ground to a paste in a glass mortar and then diluted to a standard volume. An aliquot was then taken for estimation of melanin content, and the remainder dried for zinc and copper analysis. In the second experiment an attempt was made to estimate the melanin by hydrolysing part of the retinal homogenate with 10 mls. 2 N HCl at 100° C. for 12 hours, the idea being that protein material would be

hydrolysed to amino acids whilst the melanin would be unchanged. The relative amounts of melanin in the two samples could then have been estimated spectroscopically. Hydrolysis did occur, but unfortunately humin formation made both flasks brown. The humin could not be separated from the melanin and would interfere with any spectroscopic determination of the relative amounts of pigment. It did appear, however, to the naked eye, that the homogenate and hydrolysate of the light adapted retinas was blacker than the dark adapted materials.

In the third experiment the time of light and dark adaptation used was 2½ hours. The volume of each retinal homogenate was made up to 20 mls. Five mls. of this were used for melanin separation, and the remaining 15 mls. dried, weighed, ashed, and analysed. Melanin was demonstrated by hydrolysing the portions of retinal homogenate first with trypsin (24 hrs. at 38° C. 10 mls. trypsin 1% suspension to each portion) and then with KoH (5% at 100° C. for 3 hours). The resulting solutions were diluted to 50 mls. and absorption readings taken using the Beckmann instrument.

Results.

These are shown in Tables 17 and 18 on the following pages.

TABLE 17. The Effect of Light on the Copper and Zinc Concentrations in Sheep Choroids and Retinas.

Tissue	Total Cu (ug.)	Total Zn (ug.)	Concentrations in the dry tissue Cu (ug./g.)	Zn (ug./g.)
EXPERIMENT 1.				
L.A. Retinas	3.4	23	13	88
D.A. Retinas	3.2	17	13	67
L.A. Choroids	5.5	77	21	294
D.A. Choroids	7.8	78	31	310
L.A. Retinas plus Choroids	8.9	100		
D.A. Retinas plus Choroids	11.0	95		
EXPERIMENT 2.				
L.A. Retinas	3.2	54	16	271
D.A. Retinas	3.1	18	13	76
L.A. Choroids	3.7	22	16	94
D.A. Choroids	3.8	55	13	183
L.A. Retinas plus Choroids	7.0	76		
D.A. Retinas plus Choroids	7.0	73		
EXPERIMENT 3.				
L.A. Retinas	7.3	22	33	98
D.A. Retinas	4.9	19	22	86
L.A. Choroids	9.0	71	40	297
D.A. Choroids	8.7	89	36	370
L.A. Choroids plus Retinas	16.3	93		
D.A. Choroids plus Retinas	13.6	108		

TABLE 18. Absorption Readings for the Diluted Digests of Light and Dark Adapted Retinas from Experiment 3.

Wavelength	E for L.A.Retinas	E for D.A.Retinas	Difference
300	1.895	1.13	0.765
400	0.460	0.163	0.297
500	0.150	0.072	0.078
600	0.075	0.042	0.033
700	0.070	0.040	0.030

Discussion.

A significant transfer of metal from choroid to retina is demonstrated by the results only in the case of zinc in the second experiment. Though in each experiment the copper and zinc contents of light adapted retinas are greater than those of dark adapted retinas the differences are in most cases not greater than 30% of the mean between the two figures.

Thirty per cent. is only a probable value for the maximum difference between batches of opposite eyes from six sheep. That it is correct is indicated by the fact that in no case is the difference between the sum of the figures

for L.A. retinas and choroids and D.A. retinas and choroids greater than 20% of the mean between the two. In Experiment 2 there is a difference between the zinc in L.A. retinas and D.A. retinas which is 100% of the mean between the two. There is a corresponding drop from D.A. choroids to L.A. choroids of 87% of the mean between these two figures. The difference between the total zinc content of L.A. choroids plus retinas and D.A. choroids plus retinas was only 4%. There is therefore a definite indication of transference of zinc from choroid to retina on exposure of sheep eyes to light.

There is no chemical evidence that melanin also was transferred to the retina on exposure to light. Visual observation of the two batches of retina after dissection showed in each experiment that the light adapted retinas had adhering patches of pigment on their surface, whilst the dark adapted retinas had only a few small spots of attached pigment. In the second experiment the amount of pigment adhering to the light adapted retinas was greater than in the other experiments. Table 18 shows the marked difference between the light absorption of the digests of light adapted and dark adapted retinas from Experiment 3. The figures for the relative light absorption at various wavelengths

show that the difference between the two digests can be accounted for by an end-absorption, in the light adapted digest, of the melanin type.

The present experiments were carried out under conditions which differed from those used by Shakir. He worked with eyes which had been dark adapted, and eyes which had been left in daylight. In the present work one group of eyes was dark adapted, and the other exposed to bright light. The bright light may achieve an effect on the eye not produced by daylight alone. It is thus possible that the migration of copper and zinc into the retina on dark adaptation suggested by Shakir, and the migration of zinc to the retina on exposure to bright light, are not incompatible. However, as dark adaptation of one group of eyes was carried out in each of the present experiments, it seems that the former effect, if it occurred at all, could only have been a minor one, for it would otherwise have masked the opposite movement of zinc which was observed. In Shakir's work there was a much greater difference between totals for L.A. choroids plus retinas and totals for D.A. choroids plus retinas than in the present work and the results are therefore not as significant. In addition, only two experiments showed a migration to the retina from the choroid on dark

adaptation, the third (a preliminary one) showed a movement in the opposite direction.

The observation that the amount of pigment adhering to the retina increases considerably after exposing sheep eyes to a strong light appears to contradict the view that pigment migration does not take place in mammals. Detwiler (1943) does not mention sheep as being a species in which migration has been demonstrated not to occur; it is possible therefore that this animal is an isolated case which has been overlooked. Pigment migration can only be finally demonstrated by means of histological studies, however; it is just possible that the phenomenon of extra adherence of pigment to the retina is due to some other cause than the growth of processes from the pigment epithelial cells into the retina.

Section 4. Copper and Zinc in Pigment Fractions of Mammalian Irises and Choroids.

In Chapter III, and in Section 3 of this chapter, evidence has been presented which indicates that zinc, and perhaps copper, is associated with the presence of melanin pigmentation in the iris and choroid. In the present section an account is given of work designed to show whether

zinc and copper are actually combined with the pigment fraction of irises and choroids.

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. They did not postulate a connection between zinc concentration and pigmentation.

Cattle Irises.

It was decided that the correspondence between the copper and zinc analyses for left and right irises from the same animal was near enough to permit the use of one iris from each pair as a control.

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

Whole irises (3-6) were digested with 5 ml. of an approximately 2% (w./v.) trypsin suspension and about 25 mls. of twice distilled water for 24-36 hours at 37°. The melanin pigment could then almost all be washed away from

the bulky residue of undigested fibrous, connective, and muscular tissue, which was allowed to remain in the digestion flask.

The washings containing black pigment, protein, and other material in solution were centrifuged until the supernatant liquid was yellow and this solution was then poured off. The black pigment fraction residue was stirred up with twice distilled water and re-centrifuged, 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 zinc and copper by the methods already described. The results are compared in Table 18 with the analyses for the 'control' irises, which had been dried and ashed whole. These results, plus the trypsin blank figures, should approximate to the total for the various fractions if no contamination or loss has occurred.

TABLE 18. Zinc and Copper Present in Various Fractions of Cattle Irises.

Fraction	Dry weight (g.)	Zinc (ug.)	Copper (ug.)
EXPERIMENT I			
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	0.5050	99.0	9.8
Total	0.5558	122.6	11.7
EXPERIMENT II			
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	0.4992	79.6	9.8
Total	0.5526	103.0	12.2
EXPERIMENT III (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	87.6	13.5
Trypsin blank	0.0431	12.0	2.8
Whole irises	0.4647	80.0	10.5
Total	0.5078	92.0	13.3

The results in Table 18 show at once that greater quantities of zinc and copper are associated with the pigment fraction of cattle irises than with any other fraction. On closer inspection of the results several matters are seen to require comment.

It was to be expected that the dry weight total of the fractionated irises would be less than that of the corresponding whole irises, for losses due to decomposition of organic compounds are inevitable during digestion, and in the 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 animal are also bound to occur as a result of biological variation and imperfect separations during 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 varies from one experiment to another; this is probably due to variations

in the extent of digestion of the tissue, and to small variations in the separation technique.

Only a small fraction of the total copper and zinc of the irises is not accounted for by the pigment fraction. This pigment fraction was subjected to repeated washing in the fractionation procedure. The high concentrations of zinc and copper in cattle irises must therefore be largely due to the combination of these metals in non-ionic form, with this fraction of the tissue. The precise nature of this fraction will be discussed later (Chapter VI).

Whale Irises (Humpback Whales).

As pairs of whale eyes were not available, this experiment had to be carried out without controls.

The technique of fractionation by trypsin digestion was only partially successful with whale irises. At the end of 36 hours' incubation it was found that only part of the melanin pigment could be washed away from the undigested residue. Possible explanations for this fact may be that the site of the main melanin pigmentation differs from that in cattle eyes, or that the excessively oily nature of the tissue interferes with trypsin action.

TABLE 19. Copper and Zinc in the Pigment Fraction of Whale Irises.

Fraction	Dry wt. (gs.)	Copper content (ugs.)	Zinc content (ugs.)
Pigment fraction	0.047	2.6	45
Combined washings	0.122	2.9	9.4
Undigested residue	0.165	3.5	31
Total	0.334	9.0	85.4
Trypsin blank	0.035	2.3	9.9
∴ Total for irises	0.299	6.7	75.5

It will be noticed that much less of the metal content of the whale iris was found in the pigment fraction than was the case with cattle. This can be accounted for by the pigment which remained in the undigested residue.

Cattle Choroids.

The trypsin digestion fractionation procedure did not separate all the melanin from cattle choroids, even after three days' incubation. Thus, as with whale irises, it was not possible to estimate how much of the total zinc in

the choroids was combined with the pigment. As with whole irises, however, the pigment fraction which did separate was much richer in copper and zinc than the whole tissue.

TABLE 20. Copper and Zinc in the Pigment Fraction of Cattle Choroids (Controls as for Cattle Irises).

Fraction	Dry wt.(gs.)	Copper content (ugs.)	Zinc content (ugs.)
Undigested residue	0.392	3.8	81
Pigment fraction	0.076	3.6	56
Combined washings	0.132	1.5	9.0
Total	0.600	7.9	145.0
Trypsin blank	0.053	2.3	23
6 whole choroids	0.557	5.3	116
Total	0.610	7.6	139

It is shown in Chapter VI that digestion of the pigment fraction from cattle irises with 0.1 N HCl at 100° C. splits off nearly all the zinc, but leaves most of the copper bound to the pigment material.

It was therefore considered that a hydrolysis of the choroid tissue, and separation of the insoluble pigment

material from the hydrolysate might reveal how much of the copper in the choroid is bound to pigment material. Accordingly six cattle choroids were digested with 0.1 N HCl on a water bath for 2 hours. The digest was centrifuged and washed several times till the supernatant was clear. The precipitate and the combined supernatants were dried, ashed, and analysed for zinc and copper.

TABLE 21. Copper and Zinc in the Pigment Fraction; from Cattle Choroids, insoluble after acid hydrolysis.

Fraction	Dry wt.(g.)	Copper content (ugs.)	Zinc content (ugs.)
Insoluble pigment fraction	0.162	6.1	9.9
Combined supernatants	0.379	2.0	85.5
Total	0.541	8.1	95.4

These results indicate that the copper in cattle choroids is largely bound to the pigment fraction of the tissue.

A further technique was used in an attempt to obtain a quantitative recovery of a pigment fraction from choroids.

Cattle choroids were treated for 5 hours with 0.5 N KoH solution at 100° C. At the end of this time melanin was precipitated by neutralising the digest. The precipitate was centrifuged down, washed, and part of it treated with trypsin suspension for 24 hours at 38° C. to remove unhydrolysed protein. The treated and untreated precipitates were dried, weighed and analysed. Unfortunately, the supernatants contained pigment material which remained in suspension after centrifuging and the fractionation was therefore not a quantitative one. In addition the KoH contained much copper and zinc, and the analyses for these metals in the dried supernatant were meaningless as far as the fractionation was concerned. The pigment precipitates did contain fairly high concentrations of copper and zinc, however. (Untreated precipitate 43 ug./g. Cu and 443 ug./g. Zn; precipitate after trypsin treatment 57 ug./g. Cu and 682 ug./g. Zn.).

Section 5. Discussion of the Significance of the Fractionation Results.

Section 2 is a discrete study and shows quite clearly, in confirmation of Tauber & Krause, that the corneal epithelium is richer in copper and zinc than the rest of the cornea.

Sections 1, 3 and 4 are to some extent related, and all give evidence bearing on the combination of copper and zinc with melanin pigment fractions in the eye.

It was conclusively demonstrated in Section 4 that most of the zinc and copper from cattle irises is combined with a pigment fraction of that tissue. It was also shown that most of the copper in cattle choroids is bound to pigment material. The proportion of zinc bound to pigment material in the choroid has not been demonstrated, but in Section 1 it was indicated that both in the iris and the choroid much more of the total zinc was found in the black precipitate obtained by centrifuging homogenates of these tissues than in the corresponding precipitates from retinas and lenses. In addition Section 3 indicated that a transference of melanin pigment from choroid to retina in sheep was accompanied by a corresponding movement of zinc. It can thus be inferred that in the iris and choroid of cattle and sheep, the high concentrations of copper and zinc are largely attributable to a combination of these metals with a pigment fraction of the tissues.

The pigment fraction constitutes a considerable part of the total dry weight of the iris and the ratio:

$$\frac{\text{dry weight concentration of metal in pigment fraction}}{\text{dry weight concentration of metal in iris}}$$

is only about 3.5 for copper and 4.3 for zinc.

Comparison of the Concentrations of Copper and Zinc in Pigment Fractions obtained from various Sources.

TABLE 22.

Source of Pigment Fraction	Copper Content in ugs./g.dry tissue	Zinc content in ugs./g.dry tissue
1. Trypsin digest of:- Adult cattle irises		
(a)	78	1000
(b)	65	730
(c)	80	750
(d)	87	720
Calf irises	44	520
Adult cattle choroids	48	740
Humpback whale irises	56	970
2. KoH and trypsin digest of cattle choroids	58	680
3. HCl digest of cattle choroids	38	61

The only analysis which occurs in the literature, of a comparable material, is that of Flesch (1949), who gave figures for the copper concentrations in mouse melanomas, and in pigment fractions obtained from them. The melanomas themselves contained 11-59 ugs. Cu/g. dry tissue; the dry weight concentrations of copper in a melanin fraction obtained by digestion with NaOH were 50-338 ugs./g. The dry weight concentrations in a melanin fraction obtained by trypsin digestion were twenty to ninety-five times those of the original tumour.

CHAPTER V.

ZINC AND COPPER CONCENTRATIONS IN THE TISSUES AND PIGMENT FRACTIONS OF EYES FROM FRESHWATER FISH AND FROGS

The results of Leiner & Leiner (1944) showed that zinc is present in the choroid, and other pigmented tissues, of the eyes of freshwater fishes from Lake Constance, in amounts as much as one hundred times those in similar tissues from mammalian eyes (figures for which are given in Chapter III). 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). Apart from an erroneous quotation of figures for tench and herrings, which appears to originate in an abstract (Bertrand & Vladesco, 1921b) of an original paper by Bertrand & Vladesco (1921a), the highest known concentration of zinc

in animal products until then was that of serpent venom (Delezenne, 1919), where up to 0.56% of the dry weight was accounted for by zinc. The range of zinc concentrations found by Leiner & Leiner in the dry choroids of various freshwater fish was from 0.44% to 2.96%, and the average for all the species examined was 1.18%.

It was possible that this 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 freshwater fishes everywhere.

The work described in this chapter had two main objects:-

(i) to ascertain whether the results obtained by Leiner & Leiner could 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 (cf. Chapters III and IV) can be demonstrated for freshwater 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 most 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, 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.

Section 1. 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 in Liverpool within two 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 (Rana 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.

Special features of fish eyes.

The anatomy of fish eyes follows the same general pattern found in mammalian species. All the tissues, described in Section 2 of Chapter III, which occur in mammalian eyes, are also present in fishes. In addition some species possess one or both of two tissues which are not found in mammals.

All freshwater fish eyes are lined with a Silver Membrane, or Argentea. The area covered by this membrane varies from one freshwater species to another, and it is not found at all in salt-water fish. The membrane is mainly

situated between the choroid and the sclera; it covers the inside of the sclera and also the outer surface of the iris. It contains crystals of guanine which give the tissue a silvery-white reflecting surface.

The Choroid Body or 'Gland', is found in the rear portion of the eyes of many fish. It is usually horse-shoe shaped, though sometimes it forms a complete ring round the optic nerve inside the eyeball, and sometimes it is divided into two parts by this nerve. The choroid body only occurs in fish possessing a Pseudobranch, the vestigial hyoid gill which is found on the inner side of the Aperculum or gill cover. The blood which has been aerated in the Pseudobranch is gathered into an efferent artery which enters the sclera in the neighbourhood of the optic nerve, and breaks up into a set of capillaries in the choroid body, from which blood flows into the ordinary choroidal circulation. The bulk of the tissue consists of blood (Rochon-Duvigneaud speaks of the choroid body as a "poche sereuse" or blood pocket) and for this reason it was not considered to be comparable with the other eye tissues, and was not subjected to analysis in the present work.

There are no outstanding anatomical differences between frog eyes and mammalian eyes. For a comprehensive survey of the comparative anatomy of vertebrates' eyes, reference should be made to Rochon-Duvigneaud (1943).

Dissection.

It was not possible to obtain any uncontaminated aqueous or vitreous humour from fishes or frogs, and these tissues were therefore not subjected to analysis. Difficulties in dissection arose, both for fish and frogs, from the smallness of the eyes. It was this factor which prevented more than a minute volume of aqueous humour being squeezed out of the eye before other tissues flowed through the incision. The vitreous humour was always found to be liquid, and, in cutting the sclera, became dispersed or mixed with pigment and retinal débris.

