Isolation of a Zinc Protein from the Tapetum Lucidum of the Cat

By L. R. CROFT

*Nuffield Laboratory of Ophthalmology, University of Oxford, Walton Street,
Oxford OX2 6AW, U.K.*

(Received 14 August 1972)

The tapetum lucidum of the vertebrate eye is the reflecting layer situated behind the retina. Investigations on the tapetum lucidum of the cat have been carried out over many years but without any definite conclusions being reached as to its chemical composition. Murr (1927) showed that the tapetal cells of the cat were packed with small 'rodlets', which he considered to be modified mitochondria. Hosoya (1929), in an extensive investigation, concluded that the tapetum of the cat was composed of birefringent micro-crystals. Bernstein & Pease (1959) argued that the rodlets were not crystals but modified melanin granules. Weitzel (1956) found that the tapetum of the cat had an unusually high zinc content, yet unlike the dog did not contain zinc cysteine, and it was suggested the material may be peptide in nature. The present communication describes the chemical characterization of the material found in the tapetum lucidum of the cat, which has been found to be a zinc-containing protein.

Materials and methods

The material from the tapetum lucidum of the cat was isolated by a modification of the method of Elliott & Futterman (1963). Eyes were removed immediately after death, brought to the laboratory and dissected. The retina was first removed and the tapetum gently scraped off with a sharp knife. The material from ten eyes was homogenized in 10 ml of 0.3M-sucrose–0.1M-sodium phosphate buffer, pH 7.4 (9:1, v/v). This solution was centrifuged at 500g for 10 min, to spin down cell debris. The pale-yellow supernatant was then centrifuged at 10000g for 1 h. The supernatant was discarded and the residue washed four times with sucrose buffer followed by four washings with distilled water. The material was then freeze-dried.

I.r. analysis was performed by the micro-KBr-disc method with a Perkin-Elmer 225 spectrophotometer (P.C.M.U., Harwell, Berks., U.K.). Amino acid analysis was done, after complete acid hydrolysis with 6M-HCl containing 5% (v/v) of 0.1M-thioglycolic acid for 20 h at 110°C, with a Locarte amino acid analyser with a single column. Half-cystine was determined separately as cysteic acid after performic acid oxidation. Tryptophan was determined by the method of Liu & Chang (1971). Elementary analysis

(C, H and N) was performed by Dr. F. B. Strauss (Microanalytical Laboratory, Oxford, U.K.). Zinc was determined by using atomic-absorption spectrophotometry (Yarsley Laboratories, Ashted, Surrey, U.K.). The moisture content was determined after drying to constant weight in an evacuated drying 'pistol' at 120°C. The isolated material was oxidized with performic acid as described by Croft & Waley (1971). The oxidized protein was digested with trypsin and subjected to peptide 'mapping' as described by Croft (1971).

Results

The material extracted from the cat tapetum was examined by phase-contrast microscopy, when it appeared to be composed of minute crystals of uniform size. The crystalline structure was lost on freeze-drying and also after the sample had been frozen and thawed several times. The material was only slightly soluble in 1% (w/v) NaOH and was insoluble in all other solvents tried. An i.r. spectrum of the freeze-dried material showed main absorption bands at 3352, 1650 and 1520 cm⁻¹. These latter absorption bands are the frequencies of the amide I and amide II bands and clearly indicate that the material is a polypeptide. The elementary analysis was: C, 48.2; H, 6.8; N, 14.0; Zn, 2.5% (calculated on a dry-weight basis). Amino acid analysis (recovery 98%) indicated the following composition in molar proportions, based on lysine (=1.0): Lys (1.0), His (3.5), Arg (4.5), CySO₃H (9.0), Asp (6.1), Thr (2.0), Ser (0.84), Glu (3.8), Pro (3.7), Gly (5.8), Ala (0.8), Val (1.1), Ile (1.0), Leu (2.1), Tyr (2.6), Phe (3.9), Met (absent), Trp (absent).

To render the material soluble it was oxidized with performic acid (Hirs, 1967), and the product was found to be soluble in 0.5% NH₃. The oxidized protein was purified by gel filtration on a column (45 cm × 1 cm) of Sephadex G-50, with elution with 0.5% NH₃. Fractions were collected and analysed for protein by measuring the absorbance at 280 nm. The protein emerged from the column as a single symmetrical peak; amino acid analysis indicated no significant differences from that of unoxidized material. The presence of only one peak after gel filtration suggested that the protein was composed of a single polypeptide chain. The minimum molecular weight,

Fig. 1. Peptide 'map' of a tryptic digest of performic acid-oxidized protein isolated from the tapetum lucidum of the cat

Paper electrophoresis in pyridine-acetate buffer, pH 6.5, was followed by paper chromatography carried out in butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.) on Whatman 3MM paper. The peptide 'maps' were stained with ninhydrin and the resulting colour is indicated by P (pink), O (orange) and Y (yellow). The peptide not reacting with ninhydrin is shown by solid shading. Arginine-containing peptides were detected with the phenanthraquinone reagent (Itano & Yamada, 1966) and are indicated by cross-hatching or solid shading. Aspartic acid was used as a marker in the electrophoresis direction only.

calculated from the amino acid analysis, is 6347. The performic acid-oxidized protein was digested with trypsin and the digest subjected to peptide 'mapping' (Fig. 1).