The technique of dissection used was similar to that described for mammalian eyes. The retinas from fish or frog eyes could most easily 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 the choroid with tweezers and a small scalpel. The irises of the fish were never completely separated from the 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 Rana esculenta. The variability due to dissection difficulties made it impossible to express results in terms of whole tissues, or to compare the concentrations of metals in opposite eyes of the animals.

The analytical method has already been described in Chapter II. The accuracy of the method as applied to fish eyes was not assessed but it should not be greater than that obtained with mammalian material. The percentage error due to ashing, for the tissues very rich in zinc will be less than that in mammalian tissues, where this was the main source of variation in the results, but this improvement

will to some extent have been offset by the increased importance of the error in the spectroscopic readings caused by the necessity for taking smaller aliquots which would give extinction values within the range of the Beckmann Photoelectric Spectrophotometer used.

Section 2. Results.

The main findings are displayed in Tables 23 and 24. The recorded results have been calculated directly from experimental readings; it is difficult to assess the possible deviation, but in general it is the third figure which is subject to error.

TABLE 23. The Concentrations of Copper in the Eye Tissues of Trout, Perch, and Frogs.

The plain figures show the concentrations in ug./g. dry tissue; the figures in brackets underneath (when given) show the concentration in ug./g. wet tissue corresponding to the figure immediately above.

Tissue	Trout	Perch	Frog	
			R.temporaria	R.esculenta
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 choroid	-	49.3	-	985

Note: A dash means that the tissue was absent, or not available for analysis.

TABLE 24. The Concentrations of Zinc in the Eye Tissues of Trout, Perch, and Frogs.

The plain figures show the concentrations expressed as ug./g. dry tissue; the figures in brackets underneath, when given, show the concentrations in ug./g. wet tissue corresponding to the figures immediately above.

Tissue	Trout	Perch	Frog	
			R. temporaria	R. esculenta
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	7,020	2,010	-	-
Iris plus Ciliary body	5,990 (1,560)	6,380	-	-
Choroid	15,100 (3,100)	23,800	14,400	-
Pigment-protein fraction of Choroids	-	43,800	-	28,100

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.

TABLE 25. Concentrations of Zinc in Perch Skin, Muscle and Liver.

Tissue	Zinc concentration (ug./g.dry tissue)
Skin (white)	31.0
Muscle	57.7
Liver	59.6

Proportion of the copper and zinc in the perch choroid bound to the insoluble pigment-protein fraction.

The dry matter in 73.3 mgs. of fresh perch choroid was found to weigh 8.6 mgs. When 309.2 mgs. of fresh choroids were digested with trypsin and a pigment-protein fraction isolated from the supernatant, the dry weight of this fraction was 14.9 mgs. The dry matter in 309.2 mgs. fresh choroid will be $309.2 \times \frac{8.6}{73.3} = 36.2$ mgs.

Using the concentrations of zinc and copper given in Tables 1 and 2, the proportions of copper and zinc in the choroid bound to the insoluble pigment-protein fraction can be calculated.

The percentage of total choroid zinc bound in this manner is $\frac{4.38 \times 14.9}{2.38 \times 36.2} \times 100 = 74.7\%$ and the percentage of total choroid copper $\frac{49.3 \times 14.9}{30.5 \times 36.2} \times 100 = 53.0\%$. These fractions of the total (Cu or Zn) are attached to only 41.6% of the dry weight or 4.82% of the wet weight of the choroid.

<u>Section 3</u>. <u>Discussion</u>.

It is evident from Table 26 that the high concentrations of zinc obtained by Leiner & Leiner are not due to Lake Constance as a special habitat.

In all of the fish examined, 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

TABLE 26. ***Concentrations of Zinc in the Eye Tissues of Freshwater Fish.***

The Table mainly consists of the results obtained by Leiner & Leiner for fish from Lake Constance. Figures for trout and perch obtained in the present work are placed in brackets beneath the corresponding results of Leiner & Leiner. All concentrations are expressed as ug./g. dry tissue.

Species	Zinc Concentrations					
	Choroid	Choroid Body	Argentea	Iris	Retina	Lens
1. Blue char. (Salmo lavaretus)	12,550	1,520	4,750	7,800	1,370	26
2. Sea trout. (Trutta lacustris)	9,200-11,350 (15,100)	780 - 1,490 -	6,360 (7,020)	6,960-8,700 (5,990)	540 - 990 (580)	16 (15.7)
3. Whitefish? (Gangfish) (Corregonus macrophthalmus.Nüssl)	6,760	1,080	-	4,320	810	16
4. Bream (Abramis brama L.)	7,550-10,400	470 - 910	1,620-4,430	5,360-7,330	260-940	16 - 17
5. Roach (Leuciscus rutilus)	18,300	-	-	-	1,160	-
6. Perch (Perca fluviatilis C.)	24,000-29,600 (23,800)	510-1,730 -	8,200 (2,010)	7,710-6,200 (6,380)	1,090-1,780 (728)	20 (15.6)
7. Char (Salmo salvelinus)	9,400	-	-	-	830	-
8. Lote or Burbot (Lota vulgaris C.)	9,800	640	-	10,500	2,800	32
9. Eel (Anguilla vulgaris)	15,000	-	-	-	210	40

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, 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 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 freshwater 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 530-9,800 ug./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 lens of mammals, the same order applies to results obtained on cattle, sheep, whale and rabbit eyes (cf. Chapter III). 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 tissues.

Table 25 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 fill 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 tissue in all species. The same cannot be true for copper. In general, pigmented tissues and pigment-protein fractions from all the vertebrate eyes examined in this work contain relatively high copper concentrations. In some species, however, 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 connection 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, contained a high copper concentration (672 ug./g. dry tissue). Frog eye tissues seem to have a relatively high copper content; the R. esculenta pigment-protein fraction being very 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.

At this stage it is possible to summarise in two points the position in regard to the distribution of zinc in vertebrate eyes. Firstly, combining the results obtained in the present work, and those in the literature, it can be stated as a tentative rule that the highest concentrations of zinc are found in the pigmented tissues. Secondly, the fractionation studies described in the present work have indicated that the zinc in the pigmented tissues is largely combined with a pigment-protein fraction. Though it appears that copper is also combined with the pigment-protein fraction, no such general statements can be made about this metal. The contrast between the two metals may be expressed differently by saying that it is probable that the main differences in the concentrations of zinc in the eye tissues of a particular species are due to differences in the melanin content, but that it is possible that the variations in copper concentration are due either to a multiplicity of chemical combinations, or to

none at all; no single chemical combination appears to be dominant.

The most outstanding thing about the distribution of zinc in vertebrates' eyes is the great accumulation of the metal in the pigmented parts of freshwater fish eyes. There are two possible explanations for the unique concentrations in these species. The first is that the pigment-protein fractions of the eyes have a specially strong avidity for zinc, the second that the supply of zinc to the tissues is unusually large. Both factors might, of course, be operative. The first factor will have some discussion in later chapters; only matters connected with the second factor will be mentioned here.

The concentration of zinc in lake water is probably a little higher than that in sea water, but not much. Noddack & Noddack (1940) showed that sea off the south coast of Sweden contained 14 ug. Zn per litre. Leiner & Leiner (1944) give figures for unfiltered water from Lake Constance of 30-60 ugs. Zn per litre. Filtered water will contain less than this. Zooplankton contain 200-300 ug./g. dry weight and phytoplankton much less. The possibility that the concentration of zinc in the surrounding fluid causes

the accumulation is rendered less likely by Leiner & Leiner's observation that the concentrations of zinc in the eye tissues did not increase after keeping the fish in water containing 400 ug. Zn per litre for several weeks. This, of course, does not prove that lowering the existing zinc content of a lake would not lower the concentration in the eye tissues.

There is a fundamental difference in the mineral metabolism of fresh and salt water fish. The former tend to conserve mineral salts, and the latter to excrete them. Whether this has any bearing on the difference in zinc contents of the eye tissues it is difficult to say. The need in freshwater fishes is for ionic materials to keep up the internal osmotic pressure. The zinc in the eye tissues is largely bound in non-ionic form to the pigment.

Whatever the complete explanation of the matter, it is certain that the chemical factor concerned with the actual pigment-protein complex of the eyes must play an important part, for if the supply of zinc were large, and there were no special avidity of this complex for zinc, then there would be no reason why very high concentrations of the metal are not found in the other eye tissues.

ORIGINAL WORK

Part II

CHAPTER VI.

FURTHER EXAMINATION OF PIGMENT FRACTIONS FROM THE EYES OF VARIOUS VERTEBRATE ANIMALS.

It was shown in Chapter IV and V that the zinc, and to a lesser extent the copper, of the pigmented tissues of the eyes of various animals, was largely associated with a brown-black, insoluble, fraction of these tissues. The nature of this fraction is studied in the present chapter.

The insolubility of the material made it impracticable to study effectively the chemical homogeneity of the fraction. It was thought that the most likely impurity was protein. Further trypsin digestion of the material from cattle irises showed that the zinc concentration could be slightly raised (e.g. from 870 to 1,200 ug./g. dry material) and the copper concentration slightly lowered by this process. The concentrations of the metals were never greatly changed by

trypsin digestion, however protracted. The decrease in the dry weight of pigment material was insignificant when trypsin digestion was continued for longer than three days, and it therefore appeared that a state was reached when no further protein was digestible. With perch choroid pigment, the dry weight concentration of zinc was decreased from 4.38% to 4.10% after three days trypsin digestion. Owing to the shortage of material it was not possible to discover whether this decrease was associated with a removal of organic substance. A comparison of the rapid digestion of the compact iris tissue from cattle, and choroid tissue from perch, with the slow removal of a small amount of material from the finely divided pigment fraction, indicates that there is little free protein in the latter material.

Section 1. The Nature of the Organic Material of the Pigment Fraction.

A. The non-chromogenic organic material.

Greenstein, Turner & Jenrette (1940) digested malignant melanoma tissue from mice with pancreatin, and isolated an insoluble pigment fraction from the digest. They showed that about two-thirds of this material consisted of protein or other amino acid compounds. The amino acids

were split off by hydrolysis with HCl, leaving a pigment with a nitrogen value (8.8%) which corresponds with that of melanin formed from tyrosine. They considered the non-melanin part of the pigment to be derived from the pseudo-globulin of the melanoma tissue. Melanin itself contains almost no free $-NH_2$ groups (Percival & Stewart, 1930) and is therefore not protein in nature. Serra (1945) considered that the pigment in hair is bound to keratin; he termed the whole complex 'melanokeratin' or simply melanin, and the pigment remaining after hydrolysing the protein with acid 'melanoid'.

Experiments were therefore carried out to show whether the pigment fraction of eyes is analogous to these materials.

Acid hydrolysis.

Part of pigment fractions from cattle irises and perch choroids was weighed and analysed intact, and part weighed and treated with 0.1 N HCl for 1 hour on a steam bath. The insoluble residue of the hydrolysis was centrifuged down, washed, dried, weighed, and analysed. Results are shown in Table 27.

TABLE 27. Hydrolysis of Pigment Fractions.

Pigment Fraction	Dry wt. (gs.)	Copper content (ugs.)	Zinc content (ugs.)
Cattle iris fraction (1)	0.122	9.0	100
Residue after hydrolysis of (1)	0.074	8.1	0.8
Cattle iris fraction (2)	0.129	11.3	91
Residue after hydrolysis of (2)	0.080	10.5	2.2
Perch choroid fraction (3)	0.0083	-	365
Residue after hydrolysis of (3)	0.0041	-	2.4

It is therefore clear that part of the organic material of pigment fractions is subject to solution by acid hydrolysis, and part is not. The zinc in the fractions is almost entirely split off by acid hydrolysis, but the copper in the cattle material largely remains attached to the insoluble residue.

Ninhydrin Test.

The presence of free amino groups may be detected by the use of ninhydrin, which gives a blue-purple colouration when boiled with compounds containing these groups.

The test was carried out by boiling a little ninhydrin with pigment suspensions for 2 minutes and then centrifuging till the supernatant was free of pigment.

The original cattle iris pigment fraction showed a strong blue colouration in the supernatant. A 'melanoid', obtained from this material by hydrolysing with acid till half the original dry weight was left, gave no colouration.

No reaction was observed with a pigment fraction from perch, but this may have been because insufficient material was available (about 1 mg.) to give a reaction.

This test, together with the results of acid hydrolysis, indicate that about half of the pigment fraction obtained from eyes is protein or amino acid in nature, and the whole material is analogous to the melanin complexes obtained from melanomas and from hair.

3. The chromogenic organic material.

The colour, and the resistance of the material to acid hydrolysis, indicate that the chromogenic part of the pigment fraction is melanin in nature. These two properties are common to all melanin pigments which have been examined for them, but they are not really exclusive.

Different workers have used different combinations of properties for characterising various brown and black pigments as melanins. It is therefore by no means certain that all the materials known by the name 'melanin' have the same chemical structure. It is likely, however, that most of the chromogenic parts of these materials are related to each other, and have a common chemical structure, which is probably that of a polymer whose units are indole derivatives (Raper, 1928).

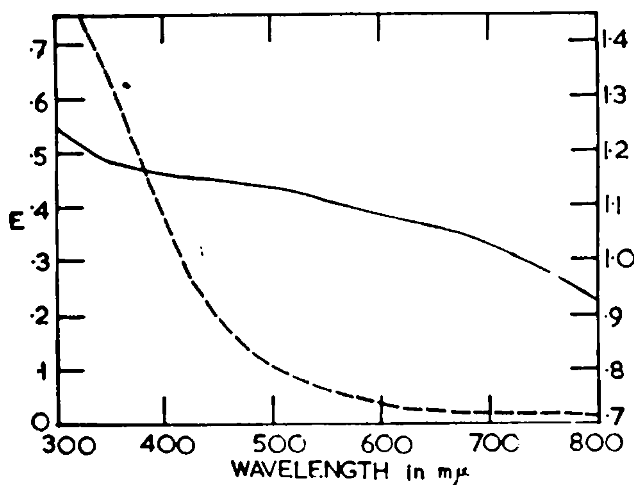
The characteristic properties of melanins are discussed by Mason (1948); the following were selected as the most convenient for settling the identity of the eye pigment.

Bleaching of the colour.

Melanins are bleached by permanganate and dichromate; Sachs (1943) showed that bleaching with hydrogen peroxide

Figure 7

Bleaching of the melanin colour by conc. HNO_3.



———————Absorption spectrum of 1 ml. of a melanoid suspension diluted to 20 mls. with water.

– – – – – –Absorption curve of 1 ml. of the same melanoid suspension treated with 1 ml. conc. HNO_3 for three days, diluted finally to 20 mls. with water, and readings taken with the Beckmann instrument using 1 ml. HNO_3 in 20 mls. water as a compensating solution.

gave a non-fluorescing substance.

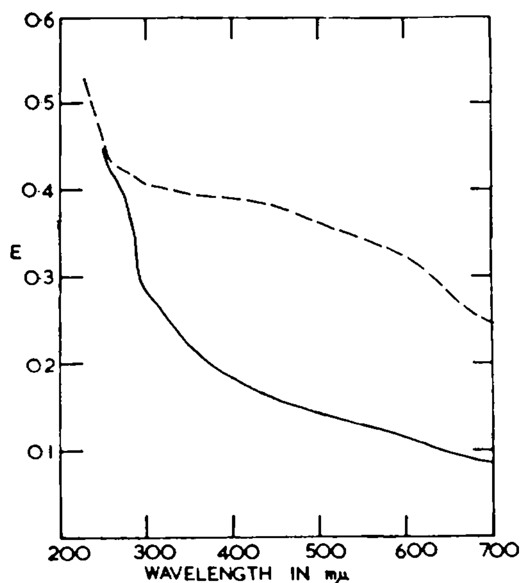
Suspensions of cattle, frog, and perch pigments obtained in the present work were found to be bleached to a yellow or light brown shade by 100 vol. H_2O_2, $K_2Cr_2O_7$ in dilute H_2SO_4, and 50% HNO_3. The nitric acid caused almost complete bleaching in a couple of hours, but the other reagents took up to 24 hours before the action was complete. The absorption curves of a cattle iris 'melanoid' fraction and an equivalent amount of the material bleached with HNO_3 are given in Figure 7.

Absorption curves.

Colour is a primary characteristic of melanin. Bloch (1927) stated that melanin may be coloured in a scale which ranges from bright yellow and red yellow through light and dark brown to deep black. Nevertheless a number of attempts have been made to characterise melanins in terms of absorption spectra (Bloch & Schaaf, 1925; Daniel, 1938; Baker & Andrews, 1944; Zwicky & Almasy, 1935; Arnow, 1938; Young, 1914). It has become clear that the mode of absorption of light by these substances is complicated by their colloidal states and by concomitants with which they are known to be associated (Mason, 1948). The curves are

Figure 8

Absorption spectra of cattle melanin suspensions, before, and after, acid hydrolysis.

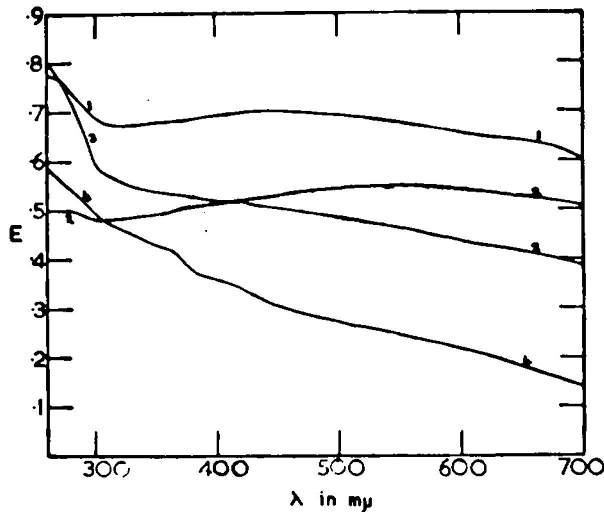


——————Original melanin fraction in water (pH 5.0).

– – – – – –Suspension of the insoluble residue after hydrolysing the original melanin fraction.

Figure 9

Absorption spectra of melanin fractions from fish and frog eyes.



Curve 1. Fresh perch pigment.

Curve 2. Fresh frog (R. esculenta) pigment.

Curve 3. Frog (R. esculenta) pigment after standing in water at 0° C. for 2 weeks.

Curve 4. Frog (R. temporaria) pigment obtained after the choroids had stood under water at 0° C. for 2 weeks.

similar, however, in that they all have the appearance of somewhat modified particulate absorption (end-absorption) curves. Serra (1945) found differences between the absorption curves of 'melanin' and 'melanoid' suspensions.

Absorption curves, measured on the Beckmann and Unicam photoelectric spectrophotometers, for the various pigment fractions from eyes examined in the present work are given in Figures 8 and 9.

The curve for cattle iris melanin fraction in Fig. 8 is similar to the curves given in the literature mentioned above. The difference between this curve, and the curve for the insoluble residue after acid hydrolysis of the original material, is similar to the difference between the 'melanin' and 'melanoid' curves of Serra (1945).

The curves for fresh perch and frog pigments in Fig. 9 are very different from that for the cattle pigment. The absorption in the higher wavelengths is almost equal to that at lower wavelengths. This difference was evident to the unaided eye, for the pigment fractions from fish and frogs were grey in shade, whereas the cattle pigment was brown. This distinction was also evident when the materials were dried. The absorption curve of a suspension of any

material in water or other fluids is a composite of two factors; the scattering of light by the particles, which depends on their size, and the absorption of light by the particles, which is dependent on their chemical structure. The scattering of light by particles cannot alone give rise to a linear absorption curve such as that observed for fish and frog material; in fact it is unlikely that the particulate absorption would have any measurable effect on the total absorption at wavelengths longer than 450 mu. The differences in the pigment curves beyond this wavelength must therefore be taken to indicate a difference in chemical structure.

Figure 9 shows that the perch and frog (R.esculenta) pigments change their absorption curves gradually on allowing suspensions of the material in water to stand at 0° for some time. The curve becomes intermediate between the original one, and the curve for cattle material.

A similar intermediate position is occupied by the curve for pigment from Rana Temporaria choroids which had been preserved for 2 weeks under water at 0° C.

Solubility.

Melanins have been reported to be soluble in a wide variety of solvents. These reports have dealt with melanins which either have been treated with strong reagents which may have degraded them, (e.g. Bloch & Schaaf, 1925), or with melanins which occur naturally in a very finely divided state (e.g. Greenstein et al., 1940), and with finely divided synthetic melanins. The reports do not distinguish between true and colloidal solution, and it seems likely that the difference between the insoluble melanins of Schmiedeberg (1897), Serra (1945), and many others, and the soluble ones, is merely a matter of particle size. Very many investigators have shown that strong alkalis render melanins soluble to some extent; this may be an effect on particle size.

The pigment material from all the eyes examined appeared to go into some kind of solution when treated with excess 1% NaOH or KoH. The material from a suspension in water could always be recovered completely by centrifugation. Only a fraction of the material suspended in NaOH solution could be recovered in this way. In experiments carried out on cattle material it was found that more of the original

material could be recovered by adding acetic acid to the alkaline mixture till the pH was 4.5. At this pH a flocculent precipitate was produced which contained more copper and zinc than the original material. The results of a quantitative experiment on these lines are given in Table 28.

TABLE 28. Fractions of a 'suspension' of Cattle Iris Pigment Fraction in 1% NaOH.

Fraction	Dry wt.(gs.)	Cu content ug./g.dry material	Zn content ug./g.dry material
Original pigment fraction	0.2964	63	771
Deposit on centrifuging the 'suspension'	0.1021	63	681
Precipitate from acid solution	0.0553	135	970

The amount of the deposit obtained by centrifuging the NaOH 'suspension' was reduced by increasing the strength of the NaOH solution, and by heating the mixture.

Hygroscopic nature of the dry pigment.

Young (1914) extracted the skin of an Australian aborigine with 5% KoH. From the extract, he separated a pigment material; after prolonged drying he found that this material was hygroscopic. In the present work it was noticed that ocular pigment materials which had been dried at 110° C. increased in weight when left to equilibrate in balances containing no drying agent, but were unaffected in balances where the air had been dried.

There are thus a number of properties which show the similarity of the chromogenic part of the black ocular pigment with melanins from other sources. Though the fresh pigments from frogs and perch differ from the cattle pigment in one property, the absorption curve, materials from these sources underwent a change on standing in contact with water, which produced pigments with curves intermediate between the original and the cattle pigment curve. In conjunction with the similarity in the other properties this indicates that all the chromogenic materials belong to the same general type, though they are not identical. The reported figures for C, H, O, N and S in melanins are so

variable (cf. Waelsch, 1932) that it was thought that an elementary analysis would not throw any light on the problem. Previous workers (e.g. Sieber, 1886; Waelsch, 1932; Krause, 1933) have mainly assumed that the black pigment of eye tissues is melanin without enquiring far into what is meant by the term. The main bases for their adoption of the term appear to have been the colour and the general insolubility and lack of reactivity of the material. Sieber obtained pigment from choroids by scraping the pigment granules out of the tissue under water. Waelsch obtained the pigment as an insoluble residue after digestion of choroids for 8 weeks with a pepsin-HCl mixture. Both these workers found their materials to be generally insoluble in organic and inorganic solvents, but to be slightly soluble in aqueous NaOH or KoH. Krause obtained his pigment by extraction with 0.5% NaOH; he stated that this material was soluble in dilute acid and alkali.

Section 2. The Composition of the Inorganic Part of the Pigment Fractions.

The amount of ash material remaining after incineration of ocular pigment fractions was always greater than the total zinc and copper content.

During the fractionation process the pigment was washed and centrifuged down from twice distilled water at least five times in order to remove free protein. During this process all soluble inorganic material was removed. This fact was shown by comparing the ash weights of two samples of dried cattle iris pigment from the one digestion experiment, both incinerated in platinum crucibles. One sample was merely dried after the washing process; the other sample was dialysed for three days against twice distilled water before being dried. The respective ash contents were 2.594% and 2.590% of the dry material. Copper and zinc accounted for 0.0063% and 0.077% of the dry weight of this sample of pigment. The ash content of various samples of cattle iris pigment varied from about 1.5% to 2.6% of the dry weight, but in no case did copper and zinc account for more than a small part of the total ash weight. The ash weight from perch pigment was just less than 10% of the dry material and the zinc content 4.38%. The zinc thus accounts for a large part of the ash, but even assuming that the metal is present in the ash as a zinc salt, there must still be other cationic elements present.

Sieber (1886) found roughly 3% ash material in pigment from choroids; Waelsch (1932) found 1.9% in material from cattle choroids. Both found that the ash contained iron. There is no record in the literature of any work on the ash from fish eye pigment, or of a complete survey of the elements present in the ash of any purified melanin material. It was therefore decided to carry out a qualitative emission spectroscopic analysis of the ash of ocular pigment materials.

Experimental Technique.

The Hilger E1.301 version of the Littrow high dispersion spectrograph was used, and an arc source for excitation of the electrode and ash. The instrument was obtained in correct adjustment from the directions given by Fore (1950).

By means of the Hartmann diaphragm three spectrograms were taken without moving the photographic plate;

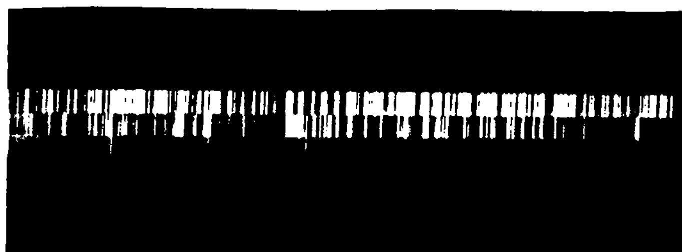
(a) a control consisting of pure graphite rods;

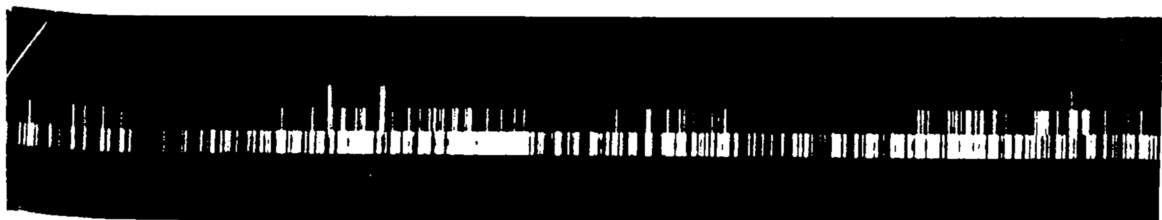
(b) the test material placed on a hollowed graphite cathode opposite a graphite anode;

(c) a reference standard consisting of two pure iron electrodes.

Figure 10

Emission spectrograms of the ash of dialysed cattle iris pigment. Contact prints obtained from the original plates.





Upper print - long wavelength range.

Lower print - short wavelength range.

Spectrograms were photographed for two wavelength ranges; 2250 - 2880 Angstrom units, and 2800 - 5050 Å. The exposure times for Ilford Ordinary plates were respectively 2 mins. (Iron 1.45) and 3 mins. (Iron 2.30).

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 spectogram was charted, and the unknown lines identified, by reference to labelled photographs in Brode (1939) and the wavelength and element tables in the Massachusetts Institute of Technology Wavelength Tables (1939).

A photograph of a typical spectrogram, taken from the original plates obtained with ash from dialysed iris pigment is given in Figure 10.

Results.

In many regions of the spectrum a given line cannot easily be assigned to a single element. In some parts of the spectrum there are few elements with any lines, but in others there are so many that even after the wavelength of the line has been determined accurately by the

scale of the Judd Lewis comparator there are often two or more elements with lines within the wavelength range which the inaccuracy of measurement and photography allow as error. It is often possible, however, to decide which element has produced the line by referring to brightness tables. Some of the possible elements may be found to have very weak lines at the particular wavelength in question, and these can be neglected. If this procedure failed to eliminate all but one of the possible elements for a particular line, the procedure followed was to search for raies ultimes of all the possible elements. The raies ultimes (r.u.) are the lines of an element which are most persistent, i.e. are the last to disappear on reducing the concentration of the element in the ash. If two or more of the main r.u.'s for an element were absent then it was usually assumed that the element was not present, though there is still a slight possibility that these particular lines were obscured for some reason. This procedure nearly always singled out the element to which a particular line must be ascribed. If it did not, the line was abandoned as being equivocal, and attention transferred to the next line in the test spectrogram not present in the control. A list of the

elements giving rise to the individual test lines was thus compiled. It was found that each element on the list occurred more than once, and that each of the elements had at least one r.u. on the spectogram.

The ashes successfully analysed were:-

(a) from dialysed cattle iris pigment;

(b) from dialysed cattle irises;

(c) from washed perch pigment.

The identified lines are listed in Appendix I, and the identified elements in Table 29.

TABLE 29.

Source of Ash	Elements definitely identified
Dialysed cattle iris pigment (Ashed in crucibles of SiO_2)	Cu, Mg, Ca, Ba, Zn, Fe, Si, Ti.
Dialysed cattle irises (Ashed in Pt. crucibles)	Cu, Mg, Ca, Ba, Zn, Fe, Si, Pt, Ti
Washed perch pigment (Ashed in Pt. crucibles)	Cu, Mg, Ca, Sr, Zn, Fe, Al, Si, Pt,

Impurities and artefacts.

Silicon and platinum were regarded as probable impurities arising as inevitable contamination from glass vessels and from crucibles.

The lines ascribed to titanium were regarded as probable artefacts. Some r.u.'s of this element were found in the control spectrogram from 'pure' graphite electrodes, as well as in the test spectrogram. The lines of titanium (amongst other elements) possess the property of being augmented in strength by the presence of other ionisable ash material. The appearance of the titanium lines in the test spectogram not present in the control is probably due to this augmentation effect.

The purpose of the analysis on cattle iris ash was to discover whether the elements in the pigment fraction were present in the tissue from which it originated, or whether they were introduced during the fractionation process. An analysis was originally carried out on fresh undialysed iris ash, but this was unsuccessful, for the vapourised sodium salts obscured much of the spectrum. The results obtained show that, apart from the possibility of contamination from the dialysis paper, which seems unlikely, there are no elements added to the pigment during the fractionation process. The fact that there was no sodium in the pigment fraction ash shows that soluble materials have been entirely removed.

The emission spectroscopic analysis is limited by the fact that the lines of the anionic elements are few and often faint; the present technique, partly because of the limited wavelength range, would not detect them.

It is fairly certain, however, that all the metallic elements present in the ash would be identified.

Discussion.

From the strength and number of their lines, iron and calcium appeared to be major, and copper, zinc, barium and magnesium, minor constituents of the ash of cattle material.

Major constituents of the perch pigment ash appeared to be zinc, calcium, and iron. Much less iron was present than in the cattle material. Al, Cu, Mg, and Sr appeared to be minor constituents.

Ramage & Sheldon (1931) found that the choroids of certain ox eyes contained barium in amounts up to 1.5% (roughly) of the dry weight. They also reported the presence of traces of strontium. The choroids of man, pigs, sheep, horses, dogs, and the common commercial fish contained no detectable quantities of barium. Barium was found in the choroids of all cattle of 3 years old, and over,

but not in calf choroids. They found barium also in irises, and the dark pigment separated from the choroid by rubbing. Other elements in the choroid ash were Na, K, Mg, Ca, Fe and Cu. Retinas, without the adhering pigment epithelium, contained similar quantities of Na, K and Mg, but less Ca and Fe than the choroids. Neither the retinas, nor any of the other tissues analysed, contained Ba.

In conjunction with the present work this therefore gives a strong indication that a number of metals are accumulated, by the process of pigment formation in eyes, which would otherwise occur either not all, or in much smaller amounts. The particular elements accumulated depend partly on the species of animal. In freshwater fishes, the work of Leiner & Leiner taken in conjunction with the present analysis of pigment fractions from perch, strongly indicates that zinc is the principal accumulation. In mammals, other metals appear to predominate.

Section 3. The Manner of Linkage between Inorganic and Organic Parts of the Pigment Fractions.

The only evidence pertaining to the problem of the forces which bind the metallic elements of the pigment

to the organic moiety, which has been obtained in this work so far, is that provided by the acid hydrolysis of the pigment fractions. This showed a difference between the binding of copper and of zinc; the latter was easily split off by treatment with hot dilute acid. Leiner & Leiner (1944) found that cysteine hydrochloride solution would extract nearly all the zinc from a dry-powdered homogenate of fish eyes.

It was decided to apply this cysteine hydrochloride procedure to a pigment fraction of eyes, and to look for further evidence concerning the binding of the metallic elements.

Cysteine hydrochloride treatment of a pigment fraction.

Leiner & Leiner extracted about 50 mgs. of material with 10 mls. 0.1 M cysteine HCl solution for several hours. In the present work a preliminary experiment was carried out by extracting 100 mgs. of cattle iris pigment for one hour with 10 mls. 0.1 M cysteine HCl solution. About 15% of the zinc was removed from the pigment, but none of the copper.

Exactly the same experiment was repeated, using an extraction time of 18 hours. The results are given in Table 30.

TABLE 30.

Material	Dry wt. (g.)	Cu content (ug.)	Zn content (ug.)
Cattle iris pigment (1)	0.1106	-	-
Insoluble residue after extracting (1)	0.1023	6.3	20
Cysteine HCl supernatant	-	1.6	42

The bulk of the ash material appeared to be in the cysteine hydrochloride crucible.

The pH of the cysteine hydrochloride solution was about 3.0; the possibility that the acidity was the active factor in removing zinc was ruled out by Leiner & Leiner, who showed that the metal was almost equally well extracted by neutralised cysteine HCl.

In the present work it was found that the pH of a suspension of the repeatedly washed residue of cysteine HCl extraction was about 3.8, whereas the original pigment suspension had a pH of 5.8.

Nature of the ash from the pigment fraction.

When the ash from cattle iris pigment material was first suspended in water the pH of the suspension was 6.5. If the mixture was allowed to stand for a few hours it was found that the pH had changed to 8.0. This can only be explained by the gradual interaction of oxides or basic salts in the ash, with the water.

Treatment of pigment with thiourea.

In solution the = S group of thiourea is in equilibrium with a - SH group. As thiourea is cheap and readily available, the possible use of this substance as a substitute for cysteine HCl in the extraction of zinc from pigment fractions was investigated. A molar solution of thiourea alone was found to have no effect on the metal content of the pigment. If the solution was made acid (0.02 N with H_2SO_4) then it was found that some of the zinc and copper were removed from the pigment after 2 days.

In addition, there was evident a difference in appearance of the treated material. The pigment had flocculated; the particles were both larger and heavier. It was later found that all these effects were due to the acid alone. The thiourea does not appear to have an action analogous to cysteine hydrochloride.

The possible linkage of the inorganic elements to sulphur groups in the protein of the pigment.

Greenstein *et al*. (1940) have shown that sulphur in the form of cysteine or cystine occurs in the protein which is not removable from a melanin fraction by trypsin digestion in much greater quantity than in normal proteins. Flesch (1949) suggested that the copper which he found in this material might be bound near the sulphur atoms. Nearly all metals are known to form mercaptides, of varying degrees of stability, in the presence of - SH groups, and the removal of zinc from ocular pigment material by cysteine hydrochloride fits in well with the hypothesis. It was therefore thought worthy of further investigation.

It had been found that dilute H_2SO_4 in the cold gradually removed zinc and other ash material from the pigment. If the hypothesis that the metals are linked to sulphur as

mercaptides is correct, then there should be a formation of -SH groups corresponding with the removal of metals. Assuming that this would give rise to an increase in the reducing power of the materials, it was decided to investigate the possible correlation between the amount of zinc and ash material removed from the pigment, and the reducing power of the material, as measured by 2-6 dichloroindophenol.

Experimental.

Four 2 ml. portions of a homogeneous suspension of cattle iris pigment in water were pipetted into four different centrifuge tubes.

To tube 2 was added also 0.1 ml. 0.1 N H_2SO_4 + 3.9 mls. H_2O.
To tube 3 was added also 1.0 ml. 0.1 N H_2SO_4 + 3.0 mls. H_2O.
To tube 4 was added also 1.0 ml. N H_2SO_4 + 3.0 mls. H_2O.
To tube 1 was added also 4.0 mls. H_2O.