Discussion

The tapetal cells of the dog are packed with crystals of zinc cysteine (Weitzel *et al.*, 1955), those of the lemur with crystalline riboflavin (Pirie, 1959) and those of the opossum with cholesterol crystals (Pirie, 1961). The alligator and many fishes have tapetal cells that contain crystals of guanine (Abelsdorf, 1898). It would appear that the tapetum of the cat is composed of crystals of a metalloprotein (Fig. 2). That the material is crystalline confirms Hosoya's (1929) original suggestion but is in disagreement with the findings obtained by Bernstein & Pease (1959). These workers based their conclusions on an electron-microscopic examination of the tapetal cells after they had been histologically fixed. They concluded that the rodlets were not crystalline, as they lacked sharp edges. It is possible that the process of fixing had

Fig. 2. Crystalline zinc protein isolated from tapetum lucidum of the cat

Phase-contrast microscopy was used (magnification $\times 200$).

destroyed the crystal form, as in this present work the crystals were found to be easily lost by freezing and thawing, suggesting a high degree of hydration. One other difficulty in accepting that it is a crystalline structure is that individually they do not exhibit birefringence under the microscope, although they do show birefringence of flow in aqueous suspension. Possibly their minute size may account for this; certainly the crystals of zinc cysteine that I have isolated from the eye of a dog have not individually shown birefringence, yet cells packed with these crystals have. The crystals from the dog are of the same shape as those from the cat, but several times larger.

That the material is a simple polypeptide is indicated from the i.r. spectrum, which shows clear amide I and amide II vibrations; the lack of any absorption near 1720cm^{-1} suggested the absence of lipid from the preparation. The high recovery from amino acid analysis (98%) confirms that the material is probably a simple polypeptide. Determination of the molecular weight of the material presents some difficulties

owing to its insoluble nature. Amino acid analysis indicated a minimum molecular weight of 6400, which was confirmed after peptide 'mapping'. Peptide 'maps' of tryptic digests showed six peptides (Fig. 1), which was to be expected from a protein containing at the most six trypsin-susceptible bonds. It would appear that 6400 is the molecular weight of the polypeptide chain, but it is likely that the zinc-complexed protein has a higher molecular weight due to polymerization via the zinc atoms or by disulphide bridges.

This peptide contains an unusually high zinc content, equal to that of metallothionein (Kägi & Vallee, 1960) and much higher than that of insulin and zinc-containing enzymes.

It is instructive to note that the tapetum of many animals has been constructed of a surplus metabolic product, e.g. guanine in fishes, uric acid in the lobster (Kleinholz, 1955) and cholesterol in the opossum. It is thus possible that this zinc-containing protein found in the cat tapetum may be related to other metalloproteins occurring in other tissues.

I have pleasure in thanking Mr. R. T. Smith, Department of Pharmacology, University of Oxford, for help in obtain-

ing material for this study and Mr. R. N. Priscott for skilled technical assistance.

- Abelsdorf, G. (1898) *Arch. Physiol. (Leipzig)* 155-167
 Bernstein, M. H. & Pease, D. C. (1959) *J. Biophys. Biochem. Cytol.* 5, 35-39
 Croft, L. R. (1971) *Biochem. J.* 121, 557-559
 Croft, L. R. & Waley, S. G. (1971) *Biochem. J.* 121, 453-459
 Elliott, J. H. & Futterman, S. (1963) *Arch. Ophthalmol.* 70, 531-534
 Hirs, C. H. W. (1967) *Methods Enzymol.* 11, 197-199
 Hosoya, Y. (1929) *Tohoku J. Exp. Med.* 12, 119-145
 Itano, H. A. & Yamada, S. (1966) *Biochim. Biophys. Acta* 130, 538-540
 Kägi, J. H. R. & Vallee, B. L. (1960) *J. Biol. Chem.* 235, 3460-3465
 Kleinholz, L. H. (1955) *Biol. Bull.* 109, 362
 Liu, T.-Y. & Chang, Y. H. (1971) *J. Biol. Chem.* 246, 2842-2848
 Murr, E. (1927) *Z. Zellforsch.* 6, 315-336
 Pirie, A. (1959) *Nature (London)* 183, 985-986
 Pirie, A. (1961) *Nature (London)* 191, 708-709
 Weitzel, G. (1956) *Angew. Chem.* 68, 566-573
 Weitzel, G., Buddecke, E., Fretzdorff, A. M., Strecker, F. J. & Roester, U. (1955) *Hoppe-Seyler's Z. Physiol. Chem.* 299, 193-213