The tubes were allowed to stand at room temperature for 20 hours and were then centrifuged, the supernatant poured off, and the solid shaken up with twice distilled water and centrifuged again. The washing was repeated and the centrifuged residues were washed into four Thunberg tubes with 4 - 1 ml. portions of Borate-Boric acid buffer (pH 7.34). To each tube was added 2 mls. of a standard

solution of 2.6 dichlorophenol indophenol. In a fifth (control) tube, 2 mls. of standard dye were mixed with 4 mls. buffer. Each tube was evacuated and filled with nitrogen, and the procedure repeated a second time. (The dye is slowly re-oxidised in air).

All five tubes were then incubated at 38° C. for 20 hours. At the end of this time their contents were poured into centrifuge tubes and centrifuged for 30 mins. at 3,500 revs. per min. The supernatants were poured into 20 ml. standard flasks and diluted to volume. These solutions, and the control similarly diluted, were then placed in an Eel colorimeter and readings taken with a red filter.

The solid residues of the reactions were dried and weighed.

The first part of the experiment, before admixture with the dye, was repeated under exactly the same conditions. From this experiment results were obtained for (a) the amounts of zinc and copper removed from the melanin fraction by various concentrations of H_2SO_4; (b) the percentage of the dry weight of the material made up by ash after the various treatments.

(a). Small amounts of organic matter which had become dissolved during the reaction were removed by shaking each supernatant from centrifugation with successive small amounts of carbon tetrachloride until no further emulsions were formed. This process allowed estimations of zinc and copper to be subsequently carried out on the solutions by the standard dithizone procedure without ashing, and without any hindrance to separation of the two layers.

(b). The solid residues were dried and weighed, ashed and weighed in platinum crucibles. Ash weights were determined in the Microanalysis Section of the Organic Chemistry Department.

The results are given in Table 31. The amounts of material used were too small to permit of significant analysis for copper.

It was evident from the results in Table 31 that the experiment had been unsuccessful in several ways.

1. The recovery of zinc as shown by the total (a + b) for each line, is not very satisfactory.

2. The measurement of ash weights is not satisfactory.

3. No correlation between the removal of ash material and the reducing power of the pigment was observed.

TABLE 31. The Removal of Zinc from, and the Reducing Power, of Pigment from Cattle Irises treated with various strengths of H_2SO_4.

Normality of H_2SO_4	ugms.Zn in 100 mgs. dry residue (a)	ugs.Zn in supernatant corresponding to 100 mgs.dry residue (b)	mgs.Ash from 100 mgs. dry residue	Eel reading of dye reduced by 100 mgs.dry residue
0.0	91.4	0	3.7	103
0.0014	-	0	3.8	-
0.0017	-	0	-	112
0.0125	-	-	1.8	-
0.017	-	23.2	-	91
0.02	80.1	30.2	1.5	-
0.17	13.3	59.7	1.8	88

A dash indicates that no analysis was made.

The minimum changes required for the experiment to give significant results are: a more reliable technique for estimating the reducing power, and a more reliable method of weighing the ash. Refinements in technique were not pursued because doubt arose as to whether, in fact, the change from a mercaptide to an - SH group would increase the reducing power of the pigment.

The results do, however, serve a useful purpose, and the experiments are recorded here because a rough relationship between the amount of zinc and ash material removed, and the strength of the sulphuric acid added, is demonstrated. An increase in the strength of acid increases the amount of inorganic material removed from the pigment in two days.

Section 4. Summary and Discussion of the Findings about the Nature of the Ocular Pigment Fractions.

It has been demonstrated in the preceding sections of this chapter:-

1. That the substance in the pigment materials obtained from the eyes of various vertebrates which is responsible for their colour belongs to the class of melanins.

2. Associated with the melanin is material of protein, or amino acid nature. This material must in some way be combined with the melanin, for it is not rendered soluble by prolonged digestion with trypsin.

3. Several metallic elements are combined in some way with the organic matter of the pigment; they are not removed by washing or dialysis. Zinc, and some other ash material, are partly split off by cold dilute H_2SO_4, and zinc is wholly removed by treatment with hot dilute HCl. Copper apparently remains attached after acid treatment. Zinc is also largely removed by treating the pigment with cysteine hydrochloride solution.

4. Melanin accounts for about half the dry weight of the pigment material. In cattle the ash weight is 1.5 - 2.6% and in perch about 10%.

5. The elements occurring in the ash vary from one species to another, and the amounts of each probably vary from one individual of a species to another; and from species to species.

The relationship between melanin and proteins in black animal pigments is not clear. Greenstein _et_ _al_. (1940) have shown that the two are associated in melanomas,

Serra (1945) and Stary & Richter (1938) likewise for hair. Adant (1932) showed that various melanins were antigenic, and this property may be due to associated protein. Abderhalden & Guggenheim (1907) and Sizer (1946) indicated that melanins could be formed by the oxidation of protein in the presence of tyrosinase, but there is nothing to show whether this is a normal physiological process or not. Regarding the linkage between protein and melanin, the work of Greenstein et al. (1940) indicated that it might be in the neighbourhood of the sulphur groups of cysteine and cystine. Stary (1949), however, treated 'leucokeratin' and 'melanokeratin' from hair with Na_2S solution, and found that the leucokeratin was then digested by trypsin, whereas the melanokeratin was not. He took this to mean that the insoluble nature of melanokeratin is not due to the interpeptidic linkage of sulphur atoms.

Pigments of the melanin type can be obtained in vitro in the complete absence of protein. Examples of this type of pigment are given by Spiegel-Adolph (1937) and Arnow (1938b). There is no evidence that these materials are really analogous with naturally occurring melanins.

The relationship between protein and melanin is only studied in the present work in so far as it is concerned with the association between melanin and various metallic elements. The relationship between the three parts of the ocular pigment material which have been identified in this chapter, melanin, protein, and inorganic constituents, is further studied and discussed in the following three chapters.

CHAPTER VII

METALS AND THE PIGMENT PRODUCED BY OXIDATION OF DI-HYDROXY PHENYL ALANINE IN VITRO.

Di-hydroxy phenyl alanine (dopa) was shown by Raper (1927) to be an intermediate in the formation of melanin from tyrosine in the presence of a tyrosinase. Dopa in solution can also be converted to a pigment of the melanin type by mild oxidising agents such as dilute H_2O_2, or by atmospheric oxygen. The melanin is obtained in the form of a brown (probably colloidal) solution. It was thought that the formation of this material might form a convenient system for the investigation of a possible combination between metallic elements and melanin in the absence of protein. Cunningham (1931) and Dubois & Erway (1946) showed that the presence of copper accelerated the oxidation of dopa. Flesch (1949) showed that a number of metals affected the rate of the reaction; the magnitude of

the effects varied in the order: Cu > Co > Ni > Mn > Pb > Fe > Zn. Copper is known to form complexes with dopa (Eicholtz, 1933) and Flesch suggested, in view of its presence in the pigment material from melanomas, that it remained in a similar association after the formation of melanin.

Before the paper of Flesch (1949) had been noticed, it was decided to investigate the effect of zinc and iron, which had both been found in natural pigments, on the rate of oxidation of dopa to melanin. It was found that zinc very slightly increased the rate of melanin formation, and had no effect on the total light absorption of the final product. Ferrous iron, however, increased considerably both the rate of formation, and the final light absorption of the completely converted material. There is therefore an indication that iron combines with the melanin, and that zinc does not.

Section 1. The Possible Combination of Metals with Melanin obtained by Oxidising Dopa.

A 0.1% aqueous solution of dopa was allowed to autoxidise in air till no further increase in the light absorption (as measured on the Eel photoelectric colorimeter) was observed.

5 mls. of the oxidised dopa solution were placed in each of eight 25 ml. standard flasks. 2 mls. of a 0.01% solution of a particular metal, as the sulphate or chloride salt, were added to each flask. The solutions were diluted to 25 mls. and readings of the general light absorption (i.e. without a coloured filter in the instrument) were taken on the Eel instrument after 15 minutes. The results are given in Table 32.

TABLE 32. Effect of Added Metals on the Light Absorption of a Melanin Pigment.

Metal salt added	Eel reading
-	25.0
$FeSO_4$	38.0
$Fe_2(SO_4)_3$	31.8
$CuSO_4$	27.0
$ZnSO_4$	25.0
$CaCl_2$	25.0
$MgSO_4$	25.0
$BaCl_2$	25.0

The solution of $FeSO_4$ had been allowed to stand for some time before the experiment; it was possible, therefore, that part of the effect of this salt might be due to ferric iron produced by oxidation. This problem was settled by the use of 0.1 M ferrous ammonium sulphate solution prepared immediately before use. The general light absorption of three solutions, diluted to 20 mls. in a standard flask, were read after 15 minutes in the Eel.

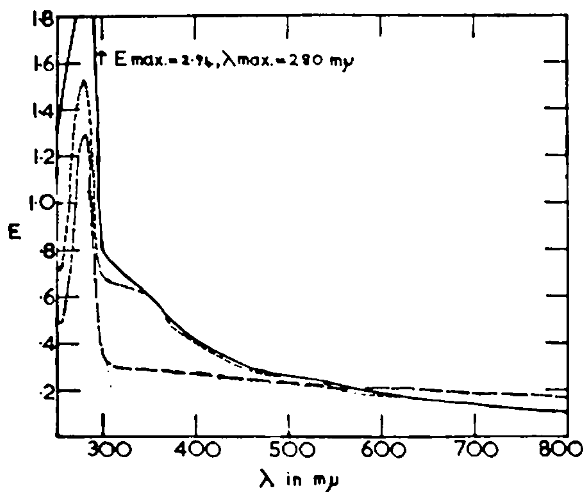
TABLE 33. Effect of a Ferrous Salt on the Light Absorption of a Melanin Pigment.

Solution	Eel reading of diluted solution
1 ml. melanin soln.	9.3
1 ml. melanin soln. plus 1 ml. $FeSO_4.(NH_4)_2SO_4$	14.9
1 ml. $FeSO_4.(NH_4)_2SO_4$ alone	0.3

Tables 32 and 33 show that ferrous and ferric iron, and copper, all affect the light absorption of the oxidised dopa melanin, and therefore can be presumed to form some kind of compound with it. The other metals have no effect on the total light absorption of the pigment, but the

Figure 11

Absorption curves of oxidised dopa solutions with added iron.



——————— Oxidised dopa solution (0.01% approx)

- - - - - - - - Oxidised dopa solution with 0.001% Fe^{+++} solution.

— — — — — Oxidised dopa solution with 0.001% Fe^{++} solution.

converse reasoning cannot be applied; it is still possible that combination takes place without affecting the light absorption.

Absorption Curves of the Compounds with Iron.

2 mls. of oxidised dopa melanin solution (approx. 0.1% w./v.) were placed in each of three 20 ml. standard flasks; to one flask was added 2 mls. of 0.01% Fe^{++} solution as ferrous sulphate, and to another 2 mls. 0.01% Fe^{+++} as $Fe_2(SO_4)_3$ (freshly prepared, but containing some $Fe(OH)_3$). Each flask was stoppered and left standing for 6 hours. The solutions were then diluted to 20 mls. and absorption readings taken on the Beckmann instrument. The solutions to which iron salts had been added contained cloudy precipitates, but they were shaken up before taking readings, and fairly homogeneous suspensions were obtained. The absorption curves are recorded in Figure 11.

The colour of the solution to which ferrous sulphate had been added was almost black, as distinct from the brown of the original melanin. This difference is illustrated in the absorption curves; that of the solution containing ferrous iron having much the flatter curve between

300 and 700 mu. The colour of the solution containing ferric iron had a stronger reddish tinge than the untreated melanin, this corresponds to a greater slope for the former between 525 mu and 550 mu. The inflection between 510 and 530 mu may perhaps be due to ferric hydroxide, but it is notable that it corresponds with the absorption band found by Flesch & Rothman (1945) for a solution of the red iron-containing pigment which they isolated from red hair.

The Compound of Ferrous Iron with Melanin from Dopa Oxidation.

The possible stoichiometric relationship between ferrous iron and melanin in the compound whose absorption curve has just been described, was investigated as follows:-

1 ml. of the melanin solution (approx. 0.1%) was placed in each of seven 10 ml. standard flasks. To these were added varying amounts of 0.01% Fe^{++} as $FeSO_4$ solution. The flasks were allowed to stand for 1 hour and then diluted to 10 mls.

Four observations were then made on each solution:-

(i) Total light absorption, measured by the Eel without a colour filter.

(ii) The presence or absence of precipitation.

(iii) The presence or absence of any colour in the supernatant after spinning down any precipitate.

(iv) The presence or absence of a blue-green or green colouration on adding Pot. Ferricyanide solution to the supernatant.

Results are given in Table 33a.

TABLE 33a. The Reaction of Ferrous Iron with Melanin from the Oxidation of Dopa.

mls. $FeSO_4$ added	Eel reading	Precipitation	Brown colour in supernatant	Ferricyanide reaction with soln.
0	32	-	+	-
0.03	34	-	+	-
0.1	40	-	+	-
0.2	33	+	+	-
0.5	29	+	-	+
1.0	30	+	-	+
3.0	32	+	-	+

Below 0.2 mls. of $FeSO_4$ there is no precipitation, No Fe^{++} detectable in the supernatant, and the brown colour of the unchanged melanin is to be observed in the supernatant.

Above this amount there is precipitation, Fe^{++} detectable in the supernatant, and the brown colour of unchanged melanin occurs in the supernatant.

It must therefore be concluded that an insoluble stoichiometric compound of melanin and ferrous iron is formed, the ratio of iron to pigment by weight being roughly 1 : 50 (0.2 x 0.01 : 1 X 0.1).

The Eel readings are most readily explained by assuming that the coagulation of the compound of iron and melanin into large particles reduced the total light absorbed. The initial increase will be due to the phenomenon previously observed; the combination of the iron and melanin to form a cloudy solution without actual coagulation. This occurred in the previous experiment, when the two were allowed to react in dilute solution, and only for 15 minutes. In the present experiment the solutions were allowed to react for 1 hour before diluting. The final rise in the Eel readings, above 0.5 mls. $FeSO_4$ added, is due to the absorption of the ferrous sulphate itself.

Section 2. The Combination of Zinc with Mixed Solutions of Dopa Melanin and a Protein Fraction from Cattle Irises.

It was known that zinc is bound to a melanin protein complex occurring naturally in eye tissues (Chapters III, IV and V) and it had been observed that zinc could affect the light absorption of melanins formed from tyrosine in the presence of protein fractions from cattle irises (see Chapter VIII). In view of the fact that zinc does not affect the total light absorption of melanin (from the oxidation of dopa) when it is added in solution, it was decided to investigate whether zinc would change the light absorption of this melanin in the presence of protein.

Experimental.

The protein used in the experiment was obtained from a homogenate of ground-up cattle irises by centrifuging to obtain a supernatant free from melanin and tissue débris, and isolating from the supernatant the protein fraction which precipitated between 10 and 30 gms. of ammonium sulphate per 100 mls. of solution. This fraction is shown in Chapter VIII to be the most active in the catalytic production of melanin from tyrosine.

The experiment was conducted as follows:-

In three 10 ml. standard flasks of pyrex glass were placed respectively:

(a) 1 ml. of oxidised dopa solution (0.1%)

(b) 1 ml. of oxidised dopa solution (0.1%) plus 1 ml. of a solution of the cattle iris protein.

(c) 1 ml. of oxidised dopa solution, 1 ml. of the same protein solution and 1 ml. of 0.01% zinc solution (as $ZnSO_4$).

In two 5 ml. standard flasks of pyrex glass were placed respectively:

(d) 1 ml. of the protein solution

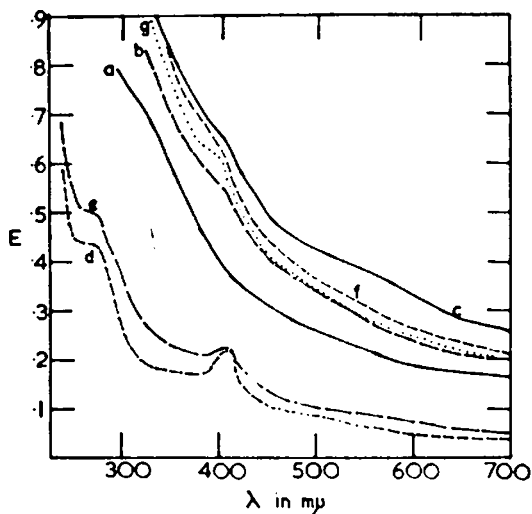
(e) 1 ml. of the protein solution plus 1 ml. of 0.01% zinc (as $ZnSO_4$) solution.

All the flasks were stoppered with glass stoppers and placed in a refrigerator at 0° C. for 4 days.

At the end of this time the solutions were made up to the appropriate volume with twice distilled water, and their absorption curves were determined on the Beckmann instrument. The absorption readings were all converted to figures for a 5 ml. dilution. These figures are given in Appendix 2, and the absorption curves of the various solutions, and combinations of solutions, are given in Figure 12 (Omitting some of the maxima between 240 mu and 350 mu).

Figure 12

The interaction of protein, oxidised dopa, and zinc, as shown by the absorption curves of mixed solutions of these substances.



The letters correspond with those in the text on page 170.

Also:-

f = a + e [calculated]

g = a + d ["]

Discussion of the Results.

The letters on the graph, which correspond with those used in the experimental description, are convenient for the preliminary discussion.

There is little notable difference between the curves for the various solutions containing melanin, in the region 240 - 350 mu. This region has been omitted from Fig. 12 owing to the high figures for the maxima, which in every case occur at approximately 280 mu. The only feature worthy of mention is the E max. (maximum extinction, or absorption, value) in curve b, which is lower than that calculated for (a + d). If zinc mixes with the protein and dopa solutions, and if dopa mixes with protein, without any reaction taking place, then the curve (c) should correspond exactly with the calculated (a + e) curve, and the curve (b) should correspond exactly with the calculated (a + d) curve. A solution of zinc sulphate was found not to absorb any light within the wavelength range under consideration here. The fact that the curve for zinc plus protein has higher extinction values than that of the protein alone shows that there is some reaction between the two. Similarly the slight difference between curve (b) and the

calculated (a + d) indicates that some combination has taken place.

The appreciable difference between curve (c) and the calculated (a + e) curve shows that a reaction can take place between zinc, protein, and melanin obtained by dopa oxidation, which increases the light absorption in the visible region, and decreases the absorption of the dopa-melanin in the 280 mu absorption band. The fact that no elaborate means were used to bring about the reaction, indicates that it could very easily be a physiological process; all that is required is the mixture of the three reactants in solution. It could not be confirmed that the reaction would take place at 37°, because the protein soon underwent changes, probably due to extraneous infection. These changes would presumably not occur *in vivo*.

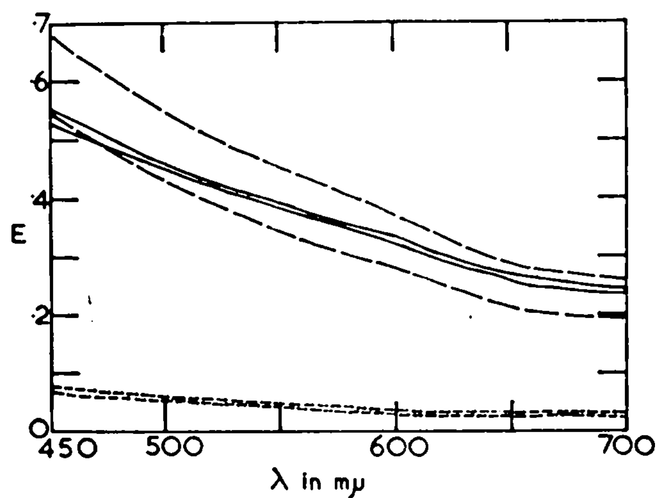
Effect of zinc on the light absorption of iris protein fractions.

Cattle irises were homogenised in the manner described in Section 1 of the next chapter. The super-natant of this homogenate was separated into fractions as follows:-

The supernatant was diluted to 100 mls. and 10 gms. crystalline ammonium sulphate added; as this salt dissolved,

Figure 20

Absorption spectra of protein fractions from the supernatant of an aqueous extract of cattle irises.



————Protein precipitated by 10 gs. Amm.sulphate/100 mls

— — —Protein precipitated by 10-20 gs. do.

--------Protein precipitated by 20-30 gs. do.

The upper curve in each case belongs to the solution to which $ZnSO_4$ was added.

the pH was adjusted to approximately 7.0 with Na_2CO_3. The mixture was allowed to remain at 0° C. for 2 hours, after which time a precipitate had begun to settle. The precipitate was centrifuged down and dissolved in 10 mls. of twice distilled water. The supernatant of the centrifugation was treated with a further 10 gs. of crystalline ammonium sulphate, the pH again being adjusted to 7.0. The resulting precipitate was allowed to settle at 0° C. for 2 hours and centrifuged down as before. A further 10 gs. of crystalline ammonium sulphate was added to the supernatant, and the previous procedure repeated.

Three solutions containing three different protein fractions in 10 mls. water were thus obtained. 1 ml. portions of each solution were placed in each of two 5 ml. standard flasks. To one flask of each pair was added 1 ml. of 0.01% Zn as $ZnSO_4$ solution. Each solution was diluted to 5 mls. with twice distilled water and absorption readings taken on the Beckmann instrument.

The absorption curves of the various solutions are shown in Figure 20.

Zinc increases the light absorption of each protein solution, but the effect is much greater for the fraction

which precipitates between 10 and 20 gs. of ammonium sulphate per 100 mls. supernatant, than for the other fractions. The effect of zinc on the 0 - 10 gs. fraction is very slight.

It seems, therefore, that zinc combines with a specific fraction of the cattle iris protein, rather than all the material. The procedure used is unlikely to give a perfect separation into fractions, and the effect of zinc on the light absorption of the two outer fractions may well be due to straying of material from the middle fraction.

CHAPTER VIII

METALS AND THE ENZYMIC SYNTHESIS OF MELANIN FROM TYROSINE

It has long been accepted that tyrosine is the natural precursor of melanin in plants, and that the presence of a system capable of oxidising tyrosine is necessary for the formation of melanin in these organisms. Till recently, the absence of any demonstration of a similar system (tyrosinase) in mammals made it seem unlikely that the same mechanism functioned in these animals. It was not until 1942, when Hogeboom & Adams first showed that extracts of melanoma tissue could catalyse oxidation of tyrosine to melanin, that the possibility of the existence of a tyrosinase enzyme in mammals was realised. The enzyme is not freely water-soluble, as is the case in plant material, but is associated with submicroscopic tissue particles. Lerner & Fitzpatrick (1950) discuss the processes of melanin formation in plants and animals, and

state: " ... the separate concepts can now be merged into a single hypothesis to account for melanin formation in man, lower animals, insects, and plants." While this is perhaps an overstatement of the present position, it is certain that a tyrosinase can occur in mammals, and other animals, and, that largely owing to the inadequacy of the theory that dopa is the general precursor, tyrosine at present appears to be the most likely starting point for physiological melanin production in animals, as well as plants.

Calkins (quoted by Lerner & Fitzpatrick (1950)) showed that extracts of beef ciliary bodies had both tyrosinase and dopa oxidase activity. It was therefore decided to try in the present work to obtain an enzyme system from cattle irises capable of oxidising tyrosine to melanin, and then to investigate the effect of the various metals found in the pigment fractions of cattle eyes on this system.

Section 1. The Presence of a Tyrosinase System in Cattle Irises, its Extraction and Purification.

A preliminary demonstration of tyrosinase activity in cattle irises was carried out on the supernatant remaining after centrifuging a homogenate of 120 irises to obtain a pigment material (which was later rejected as impure).

1 ml. of a dilute tyrosine solution exposed to the air did not darken at all in 2 days. If 1 ml. of the supernatant from irises was added, darkening began to occur after 6 hours exposure.

Extraction procedure.

On repeating the extraction process first used, it was found that it did not yield an active solution with any certainty. Sometimes activity was present, sometimes it was not. Several modifications of the process were then tried to find a method which gave a reproducible extraction.

The following procedure was found satisfactory:

Cattle irises, or cattle irises and choroids, were washed with 0.1% acetic acid. The residue was placed in a conical flask and frozen to - 40° C. in solid CO_2 and alcohol, keeping it at this temperature for 1 hour. (An attempt to break up the melanophore cells which contain the enzyme.) The tissue was then allowed to warm up, and crushed with quartz in a glass mortar. The pH of the homogenate was adjusted to 7.5 with a solution of A.R. sodium carbonate in twice distilled water. The whole mixture was centrifuged at 2,000 r.p.m. till all the black

material was precipitated, and the supernatant again adjusted to pH 7.5 if necessary. Throughout the manipulations precautions were taken to exclude contamination from any stray sources of metallic elements.

It was later found by experience that the preliminary washing of the tissue was unnecessary. It was also doubtful whether any increase in yield was brought about by the freezing process; it was not used in all the experiments, and the extracts were still sufficiently active when it was omitted.

Quantitative comparisons of tyrosinase activity in the extracts were not used. A qualitative test, based on the work of Onslow (1917) was applied.

In a 25 ml. pyrex conical flask were placed 2 mls. of the enzyme preparation, 1 ml. of an approximately 0.1% (w./v.) suspension of tyrosine, and 0.1 ml. of 0.05% H_2O_2. The hydrogen peroxide is unnecessary for the production of blackening in the solutions, and it did not affect the total light absorption of the final product. It was found to reduce the time between mixing the solutions, and the first appearance of darkening. This time is the 'induction period', which is always a feature of the conversion of tyrosine to melanin in the presence of tyrosinase (Lerner,

Fitzpatrick, Calkins & Summerson, 1949).

Purification of the enzyme.

Various methods have been used to purify tyrosinase, (Lerner *et al*. 1949). Examples are:

(a) Precipitation at pH 4.75 with 0.1 M acetate-acetic acid buffer,

(b) Precipitation with 40% alcohol, removal of the precipitate, and precipitating again with 60% alcohol. The enzyme should be in the second precipitate.

(c) Ammonium sulphate, and other salt precipitation methods.

Only the last group was found to produce fractions with any activity at all. The precipitate with 20 gms. ammonium sulphate per 100 mls. solution had some activity, but not as much as the original solution.

It was found that animal tyrosinase (in crustacea) has been reported to be much more sensitive to inactivation at pH's below 5.0, than is the plant material. The ammonium sulphate fractionation was therefore repeated, with the addition of Na_2CO_3 during the process, so that the pH was kept above 6.5. This procedure was relatively successful.

It was found:-

(i) That proteins precipitated by up to 18 gms. of crystalline ammonium sulphate per 100 mls. solution contained no activity.

(ii) The range, from 18 - 35 gms. ammonium sulphate added to 100 mls. of solution gives protein precipitates which are active.

(iii) The most active fraction (as measured by the amount of melanin formed after 16 hours at 37° C.) is precipitated by between 25 and 30 gms. added to 100 mls. solution.

(iv) The supernatant of the 30 gms. precipitation had some activity, but no activity was found in either the precipitate, or the supernatant, of 35 gms. precipitation. The process of precipitation must therefore be capable of destroying some activity.

A solution of the 25 - 30 gs. precipitation process was adjusted to pH 7.5 and used as Preparation 1.

Using the information obtained in the above fractionation, further enzyme preparations were obtained as follows, when required for a series of experiments.

The supernatant of a homogenate of irises, obtained in the manner previously described, was diluted to 50 or

100 mls., and 18 gms. ammonium sulphate (crystalline A.R.) per 100 mls. added, at the same time adjusting the pH with dilute sodium carbonate solution to between 6.5 and 7.5. The resulting precipitate was centrifuged down, the supernatant poured off, and 17 gms. more ammonium sulphate added. The pH was again adjusted to pH 6.5 - 7.5, and the precipitate allowed to form for 3 hours in the refrigerator. The mixture was centrifuged, the precipitate dissolved in twice distilled water, and Na_2CO_3 solution added till the pH was 7.5. The solution was dialysed against twice distilled water containing Na_2CO_3 for 18 hours.

Preparations 2, 3, 4 and 5 were obtained in this way.

The enzyme preparations were stored in a refrigerator at 0° C. They were never found to keep their activity for more than a few days even under these conditions; the rate of deterioration of the preparations was variable.

A part of Preparation 5 was treated to remove sodium carbonate, and substitute sodium citrate buffered to pH 6.8 with NaOH. The enzyme solution, containing a small amount of sodium carbonate, was dialysed against a pH 6.8 mixture of sodium citrate and NaOH, in the refrigerator for

3 days, the outer solution being changed 6 times. The resulting solution was called Preparation 5a.

Section 2. Manometric Experiments with Enzyme Preparations from Cattle Irises.

Though the term 'tyrosinase' has been applied often to systems whose only known property is that they catalyse the conversion of tyrosine to melanin, there are several properties necessary to characterise the system as the chemical entity 'tyrosinase', as it has been isolated by various workers (cf. Lerner & Fitzpatrick, 1950).

The necessary properties are:-

(i) The material must catalyse the oxidation of tyrosine by molecular oxygen, the final product of the reaction being melanin.

(ii) Copper must be demonstrated to be an essential part of the enzyme material.

The second property has often been identified by inference from the results of inhibition studies (e.g. Lerner _et_ _al_., 1950). The catalysis of the processes in (i) are inhibited by such compounds as Thiouracil and Di-ethyl dithiocarbamate; if this inhibition is reversed by the addition of copper salts, but no other metals, then

there is indication that copper is part of the enzyme complex.

The uptake of atmospheric oxygen, and the effect of inhibitors on the reaction, can both be most easily investigated by manometric methods.

In the present work the Gallenkamp version of the Warburg apparatus was used for the manometric work.

The general principles, and the experimental details for the use of the Warburg apparatus are well known; reference was made to Dixon (1934) when necessary.

No uptake of oxygen on mixing a 'brei' of cattle irises with tyrosine and phosphate buffer in the Warburg flasks occurred after 6 hours at 38° C.

Some success was obtained using Preparation 2 (described in Section 1 of this chapter).

<u>Experimental Details.</u>

Six Warburg flasks of the single stoppered side-arm type were used for the experiment. They were numbered 1, 2, 5, 7, 13 and 23.

Flask No.	Contents of the main compartment	Contents of the side-arm
23	3 mls. of water	-
1	1 ml. of tyrosine suspension (0.1%) 1 ml. phosphate buffer (pH 6.8)	1 ml.enzyme soln. (Prep. 2)
7	1 ml. tyrosine suspension (0.1%) 1 ml. phosphate buffer (pH 6.8)	1 ml. of water
5	1 ml. dopa solution (0.1%) 1 ml. phosphate buffer (pH 6.8)	1 ml. enyzme soln. (Prep. 2)
13	1 ml. dopa solution (0.1%) 1 ml. phosphate buffer (pH 6.8)	1 ml. of water.
2	1 ml. of water 1 ml. of phosphate buffer (pH 6.8)	1 ml. of enzyme soln. (Prep. 2)

The six flasks were fixed to their respective manometers, stoppered, and placed in the water bath of the Warburg apparatus, which was thermostatically maintained at 38° C. With the vent to the atmosphere left open, the flasks were allowed to equilibriate for 15 minutes with the shaker switched off. The shaker was then switched on, and the flasks oscillated for 2 minutes. The shaker was stopped, the left hand column of manometer fluid adjusted to the middle (15 cm.) mark in each case, and the taps turned

so that the flasks communicated only with the manometers. The shaker was switched on, and the rate of oscillation adjusted to 75 per minute. Shaking was stopped every half an hour to adjust the left-hand columns of the manometers to the 15 cm. mark again, and to record the readings in the right-hand column. After the first three hours, during which time hardly any change took place in the readings (presumably an induction period was operating), the apparatus had to be left, and readings taken only at times of 7½ and 8 hours from the start of the experiment.

Results.

The difference in cms. between the level of fluid in the right-hand limb and in the left-hand limb is recorded as 'h'. In every case except one the left-hand column was lower than the right-hand column, so that 'h' is nearly always positive. 'δ' is the calculated difference between 'h' for flask 23 (the control which measures only the effect due to atmospheric pressure), and the flask being considered.

Using these conventions, the results are tabulated below.

TABLE 34. Readings with Warburg Manometers in an Experiment on Enzyme Preparation 2.

Time (hrs.)	FLASK 1 h	1 δ	7 h	7 δ	5 h	5 δ	13 h	13 δ	23 h	23 δ	2 h	2 δ
3	0.25	0.2	0.1	0.05	0.15	0.10	0.15	0.10	0.05	-	0	-0.05
$7\frac{1}{2}$	5.20	3.05	1.5	0	2.85	0.70	1.80	0	2.15	-	1.8	-0.35
8	5.80	4.10	1.6	0	2.85	1.15	1.95	0.25	1.7	-	1.55	-0.15

To calculate the changes in volumes of gases in the various flasks from the change in pressure, the flask constant (k) is employed. The flask constant depends on a number of factors which are the same for each flask, and one, the flask volume, which is an individual characteristic.

$$k = \frac{(V_g - V_F) \times \frac{273}{t} + V_F \times \alpha}{Po}$$

where V_g = the volume of the flask,
V_F = the volume of liquid in the flask (3 mls. here),
t = absolute temp. of the bath (292° here),
α = the absorption of O_2 in mls./ml. fluid at the temperature of the bath (0.0235 here),
Po = the atmospheric pressure (760 mms. approx.).

V_g was estimated by filling the flasks with mercury, according to the method of Dixon (1934).

TABLE 34. Readings with Warburg Manometers in an Experiment on Enzyme Preparation 2.

Time (hrs.)	FLASK 1 h	1 δ	7 h	7 δ	5 h	5 δ	13 h	13 δ	23 h	23 δ	2 h	2 δ
3	0.25	0.2	0.1	0.05	0.15	0.10	0.15	0.10	0.05	-	0	-0.05
7½	5.20	3.05	1.5	0	2.85	0.70	1.80	0	2.15	-	1.8	-0.35
8	5.80	4.10	1.6	0	2.85	1.15	1.95	0.25	1.7	-	1.55	-0.15

To calculate the changes in volumes of gases in the various flasks from the change in pressure, the flask constant (k) is employed. The flask constant depends on a number of factors which are the same for each flask, and one, the flask volume, which is an individual characteristic.

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α = the absorption of O_2 in mls./ml. fluid at the temperature of the bath (0.0235 here),
Po = the atmospheric pressure (760 mms. approx.).

V_g was estimated by filling the flasks with mercury, according to the method of Dixon (1934).

Expressing volumes in uls. (cu.mms.) the k values for the flasks were found to be:-

k_1 - 1.679; k_2 - 1.457; k_5 - 1.847;

k_7 - 1.543; k_{13} - 1.457.

Using these values, the volumes of oxygen absorbed in the various flasks were calculated, and are recorded below.

TABLE 35. The Volumes of Oxygen in uls. absorbed by various mixtures during an experiment with Enzyme Preparation 2.

Time (hrs.)	FLASK 1 (Tyrosine plus enzyme)	FLASK 7 (Tyrosine alone)	FLASK 5 (Dopa plus enzyme)	FLASK 13 (Dopa alone)
3	3.4	0	1.8	-
7½	50.5	0	12.9	-
8	68.8	0	21.2	3.5

The small increase in pressure in the flask containing enzyme and buffer alone was probably not significant.

Flasks 1 and 5 were the only ones which showed any blackening.

It is evident from this experiment that the material obtained from cattle irises contains an enzyme system which catalyses the oxidation by molecular oxygen of tyrosine and dopa to melanin. The particular fraction used for the experiment (Preparation 2) appeared to be more active towards tyrosine than dopa.

To show that this enzyme system is actually a single copper-containing protein analogous to the materials obtained from plant sources requires further work.

An attempt was made to investigate the effect of various inhibitors on the reaction, to see whether the enzyme system reacted analogously to tyrosinase. No inhibition of the reaction was demonstrated either for thiourea, or di-ethyl dithio-carbamate. However, the uptake of oxygen was in every case very slow and, if there were any tyrosinase present it could only have constituted a small fraction of the total weight of protein in the preparation. It is doubtful, therefore, whether it can be concluded that the inhibitors would have no effect on the pure enzyme system; they may have been diverted from their action on the enzyme by combination with other materials in the preparation studied.

Whatever the chemical nature of the enzyme system, at the very least it is one means by which tyrosine can be converted to melanin in cattle irises. As such, it was used in the studies into the possible functions of various metals in the formation of melanin pigment in eyes, which are described below.

Winternitz (1918) described an enzyme from the uveal tract of hogs which darkened a solution of tyrosine. He did not investigate whether the reaction was one involving molecular oxygen. The uptake of oxygen from the air is a feature which is characteristic of all the other tyrosinase systems described, and to this extent the process of melanin formation described in the present work is certainly analogous to them.

Section 3. The Effect of Various Metals on the Pigment formed from Tyrosine in the presence of Enzyme Preparations from Cattle Irises.

The experiments described in this section were intended to show whether the metals found in pigment fractions of cattle irises, when added to a mixture containing tyrosine and a protein fraction from irises which catalyses the formation of melanin, had any effect on the light absorption of the pigment formed. Observations on the effect of these

metals on the rate of colour production in this system were also recorded, but as the number of factors controlling the rate of reaction are known to be numerous, it was not considered practicable to study these effects definitively.

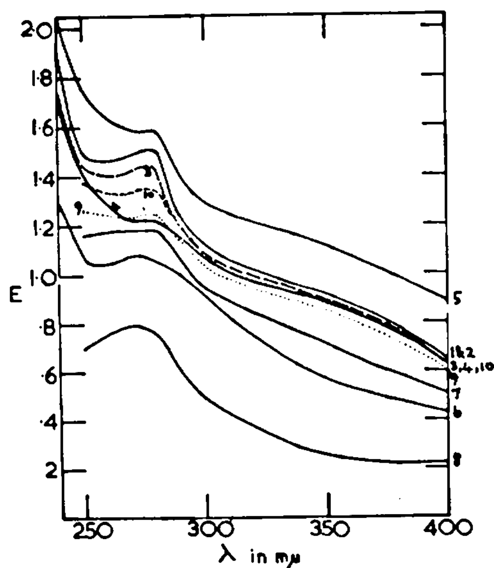
The principle of the experiments was to compare the absorption curves of solutions to which metals had been added, with others to which they had not, in every case taking readings when pigment formation was complete.

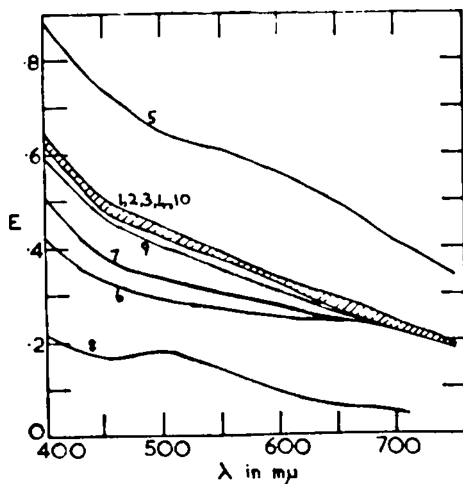
The conditions of the experiment were those described in Section 1 of this chapter for the assay of the activity of various enzyme preparations.

By measurement of the total light absorption of the solutions in the Eel, it was observed that colour production stopped under these conditions usually after one or two days, and was always complete before 3 days had elapsed. If the solutions were left for 6 or 7 days after mixing, the pigment particles began to agglomerate, and the colloidal solution was destroyed. Up to 4 or 5 days the particles of pigment were so small that they were not precipitated by prolonged high speed centrifugation. At 5 or 6 days the effects of infection by various organisms often became apperent. It was decided therefore to take absorption readings three days after mixing the solutions.

Absorption spectra of melanins enzymically synthesised in the presence of various metals.

Figure 13 - u.v. region.
Figure 14 - visible region.





The numbers correspond with those in the text for Experiment 1 (page 195).

diluted to volume, the contents mixed thoroughly, and absorption readings for each solution taken on the Beckmann instrument.

Experiment 1.

The enzyme solution used was Preparation 1.

To flask 1 was added: nil.

To flask 2 was added: nil.

To flask 3 was added: nil.

To flask 4 was added: 50 ugs. Ca as $CaCl_2$.

To flask 5 was added: 40 ugs. Zn as $ZnSO_4$.

To flask 6 was added: 20 ugs. Fe^{++} as $FeSO_4$.

To flask 7 was added: 20 ugs. Fe^{+++} as $Fe_2(SO_4)_3$.

To flask 8 was added: 20 ugs. Cu as $CuSO_4$.

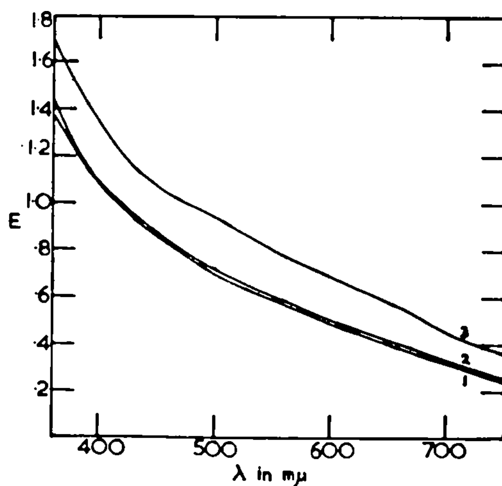
To flask 9 was added: 50 ugs. Ba as $BaCl_2$.

To flask 10 was added: 50 ugs. Mg as $MgCl_2$.

The absorption curves of the pigments obtained by incubating these solutions are given in Figure 13 (u.v.) and Figure 14 (visible region). It will be noted that several of the curves correspond very closely on the photograph. An even closer correspondence rendered useless the full representation of some of the curves; the extinction readings of all the solutions are therefore given in Appendix 3(a)

Figure 15

Absorption spectra of melanins enzymically synthesised in the presence of various metals (visible region).



The numbers correspond with those in the text for Experiment 2 (page 197).

Experiment 2.

Only a small part of the enzyme preparation 2, to which an excess of Na_2CO_3 had been added (making the pH about 7.8) was preserved from deterioration. This preparation was used here.

To flask 1 was added: nil.

To flask 2 was added: nil.

To flask 3 was added: 100 ugs. Ca as $CaCl_2$.

The absorption curves of the resulting pigments are given in Figure 15 (visible region only).

Experiment 3.

Enyzme Preparation 4.

To flask 1 was added: nil.

To flask 2 was added: nil.

To flask 3 was added: 10 ugs. Cu as $CuSO_4$.

To flask 4 was added: 20 ugs. Fe^{++} as $FeSO_4$.

To flask 5 was added: nil.

To flask 6 was added: 20 ugs. Fe^{+++} as $Fe_2(SO_4)_3$.

To flask 7 was added: 100 ugs. Ca as $CaCl_2$.

To flask 8 was added: 80 ugs. Ca as $CaCl_2$.

To flask 9 was added: 100 ugs. Zn as $ZnSO_4$.

To flask 10 was added: 80 ugs. Zn as $ZnSO_4$.

To flask 11 was added: 50 ugs. Zn as $ZnSO_4$.

To flask 12 was added: 100 ugs. Mg as $MgCl_2$.

To flask 13 was added: 80ugs. Mg as $MgCl_2$.

To flask 14 was added: 100 ugs. Ba as $BaCl_2$.

To flask 15 was added: 100 ugs. Co as $CoCl_2$.

The contents of flask 16 were mixed just before diluting for reading on the Beckmann and there was therefore no melanin produced; besides the standard constituents, 100 ugs. Zn as $ZnSO_4$ were added.

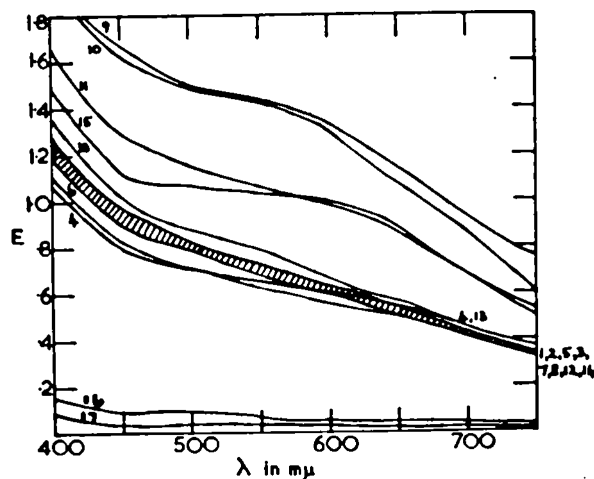
The contents of flask 17 were similar to those of 16, but no zinc was added.

In order to test that the effects of the metals on the absorption curves were not due to differences in pH, the pH's of the various pigment solutions were recorded by the use of a B.D.H. capillator set.

The results are given in Table 36.

Figure 16

Absorption spectra of melanins enzymically synthesised in the presence of various metals (visible region).



The numbers correspond with those in the text for Experiment 3 (page 197).

TABLE 36.

Metal added to the reaction mixture	pH of the melanin solution produced
-	6.65 ± 0.05
-	6.6 " "
-	6.5 " "
10 ugs. Cu	6.5 " "
20 ugs. Fe^{++}	6.6 " "
20 ugs. Fe^{+++}	6.55 " "
100 ugs. Ca	6.6 " "
80 ugs. Ca	6.7 " "
100 ugs. Ba	6.6 " "
100 ugs. Co	6.6 " "
100 ugs. Zn	6.7 " "
80 ugs. Zn	6.6 " "
50 ugs. Zn	6.6 " "
100 ugs. Mg	6.5 " "
80 ugs. Mg	6.65 " "

The absorption curves of the solutions are given in Figure 16 (visible region) and the extinction readings in Appendix 3 (b).

Visual observations during Experiment 3 are recorded below for various stages of the reaction.

12.30 p.m. Wednesday.

Experiment started. Flasks containing Zn slightly more opalescent than the others.

4.0 p.m. Wed.

100 and 80 ugs. Zn flasks begin to go grey.

4.45 p.m. Wed.

50 ugs. Zn flask begins to go grey.

5.0 p.m. Wed.

Control flasks slightly darker than all others apart from zinc.

6.15 p.m. Wed.

Controls dark brown. Zn flasks continually growing more grey. Others at 5.0 p.m. stage of controls.

7.30 p.m. Wed.

Controls brown-black. Zn flasks dark grey. Others dark brown.

8.45 p.m. Wed.

Co flask light grey. Cu, Fe^{++}, Fe^{+++} grey-brown. Zn flasks dark grey. Others brown black.

9.45 p.m. Wed.

Co flask dark grey. Fe^{+++} flask reddish-brown. Others merely deepened their former shades.

9.45 a.m. Thursday.

Cu flask light grey-brown. Zn flasks grey-black. The rest appear brown-black.

Experiment 4.

Enzyme preparation 4.

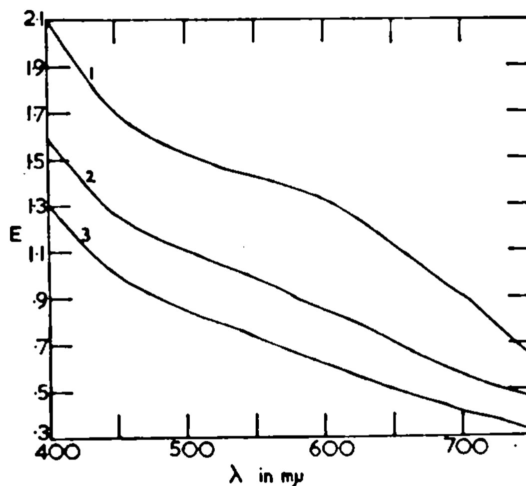
To flask 1 was added:100 ugs. Zn at the start of reaction.

To flask 2 was added:100 ugs. Zn 60 hrs. after the start, (i.e. 12 hrs. before end).

To flask 3 was added: nil.

Figure 17

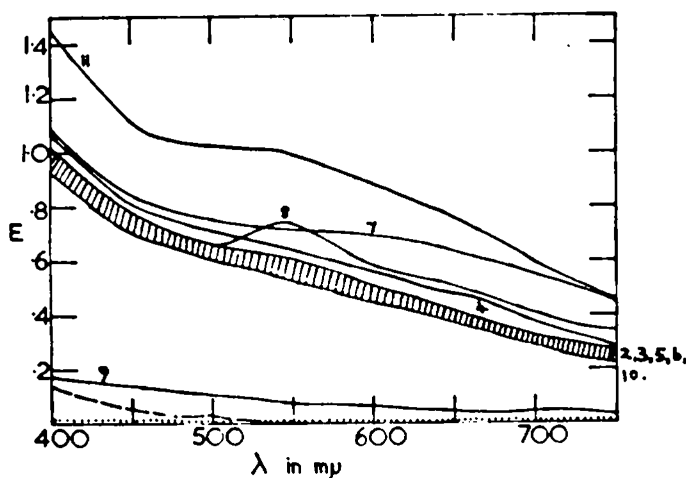
Absorption spectra of melanins enzymically synthesised in the presence of various metals (visible region).



The numbers correspond with those in the text of Experiment 4 (page 201).

Figure 18

Absorption spectra of melanins enzymically synthesised in the presence of various metals.



The numbers correspond with those in the text of Experiment 5 (page 204).

— — — — — 100 γ Fe^{+++} as $Fe_2(SO_4)_3$ in 20 mls. water.

··············· 100 γ Zn as $ZnCO_3$ in 20 mls. water.

The absorption curves of the pigments produced are given in Figure 17.

Experiments in which sodium citrate and citric acid were added to the enzyme solution as a buffer.

Experiment 5.

Enzyme preparation 5(a). pH 7.0 with sodium citrate and citric acid.

To flask 1 was added: nil.

To flask 2 was added: 100 ugs. Zn as $ZnSO_4$.

To flask 3 was added: 100 ugs. Mg as $MgCl_2$.

To flask 4 was added: 70 ugs. Zn as $ZnSO_4$.

To flask 5 was added: 100 ugs. Ca as $CaCl_2$.

To flask 6 was added: 100 ugs. Ba as $BaCl_2$.

To flask 7 was added: 100 ugs. Fe^{++} as $FeSO_4$.

To flask 8 was added: 100 ugs. Fe^{+++} as $Fe_2(SO_4)_3$.

To flask 9 was added: 50 ugs. Cu as $CuSO_4$.

To flask 10 was added: 100 ugs. Co as $CoCl_2$.

To flask 11 was added: 100 ugs. Zn as $ZnCo_3$ suspension.

The absorption curves of the resulting pigment solutions are given in Figure 18, and the extinction readings in Appendix 3 (c).

The pH values of the various pigment solutions are given in Table 37. (B.D.H. Capillator set).

TABLE 37.

Metal added to the reaction mixture	pH of the melanin solution produced
-	7.0 ± 0.05
100 ugs. Mg	7.3 " "
100 ugs. Zn as $ZnSO_4$	7.1 " "
70 ugs. Zn	7.4 " "
100 ugs. Ca	7.1 " "
100 ugs. Ba	7.3 " "
100 ugs. Fe^{++}	7.1 " "
100 ugs. Fe^{+++}	6.6 " "
50 ugs. Cu	7.2 " "
100 ugs. Zn as $ZnCO_3$ suspension	7.4 " "
100 ugs. Co	6.8 " "

Experiment 6. Possible effect of particles.

Enzyme preparation 5(b).

To flask 1 was added: nil.

To flask 2 was added: nil.

To flask 3 was added: nil.

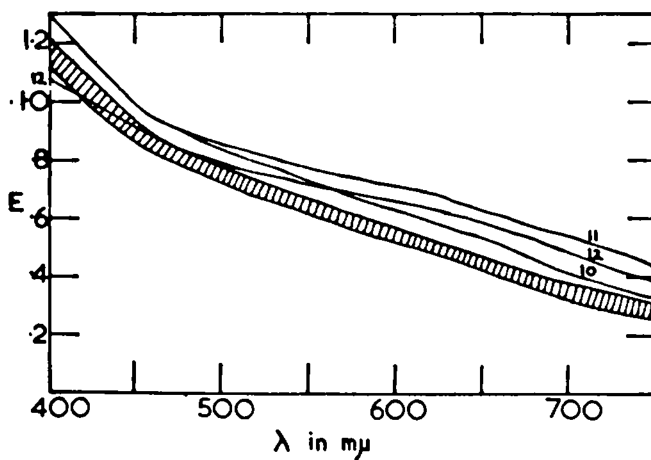
To flask 4 was added: 100 ugs. Ca as $CaCO_3$ suspension.

To flask 5 was added: 100 ugs. Zn as $ZnCO_3$ suspension.

To flask 6 was added: 100 ugs. Si as SiO_2 suspension.

Figure 19

Absorption spectra of melanins enzymically synthesised in the presence of various metals (visible region).



The numbers correspond with the text of Experiment 6 (page 205).

Curves 1 - 9, inclusive, occur within the shaded area.

To flask 7 was added: 100 ugs. Ba as $Ba(OH)_2$.

To flask 8 was added: 100 ugs. Ba as $BaCO_3$.

To flask 9 was added: 100 ugs. Al as Al_2O_3.

To flask 10 was added: 100 ugs. Zn as $ZnSO_4$ plus 1 mg. Na_2CO_3.

To flask 11 was added: 50 ugs. Fe^{++} as $FeSO_4$ plus 1 mg. Na_2CO_3.

To flask 12 was added: 100 ugs. Fe^{++} as $FeSO_4$.

The absorption curves of the resulting pigment solutions are given in Figure 19.

Summary and Discussion of the Results in this Section.

1. It is evident that the addition of various metals to the reaction mixture can affect the absorption curve of melanin formed from tyrosine, in the presence of protein from cattle irises which contains a tyrosinase system.

2. The anions of the buffer solutions used play a part in determining the effects which the various metals produce.

3. In solutions at pH 6.5 - 6.7, buffered only by the addition of Na_2CO_3 to the protein solution, 10 - 25 ug. Zn/ml. enhanced the light absorption of the melanin produced at all wavelengths. 25 ug. Co/ml. also enhanced the light absorption, but 5 ug./ml. of Fe^{++} and Fe^{+++} reduced the light absorption below 650 mu. [Figs. 13, 14, 16]

4. In solutions at a pH above 7.0, 25 ug. Ca/ml. enhanced the light absorption, in the presence of protein and Na_2CO_3 only. [Fig.15]

5. In solutions containing sodium citrate and citric acid (and probably NaOH also), zinc has no effect unless carbonate is also added. 12.5 ug. Fe^{++} and Fe^{+++}/ml. enhance the light absorption without adding carbonate. [Fig.18]

6. 2.5 ug./ml. of copper has no effect on the melanin absorption curve, but 5 ug./ml. and 12.5 ug./ml. inhibit melanin formation entirely. An absorption band at 500 mu, possibly due to the intermediate compound hallachrome, occurred in the solution containing 5 ug. Cu/ml. [Fig.14]

7. The absorption curve for the solution containing 12.5 ug./ml. of zinc was much lower than that for the [Fig.16] solution containing 20 ug./ml., but the latter curve was very little different from a solution containing 25 ug. Zn/ml.

8. The absorption curves of melanins produced in the presence of a metal are characteristic, and differ from those obtained with another metal. It is notable that the pigment formed in the presence of ferric iron* shows an absorption band with a maximum which corresponds roughly with the inflexion found for the compound between dopa and ferric iron (cf. Chapter VII), and with the absorption band

* This experiment has not been reproducible, and it seems likely that the absorption band is due either to an intermediate in melanin formation, or to an extraneous contaminant.

of the iron-containing pigment which Flesch isolated from red hair. There is no such band in a solution of $Fe_2(SO_4)_3$ partially hydrolysed to $Fe(OH)_3$.

9. Experiment 5 (Fig. 18) raised the possibility that the enhancement of the light absorption of the pigment might be due to the presence of inorganic particles in the reaction mixture. Experiment 6 showed that particles of $BaCO_3$, $Ba(OH)_2$, Al_2O_3, SiO_2, and $CaCO_3$ had no effect on the melanin. Zinc in the form of a suspension of $ZnCO_3$ had less effect than zinc in the form of $ZnSO_4$, to which Na_2CO_3 had been added.

10. Experiment 4 (Fig. 17) showed that zinc added at the start of the reaction has a greater effect than zinc added towards the end.

The inhibition of melanin formation by concentrations of copper higher than 5 ug./ml. is interesting in view of the facts that copper is known to be part of the tyrosinase molecule, and that it is known to accelerate the formation of melanin from dopa. Figge (1948) showed that various reversibly oxidisable substances could enhance the rate of dopa oxidation by atmospheric oxygen, but could completely inhibit the oxidation of tyrosine by tyrosinase. The

inhibitory effect was due to a change in the redox potential of the reaction mixture. It seems possible that a similar effect is operating in the present case.

It is possible that the lack of any enhancement of the melanin light absorption by iron in solutions containing Na_2CO_3, is also due to a redox effect. In the presence of carbonate only, 25 ug./ml. of Fe^{++} and Fe^{+++} were found, in a qualitative experiment, to inhibit melanin formation almost entirely.

The experiments carried out in citrate-citric acid solution were not entirely satisfactory, because the buffer was used at the extremity of its effective pH range, and the pH was therefore not rigidly controlled. Qualitative experiments were carried out in which various other buffer solutions were added to the reaction mixture. Borate-boric acid buffer (pH 7.3) inhibited the reaction completely. Melanin was formed in the presence of a Britton & Robinson standard buffer (pH 6.8) but the induction period was lengthened. Phosphate buffer (pH 6.8) allowed melanin formation to occur, and it was qualitatively observed that both zinc and ferrous iron slightly enhanced the light absorption of the melanin formed.

Section 4. The Amount of Zinc combining with Pigment enzymically synthesised from Tyrosine, and with the Protein Fraction from Cattle Irises responsible for this Reaction.

It has been shown that very high concentrations of zinc occur in melanin-protein fractions from some fish eyes; this zinc is non-dialysable. The fact that zinc can affect the absorption spectrum of pigments formed from tyrosine in the presence of cattle iris proteins shows that some kind of zinc combination is formed. No such combination occurs with melanin formed from dopa in the absence of protein, but if protein is added to a mixture of zinc and the dopa-melanin, the light absorption of the mixture of the three becomes greater than the sum of the individual components. It therefore seems possible that the increased light absorption of the reaction products observed on the addition of zinc to the reaction mixtures, described in the last section, is due to the formation of a complex of melanin, protein, and zinc, analogous to the materials obtained from various eyes by the trypsin digestion procedure.

To test whether the combination of melanin and protein really forms a complex with zinc during the melanisation reaction, the following experiment was carried out.

The object of the experiment was to find out how much of a given excess of zinc was taken into non-ionic combination with: (a) melanin formed in the absence of protein; (b) melanin formed in the presence of protein; (c) the same protein without melanin.

Experimental Procedure.

The reactants were placed in three 250 ml. beakers, the ratio of surface area to total volume of reactants was then large enough to permit ready absorption of oxygen.

Beaker A contained 10 mls. tyrosine suspension (0.1%), 10 mls. citrate buffer (pH 6.8) containing 2,000 ugs. Zn as $ZnSO_4$ and 2,500 ugs. of (CO_3) as Na_2CO_3, and 5 mls. of 0.1% dopa solution.

Beaker B contained 10 mls. tyrosine suspension (0.1%), 10 mls. citrate buffer (pH 6.8) containing the same amounts of $ZnSO_4$ and Na_2CO_3 as in A, and 5 mls. of a suspension in phosphate buffer (pH 6.8) of a protein fraction (about 0.25 gms.), precipitated between 10 and 30 gms. of crystalline ammonium sulphate per 100 mls. of an extract of cattle irises obtained in the manner described in Section 1 of this chapter.

Beaker C contained 10 mls. of twice distilled water, 10 mls. of citrate buffer (as for A and B), and 5 mls. of the protein suspension used in B.

All three beakers were incubated at 38° C. for 4 days, diluting each with 10 mls. of water on the second day. On the fourth day 50 mgs. of B.D.H. trypsin were added to beakers B and C, and the beakers incubated for a further 3 days at 38° C.

Beaker A was left in the incubator at 38° C. for a further 30 days (diluting with twice distilled water at appropriate intervals), when the formation of pigment appeared to have ceased.

After trypsin digestion the contents of beakers B and C were centrifuged, first at low speed to bring down the fraction which separates with the pigment, then at high speed to bring down as much protein as possible. The 'pigment fractions' were washed and re-centrifuged several times, and then dried, weighed, ashed, and analysed for zinc. The rest of the protein was discarded. The supernatants were evaporated at 100° C. to about 10 mls. volume and centrifuged again; the precipitates were discarded.

10 mls. of acetate buffer (pH 4.75) were added to each supernatant and the volume of each mixture made up to 50 mls. with twice distilled water. Two 0.5 mls. aliquots

from each solution were taken for zinc analysis. Each aliquot was diluted to about 7.5 mls. with twice distilled water and shaken with excess carbon tetrachloride in several portions to remove the remaining proteins in solution. The CCl_4 layers (droplets) were run off, and the zinc extracted from the solution by dithizone in carbon tetrachloride after adding 2.5 mls. more acetate buffer, and 1 ml. of sodium thiosulphate solution, as in the standard procedure used for zinc estimations.

After 30 days' incubation, beaker A was treated in a similar way, except that no trypsin digestion was used. Hardly any material precipitated during centrifugation.

Results.

Solution	Total ionic zinc (ugs.)	Total bound ∴ zinc (ugs.)
A	2,000	-
B	1,550	450
C	1,800	200

Zinc concentrations in the 'pigment fractions' separated by slow centrifugation:-

Solution	Dry weight (mgs.)	Zinc concentration (ug./mg.)
A	-	-
B	18.7	4.6
C	9.7	0.83

It is evident from this experiment that no zinc is bound in non-ionic form by melanin formed in the absence of protein, some is bound by the protein fraction from cattle iris, but much more is bound by a complex of this protein with melanin formed from tyrosine.

It is probable that the 10 mgs. of tyrosine present in solution B was not entirely converted to melanin. Assuming, however, that the conversion was complete, and that the melanin formed was combined with its own weight of protein (cf. Chapter VI) to give a total pigment fraction of 20 mgs., the amount of zinc combined with this must be 250 ugs., (450 total in B, minus 200 combined with protein) giving a concentration of 1.25%. This is of the same order as the zinc concentrations found in the pigment fractions obtained from frog and perch eyes (2.8 and 4.38% respectively). Trypsin digestion of a solution of melanin formed enzymically in the presence of zinc, in

Experiment 4 of the last section of this chapter, gave, after further fractionation, a pigment material containing 1.01% of zinc.

Some of the suspension of cattle iris protein precipitated between 10 and 30 gms. of ammonium sulphate per 100 mls. iris extract, was mixed with Na_2CO_3, and 200 ugs. zinc, in the form of $ZnSO_4$, added to it. The mixture was left in the refrigerator at 0° C. for 24 hours, and then dialysed against twice distilled water at 0° C. for 3 days. The protein material was precipitated again with Specpure ammonium sulphate, washed with a little twice distilled water, dried, weighed, and analysed for zinc. The total weight of material was 32.3 mgs. and the zinc content 27 ugs. The concentration of zinc is therefore 0.083%. This corresponds exactly with that for the material separated by slow centrifugation from solution B in the experiment described above. It seems, therefore, that this protein fraction from cattle irises is only capable of combining with a small amount of zinc, and that it then becomes 'saturated' with the metal. The amounts of zinc bound to the melanin-protein material show that a different factor must be operative in binding the zinc to this complex.

CHAPTER IX

DISCUSSION OF THE EVIDENCE FOR AN ASSOCIATION BETWEEN VARIOUS METALS AND MELANIN PIGMENTATION IN ANIMALS.

The chemical nature of the melanin pigment in animals is complex, and at present only partially elucidated. The number of factors which have been reported to be necessary for the normal physiological formation of melanin is very great, but there is as yet no comprehensive scheme which explains how, and when, all these factors are operative in the natural synthesis of the pigment. It can therefore only be surmised which of the factors are primary, and which are secondary to the main process.

It seems likely that one metal, copper, is a primary factor in melanin formation in animals. It cannot yet be regarded as settled that tyrosine is the natural precursor of melanin in animals, though it seems likely to prove so. Whatever the precursor, the only two enzymes known to occur in pigmented tissues and not in other animal

tissues, and which are capable of producing melanin, are tyrosinase and dopa-oxidase (Hermann & Boss, 1945). Lerner, Fitzpatrick, Summerson & Calkins (1950) showed that both the tyrosinase and the dopa-oxidase activity of melanoma extracts were dependent on the presence of copper. Complete removal of copper (as the cyanide salt) entirely stopped their activity; restoration of the original amount of copper restored 90% of the activity. Further evidence for the primary rôle of copper in melanin pigmentation is given by Lerner & Fitzpatrick (1950).

The present work has been concerned with various metallic elements in what seems likely to be a secondary rôle in the formation of animal melanin. There is considerable evidence that a secondary association between melanin pigmentation and various metals does exist, but often the individual results which go to make up this evidence appear to have been considered by the authors to be artefacts, and, through lack of any co-ordinating review, the significance of the whole body of results has not been examined.

The evidence for this secondary association comes under two headings:-

(i) Evidence obtained from studies on the composition of animal melanins and black animal tissues.

(ii) Evidence obtained from studies on the colour of pigments produced *in vitro* by the action of melanin-forming enzyme systems.

Several metals have been shown to occur in melanin preparations from animal tissues, or to occur in greater concentration in black and brown tissues than in other tissues.

Sieber (1886) divided animal melanins into different classes according to whether or not they contained iron or sulphur. Preparations of melanin from the uveal tract of the eye, and from melanomas, contained iron.

Gortner (1911) showed that pigment from the black hair of rabbits and from black feathers contained 2-3% ash, which was chiefly iron oxide (Fe_2O_3). He found two kinds of pigment in dark horse hair, one being a melano-protein with a very low ash content, and the other containing about 3% ash, which was chiefly iron oxide.

Waelsch (1932) obtained melanin from choroids by digesting the tissue with a pepsin-HCl mixture. The black insoluble residue contained 1.9% of ash, part of which was iron.

Many reviews mention that melanins have often been found to contain iron.

In the present work iron has been shown to occur in melanin preparations from cattle irises and perch choroids, the ash of the former containing a greater proportion of iron than the latter.

Flesch & Rothman (1945) isolated a red, ferric iron-containing pigment from human red hair by boiling and extracting it with HCl. The pigment also contained traces of Si, Cu, and Mg. The possibility that the pigment is analogous to melanins is indicated by the fact that the absorption band of a solution of his material corresponds with those found for complexes of Fe^{+++} with melanins in the present work. Nickerson (1946) isolated a red melanin from poultry feathers which he believed to be identical with that obtained by Flesch & Rothman.

Flesch (1949) showed that the white hair of rats and guinea-pigs contained considerably less iron than red hair. He also found that white hair contained slightly less copper than black hair from the same animal. Melanin fractions from melanomas were found to contain many times as much copper as the whole tissue.

Santaolalla (1949) showed that brown feathers contained slightly more copper than grey ones.

Guiliani (1938) examined the ash of the ink sac from Sepia Officinalis. The dried ink gave a total ash

of 1.86% and a copper analysis of 1.17%. The washed and dried empty ink bladder contained 3.9% ash, 2.02% Cu, and 0.31% Fe.

In the present work copper has been shown generally to occur in higher concentrations in pigmented eye tissues than in non-pigmented tissues, and to be concentrated in pigment fractions of the eyes.

The concentrations of copper found in pigmented materials by all the various workers are too high to be accounted for merely by the small amount of copper contained in tyrosinase. Also, the copper in the various pigment fractions is bound to highly insoluble material, whereas tyrosinase even in animals is a fairly soluble protein.

Ramage & Sheldon (1931) showed that barium occurred in the choroids and irises, and pigment fractions from these tissues, but not in other tissues of cattle eyes. In addition, the choroids contained more Ca and Fe than retinas which had been separated from pigment. Barium was not found in the choroids of a number of other animals.

The presence of barium in cattle irises and pigment fractions has been confirmed in the present work. This metal was not found in pigment from perch eyes.

It has been shown in the present work that the concentration of zinc in the pigmented eye tissues of a

number of animals is invariably higher than the concentrations in the parts of the eye which do not contain melanin. These results confirm those obtained by Leiner & Leiner (1944) for a number of species of fish. These high concentrations of zinc have been shown in the present work to be due to combination of the metal with a melanin-protein material of the pigmented tissues.

Complete analyses of the ash from pigment materials or pigmented tissues have rarely been carried out. Eckardt, Stolzar, Adam & Johnson (1943) give a list of the metals found in normal human corneas, and the corneas of patients suffering from hepato-lenticular degeneration (Wilson's disease). In this condition a brownish-green pigment is deposited in Descemet's membrane of the cornea. In normal corneas they found Na, Ca, and Mg, whereas in the corneas of patients suffering from Wilson's disease they found Na, Ca, Mg, Zn, Cu, Fe, Al, and Ag.

In the present work it has been shown that Fe, Zn, Ca, Mg, Ba, and Cu occur in pigment material from cattle irises, and Fe, Zn, Ca, Mg, Sr, Al, and Cu, in perch choroid pigment. Some of these elements were probably only present as traces.

Fore (1950) observed that white rabbit hair gave rise to much less ash than black hair from the same animal.

Experiments were carried out in the present work on hair from rabbits and rats. Rats with both black and white hair had been guillotined for experiments on their eyes, and hair was cut from the corpses of these animals. Hair from a black and white variety of rabbit was removed with the aid of an electrical cutter. The samples of black hair and white hair from the two species were washed with 20% alcohol (to produce wetting of the surface) and then several times with twice distilled water. The samples of hair were dried, weighed, ashed, and analysed. Analyses for copper and zinc were carried out by the standard procedure described in Chapter II. Analyses for iron in the ash from rat hair were carried out by the o-phenanthroline technique described by Sandell (1944). The results are given in Table 38.

Though the ash content of the white hair analysed is much lower than that of the black hair, the concentrations of iron, copper and zinc differ very little. Fore (1950) showed that the manganese contents of the two types of hair from rabbits were roughly the same. Some other ash constituent must therefore account for the big difference between the two types of hair.

TABLE 38. Results of an Examination of the Ash from Black and White Hair.

	Black Hair	White Hair
(a). Rabbits.		
% dry weight recovered as ash	1.153	0.381
Copper concentration (ugs./g. dry hair)	13.3	13.6
Zinc concentration (ugs./g. dry hair)	345.5	328.0
(b). Rats.	(Grey Hair)	
% dry weight recovered as ash	0.83	0.52
Iron concentration (ugs./g. dry hair)	30.9	28.8

It was shown in Chapter III that the zinc content of albino rabbit irises and choroids was much lower than that of the corresponding tissues from coloured rabbits.

There is thus a great deal of evidence which shows that various metals occur in higher concentration in tissues containing melanin pigment than in those which do not. All these metals have also been shown to occur in melanin pigment materials from various sources. It has been shown in the present work that the zinc bound to such materials can account for nearly all of the zinc in the pigmented eye tissues.

As far back as 1901, Gessard studied the action of various salts on the formation of melanin from tyrosine in the presence of tyrosinase. He stated that the precipitation phase, at the end of the tyrosine-tyrosinase reaction, was under the influence of various natural salts in the aqueous medium. The alkaline earth salts were found to be very much more active than the alkali metal salts. A red colour usually occurred as an intermediate stage in melanin formation; in the presence of an excess of calcium salts this was suppressed, and black was the first colouration to appear. However, he found also that the presence of various salts could cause a considerable retardation of the first

appearance of the black colouration.

Wolff (1910) stated that in the presence of disodium phosphate he obtained black melanin, and in its absence grey melanin.

Piettre (1912) confirmed that various salts were influential in the precipitation of melanin prepared by the use of animal tyrosinase. Durham (1905) found it expedient to add ferrous sulphate to the reaction mixture when she was testing various animal tissues for tyrosinase activity. Wager (1949) found that iron increased the colour of the pigment responsible for stem-end blackening in the potato.

There is thus much support in the literature for the view that various metals can combine with, and influence the colour of, melanins synthesised *in vitro* by the agency of either plant or animal tyrosinase. In the present work it has been shown that several metals can increase the colour, and influence the shape of the absorption curve, of melanin synthesised by the agency of an iris enzyme system. It has been shown that iron is the only one of these metals which will effect the light absorption of a melanin in the absence of protein, and in the case of zinc, it has been shown that zinc is combined in considerable amount by a

melanin-protein complex. It is notable that the percentage of zinc in such a synthetic complex of melanin with cattle iris protein approaches that found in natural melanin complexes from fish eye tissues. It has not been shown in what quantities metals other than zinc will combine with a melanin-protein complex, but it seems possible that some such combination is responsible for the presence of various metals in non-dialysable form in pigment materials from various animal sources.

In regard to the form of the metals in combination with synthetic melanin complexes, Gessard(1901) thought there was no true chemical linkage between metals and the pigment. He found that various insoluble alkaline earth metal salts could adsorb the rose-coloured intermediate in melanin formation, and that conversion to the black pigment could occur in the absorbed state. Piettre (1912) confirmed that a loose complex or co-precipitate, of variable composition, could be formed between melanin and the solid material of baryta-water. He also showed, however, that a compound between melanin and barium, other than an adsorption complex, could be formed. Addition of a solution of $BaCl_2$ to a tyrosine-tyrosinase reaction mixture gave a melanin product containing about 6.6% Ba. No chlorine was detected in

this material. In the present work, it has been shown that many insoluble metallic compounds with adsorptive properties have no effect on the melanisation reaction, whereas soluble salts of some of the same metals can have an effect.

There is something of an analogy between the combination of various metals with a melanin complex, and the combination of various metals with the peptidase enzymes (vide Smith, 1949). In both cases a number of metals can be involved, and the combination can be influenced by the pH and nature of the buffer.

Further investigation is required before a picture of the linkage between metal and melanin complex can be obtained. It seems possible, however, that sulphur groups may be involved in the case of zinc. Eisenbrand & Wegel (1941) showed that a complex between zinc and cysteine, cystine, or glutathione is formed on mixing $ZnCl_2$ with a solution of these compounds.

From the viewpoint of function, it seems fairly certain that no metal apart from copper is essential for the formation of the actual melanin molecule in living organisms. It seems probable, however, that various other metals can influence the intensity, and the shade, of the

melanin after it has been formed, and thus of the dark animal colours for which melanin is essentially responsible.

SUMMARY

OF THE RESULTS OF THE WORK,

THEIR IMPLICATIONS AND LIMITATIONS

CHAPTER X

SUMMARY OF THE RESULTS.

1. It has been shown (partly in confirmation of Tauber & Krause and Shakir) that the various tissues of the eyes of several mammals differ from each other in respect of their copper and zinc concentrations. Similar differences were found for the eye tissues of perch, trout, and frogs. These latter results confirmed the extensive studies of Leiner & Leiner on the zinc contents of fish eyes.

2. In all the species examined, the parts of the eye which contain melanin pigment had higher zinc contents than the other parts. The concentration of zinc in the choroids and irises of fish and frogs was very much greater than in the same tissues of mammals. The distribution of copper in the eye does not follow exactly the same pattern in all the species examined, as is the case for zinc. In general, however, the pigmented tissues are richest in copper.

3. The zinc content of the choroids and irises of albino rabbits is much less than the same tissues of coloured rabbits. There is little difference between the non-pigmented eye tissues of the two varieties.

4. In confirmation of Tauber & Krause it has been shown that the corneal epithelium contains higher concentrations of copper and zinc than the rest of the cornea.

5. The copper and zinc in cattle irises is largely bound to a melanin-protein fraction of the tissue. The concentrations of copper and zinc, especially the latter, in similar pigment fractions obtained from cattle, perch, and frog choroids, and from whale irises, were always much greater than the concentrations in the whole tissue, and it is therefore indicated that this material is responsible for the specific accumulation of these metals in pigmented tissues.

6. The melanin of cattle irises and perch choroids was shown to occur in the form of a complex containing (in addition to the coloured melanin compound) material of protein or amino-acid nature, and an inorganic part.

7. The inorganic part of the pigment from cattle irises contained Fe, Ca, Mg, Ba, Zn, and Cu, of which iron and calcium appeared to be major constituents. The perch pigment complex contained Zn, Ca, Fe, Mg, Sr, Al, and Cu, of which zinc, calcium, and iron appeared to be major constituents.

8. Ferric and ferrous iron, and copper, but not Ca, Mg, Zn, or Ba, affect the light absorption of a melanin material in the absence of protein. Ferrous iron forms a stoichiometric compound with the melanin. The mixture of ferric iron with the melanin shows an inflexion in the absorption curve which coincides with the absorption band found for the red pigment from human hair, which was shown by Flesch to contain ferric iron.

9. Zinc increases the absorption of light by a mixed solution of protein and the melanin obtained by oxidising dopa.

10. An enzyme system from cattle irises which catalyses the oxidative conversion of tyrosine, and to a lesser extent dopa, to melanin, was extracted and partially purified.

11. Zn, Ca, Co, Fe^{++}, and Fe^{+++}, were all found to be capable of increasing the light absorption, and modifying the absorption curves of melanins formed from tyrosine in the presence of the enzyme system from cattle irises. The nature of the buffer used for the reaction influenced the effects obtained with the metals.

12. Zinc combines with the compound of melanin and the protein fraction from cattle irises which contains the enzyme system, to a much greater extent than with the protein alone. It does not combine at all with melanin formed in the absence of protein.

Limitations of the Results. Desirable extensions of the work which arise from the limitations.

There are several parts of this work where the experiments have been inconclusive, or where the full investigation of a problem has not been completed.

In Section 3 of Chapter IV, further experiments which would put the results on a better statistical basis, are necessary to show conclusively whether zinc and copper are transferred to

the retina of sheep with melanin pigment, under the influence of light. A histological study of the choroid and retina would be necessary to show whether the transference of pigment from one tissue to the other is actually a pigment migration.

The investigation of the nature of the linkage of the inorganic with the other part of the melanin complex, given in Section 3 of Chapter VI, contains only preliminary experiments. A great deal of further work on the subject is required before any sound idea of the kind of linkage involved can be formed.

In continuance of the work of Chapter VII, a survey of the effect of metals, other than those investigated, on the light absorption of melanin solutions is desirable. A study of the effect of metals other than zinc on the absorption curves of mixed solutions of melanin and protein might also yield useful results.

In continuance of the work of Chapter VIII, further investigation of the influence of different buffers on the combination between metals and melanin pigment is necessary before a clear picture of the nature of the reactions taking place can be obtained.

Further work on the properties and composition of the compound of ferric iron with melanin obtained by the oxidation of dopa and tyrosine are desirable to show whether it is identical or analogous with the red pigments which occur in human hair, and poultry feathers.

General Implications of the Work.

For some time past, it has generally been considered that biological trace elements are associated with, and function in conjunction with, enzymes or enzyme systems (cf. Green, 1941). The present work shows that zinc, and to a lesser extent copper, can be combined with specific non-enzymic constituents of animal tissues in amounts which are beyond the limits usually set to the concentrations of 'trace elements' in tissues. A rigid distinction between 'trace' and other metallic elements seems therefore to be untenable. The fact that an element may occur in traces in some species, and in large quantities in others (vide Chapter I) indicates that the distinction has, even in the past, been mainly a theoretical one.

The observation that various metals are associated with, and can influence the colour and absorption curves of

melanin complexes, adds yet another to the factors which are involved in the formation of the black, brown, and grey pigments of animals.

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APPENDIX 1. (Refer to Chapter VI, Section 2).

Identified emission lines in the spectra of various ash materials under arc excitation.

a. Ash from dialysed cattle iris pigment.

Wavelength of line in A°.	Element responsible	Wavelength of line in A°.	Element responsible
2335.3	) Ba	2802.7	Mg
2347.6	)	2824.4	Cu
2398.6	Ca	2852.1	Mg
2435.16	Si	2915.5	"
2506.90	"	2997.4	Cu
2514.3	"	3066	Ti
2516.1	"	3072.1	Zn
2519.2	"	3096.9	Mg
2524.1	"	3158.9	Ca
2528.52	"	3179.33	"
2569.9	Zn	3181.3	"
2634.8	Ba	3247.55	Cu
2647.3	"	3273.96	"
2679	Ti	3302.6	Zn
2776.7	Mg	3303.0	"
2779.9	"	3332.2	Mg
2781.4	"	3336.7	"
2783.0	"	3501.1	Ba
2790.8	"	3644.39	Ca
2798.0	"		

b. Ash from dialysed cattle irises.

Wavelength of line in A°.	Element responsible	Wavelength of line in A°.	Element responsible
2335.3	Ba	2790.8	Mg
2347.6	"	2794.2	Pt
2398.6	Ca	2795.5	Mg
2514.3	Si	2798.0	"
2516.1	"	2802.7	"
2519.2	"	3072.1	Zn
2528.5	"	3158.9	Ca
2552	Pt	3179.33	Ca
2634.8	Ba	3181.3	Ca
2776.7	Mg	3247.55	Cu
2778.3	"	3273.96	"
2779.9	"	3302.34	Na
2781.4	"	3302.94	"
2783.0	"		

In the spectra of both (a) and (b) the lines due to iron were too numerous to list.

c. Ash from washed perch pigment.

Wavelength of line in A°.	Element responsible	Wavelength of line in A°.	Element responsible
2881.55	Si	3372	Ti
2929.79	Pt	3383.77	"
2947.66	Fe	3642.68	"
2966.90	"	3644.39	Ca
2973.14	"	3653.49	Ti
2983.57	"	3838.69	Mg
2994.44	"	3832.21	Mg
2997.96	Pt	3933.67	Ca
3020.5	Fe	3968.48	"
3021.08	"	3944.11	Al
3064.71	Pt	3961.5	"
3075.88	Zn	4077.71	Sr
3179.33	Ca	4226.73	Ca
3247.55	Cu	4283.1	"
3282.32	Zn	4289.36	"
3273.96	Cu	4299	Ti
3302.6	Zn	4302	Ca
3303.0	"	4318	"
3345.0	"	4425.44	"
3346.3	"	4434.96	"
3371	Ti	4454.78	"

APPENDIX 2. (Refer to Chapter VII. section 2)

Extinction readings (Beckmann instrument) for various solutions (reacted for 4 days at 0° C.) diluted to 5 mls. with water. (5)
" " 10 " " " (10)

Wavelength in mu.	1 ml. Melanin (10) (a)	1 ml. Melanin + 1 ml. Protein (10) (b)	1 ml. Melanin + 1 ml. Protein + 1 ml. $ZnSO_4$ (10) (c)	1 ml. Protein (5) (d)	1 ml. Protein + 1 ml. $ZnSO_4$ (5) (e)
250	0.54	0.702	0.791	0.438	0.547
260	0.64	0.799	0.880	0.438	.511
270	1.04	1.22	1.263	0.413	.506
280	1.97	1.61	1.69	0.348	.488
290	0.91	1.07	1.14	.262	.423
300	.590	0.480	0.542	.198	.386
350	.304	.358	.422	.167	.235
400	.203	.278	.330	.210	.228
410	.190	.268	.320	.218	.228
420	.179	.245	.295	.158	.189
450	.155	.204	.250	.104	.138
500	.130	.171	.212	.086	.104
550	.111	.142	.192	.063	.088
600	.094	.124	.166	.050	.074
650	.088	.102	.141	.039	.056
700	.082	.100	.129	.036	.050

APPENDIX 3 (a). (Refer to Chapter VIII, Section 3).

Extinction readings (Beckmann) for melanins enzymically synthesised in the presence of various metals. Experiment 1.

Wavelength in mμ.	1 Blank (a)	2 Blank (b)	3 Blank (c)	4 50γCa	5 40γZn	6 20γFe^{++}	7 20γFe^{+++}	8 20γCu	9 50γBa	10 50γMg
750	.195	.181	-	.176	.343	.185	.178		.178	.185
700	.240	.231	.227	.215	.410	.218	-	.043	.216	.230
650	.293	.275	-	.263	.496	.243	.215			
600	.333	.328	.323	.320	.555	.252	.273	.092	.303	.330
550	.390	.383	-	.378	.608	.271	.301		.352	
500	.445	.438	.430	.420	.648	.291	.335	.180	.401	.427
450	.505	.498	-	.478	.739	.331	.382	.163		
400	.645	.640	.630	.620	.881	.423	.505	.216	.592	.620
350	.920	.900	.900	.880	1.120	.568	.705	.255	.850	.880
300	1.12	1.09	1.07	1.05	1.290	.710	.840	.485	1.025	1.060
280	1.502	1.430	1.35	1.221	1.585	1.050	1.18	.755	1.25	1.350
270	1.485	1.415	-	1.221	1.59	1.030	1.18	.790	1.23	1.340
250	1.490	1.430	1.35	1.411	1.75	1.060	1.16	.695	1.26	1.375
240	1.920			1.71	2.05	1.300				

APPENDIX 3 (b). (Refer to Chapter VIII, Section 3).

Extinction readings (Beckmann) for melanins synthesised in the presence of various metals. Experiment 3.

Wavelength in mu.	1 Blank a	2 Blank b	3 10γCu	4 20γFe^{++}	5 Blank c	6 20γFe^{+++}	7 100γCa	8 80γCa	9 100γZn	10 80γZn	11 50γZn	12 100γMg	13 80γMg	14 100γBa	15 100γCo	16 Blank + 100γZn. No Oxidation.	17 Blank. No Oxidation
400	1.253	1.250	1.075	1.07	1.288	1.11	1.09	1.25	2.03	2.03	1.66	1.19	1.36	1.23	1.49	.163	.091
450	.950	.950	.820	.800	.980	.830	.815	.952	1.675	1.64	1.29	.901	1.025	.935	1.115	.098	.040
500	.815	.815	.710	.705	.825	.705	.707	.827	1.50	1.49	1.15	.782	.880	.805	1.065	.088	.043
550	.705	.703	.625	.655	.718	.622	.635	.736	1.44	1.425	1.06	.687	.790	.708	1.030	.064	.038
600	.600	.601	.541	.605	.615	.550	.555	.639	1.32	1.30	.974	.600	.668	.615	.990	.048	.020
650	.499	.500	.460	.541	.510	.465	.478	.539	1.15	1.075	.857	.502	.565	.510	.874	.043	.016
700	.402	.401	.373	.455	.401	.377	.388	.433	.930	.870	.680	.400	.453	.405	.675	.036	.016
750	.321	.320	.298	.367	.325	.301	.295	.345	.757	.695	.530	.308	.350	.317	.510	.034	.015

APPENDIX 3 (c). (Refer to Chapter VIII, Section 3).

Extinction readings (Beckmann) for melanins synthesised enzymically in the presence of various metals. Experiment 5.

Wavelength in mu.	1 Blank a	2 100γZn	3 100γMg	4 70γZn	5 100γCa	6 100γBa	7 100γFe^{++}	8 100γFe^{+++}	9 50γCu	10 100γCo	11 100γZn as $ZnCO_3$
400	1.00	1.00	.970	1.075	.976	1.015	1.090	1.060	.197	1.025	1.453
450	.752	.762	.720	.818	.735	.781	.848	.765	.145	.801	1.114
500	.662	.661	.620	-	-	.650	.739	.654	.115	.685	1.03
550	.581	.598	-	.713	.635	.560	.689	.732	.073	.590	.991
600	.502	.536	.460	.558	.467	.480	.638	.525	.062	.511	.882
650	.416	.446	.387	.481	.396	.401	.583	.479	.048	.432	.760
700	.327	.341	.307	.377	.317	.322	.500	.405	.045	.356	.592
750	.264	.272	.245	.298	.252	.261	.420	.343	.035	.270	.461

SUMMARY OF THE AIMS AND RESULTS OF THE WORK

AIMS

The knowledge of the distribution of zinc and copper in the eyes of vertebrate animals was incomplete. Shakir (1948) began an investigation into the distribution of trace metals in eye tissues, and paid particular attention to zinc and copper. The present work was initiated with the intention of confirming and extending his results, and, if possible, determining whether or not all the zinc and copper in the eye is associated with the known functions of these elements.

RESULTS

1. The tissues of the eyes of each of several species of vertebrates differ from each other in their zinc and copper concentrations. In each of the species examined the order of the tissues, arranged according to the magnitude of their zinc contents, was roughly the same, the highest concentrations occurring in the tissues containing melanin pigment. The distribution of copper does not follow exactly the same pattern in all the species examined, but in general the pigmented tissues are richest in copper.

2. In cattle irises it has been shown that the high concentration of zinc and copper is due largely to an association of these metals with a pigment-protein fraction

of the tissue. Similar fractions from the irises and choroids of other animals always contained higher concentrations of copper and zinc than the whole tissue.

3. The concentrations of zinc in the pigmented eye tissues andpigment-protein fractions of perch, trout, and frogs are very much higher than those encountered in the corresponding mammalian tissues, and higher than any figures given in the literature for other animal material. The findings of Leiner & Leiner for freshwater fish from Lake Constance are thereby confirmed for fish from Lake Windermere.

4. The concentrations of zinc in the choroid and iris, but not the other eye tissues of albino rabbits are lower than in the same tissues of coloured rabbits.

5. The corneal epithelium contains higher concentrations of copper and zinc than the rest of the cornea.

6. The melanin of cattle irises and perch choroids occurs in the form of a complex containing, in addition to the melanin itself, material of protein or amino-acid nature, and an inorganic part.

7. The inorganic part of the pigment from cattle irises contains Fe, Ca, Mg, Ba, Zn, and Cu, of which iron and calcium appeared to be major constituents. The perch pigment complex contained Zn, Ca, Fe, Mg, Sr, Al, and Cu. of which zinc, calcium, and iron appeared to be major constituents. Of these metals ferric and ferrous iron, and copper were the only ones

found *in vitro* to affect the light absorption of a melanin material in the absence of protein.

8. An enzyme system is present in cattle irises which catalyses the oxidative conversion of tyrosine to melanin.

9. Zn, Ca, Co, Fe^{++} and Fe^{+++}, under various conditions, are all capable of increasing the light absorption, and modifying the absorption curves of melanins formed from tyrosine, using the enzyme system from cattle irises.

10. Zinc combines with the complex of melanin and cattle iris protein to a much greater extent than with the protein alone, but it does not combine at all with melanin formed in the absence of protein.

It is clear from these results that zinc and copper in the vertebrate eye are largely associated with non-enzymic tissue constituents, and their presence in the eye tissues is therefore not more than partially concerned with their previously known functions.

Though the melanin molecule can be formed in the body in the absence of any metal other than copper it has been shown that zinc and other metals can influence the shade and

colour intensity of melanins synthesised _in vitro_ by the oxidation of tyrosine in the presence of an enzyme preparation from cattle irises.