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Cholesterosis bulbi

Case report with modern chemical identification of the ubiquitous crystals*

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Although cholesterosis bulbi has a long history in the ophthalmic literature, beginning with Parfait-Laudrau (1828), very little definitive chemical investigation has been done on the identity of the crystalline material which characterizes this disease. Earlier investigators identified the crystals as cholesterol by their characteristic microscopical appearance and, more recently, colorimetric tests have been used which substantiate earlier results (Forsius, 1961; Kumar, 1963). However, the colorimetric tests neither differentiate between molecules related in structure to cholesterol nor examine the question of other constituents present in these crystals.

The investigation described below attempts to answer these questions and further investigates the level of cholesterol dissolved in the aqueous of a patient with cholesterosis bulbi.

Case report

A 31-year-old caucasian male was referred to the Eye Clinic by a private ophthalmologist; he had had intermittent pain in the left eye for 3 months and this had been treated with atropine and topical corticosteroids with only partial relief of symptoms. He stated that he had always had poor vision in the left eye but could not recall any trauma; 5 years before he had noted a white pupil in the left eye due to cataract formation.

Examination

The visual acuity was 20/20 in the right eye and no perception of light in the left. The right eye was normal.

There was mild ciliary injection in the left eye. The pupil was 4 mm. and irregular due to posterior synechiae. It constricted to 3 mm. consensually but did not react directly to light. The cornea was clear. A mature cataract with a broken anterior capsule was present. Posterior synechiae were noted at 10 o'clock and from 2 to 5 o'clock. A blood vessel from the iris had extended 1 mm. into the lens at 4 o'clock. The anterior chamber was filled with circulating, flat, rectangular crystals which layered inferiorly into a 1 mm. pseudohypopyon (Fig. 1, opposite). The posterior chamber and fundus could not be visualized because of the cataract.

Applanation tonometry showed an intraocular pressure of 16 mm.Hg in each eye. All other clinical tests were within normal limits.

The left eye was enucleated and pathological examination of the tissues surrounding the anterior chamber revealed the typical "cholesterol clefts" characteristic of cholesterosis bulbi (Fig. 2, overleaf).

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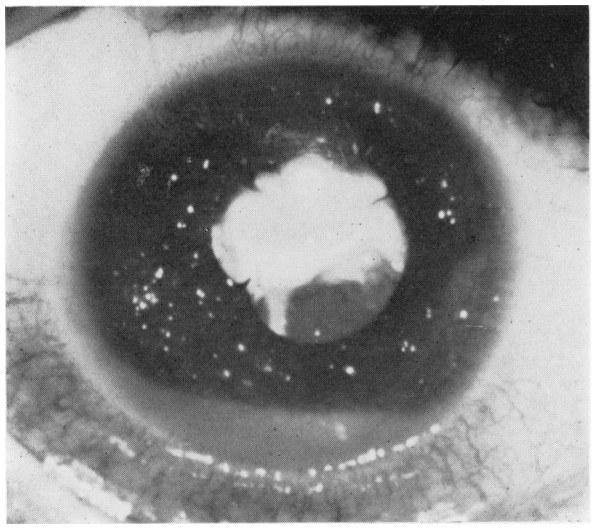

FIG. 1 *Anterior view of eye* in situ

Methods and results of chemical investigations

Immediately after enucleation, the aqueous was removed by paracentesis and transferred to a graduated centrifuge tube. The vitreous was removed to a similar tube by incising the eye at the midline and everting the back half. A drop of aqueous was taken immediately for microscopical examination of the suspended crystalline material. The aqueous volume was estimated to be 670 µl. and the size of this figure suggests that the sample was contaminated with liquefied vitreous. The vitreous volume was 2·8 ml. and the material was yellow in colour.

Both samples were centrifuged and the precipitates isolated by decantation (see Fig. 3). The aqueous precipitate was partitioned between equal volumes of water and chloroform (0·2 ml.), then 5·6 ml. of chloroform: methanol (2 : 1) were added, making a total volume of 6 ml. this extraction solution was added to the vitreous precipitate and both samples were centrifuged. The minute amount of debris was discarded by carefully transferring the supernatant fluid to clean tubes. Both supernatant fluid samples were then taken to dryness under nitrogen, redissolved in a known volume of chloroform, and a 5 µl. sample taken for thin-layer chromatography. The samples, along with a standard lipid mixture, were chromatographed on Silicagel G with 90 : 10 : 1 (petroleum ether: ether: acetic acid) as the mobile phase. The spots were developed by charring with concentrated sulphuric acid and heat. The results are shown in Fig. 4. Although both samples show large spots in the cholesterol region, only the vitreous precipitate demonstrates sigificant amounts of cholesterol ester. Spots at the origin indicate the presence of either phosphatides or polar oxidation products.

The remainder of the aqueous precipitate (about 80 per cent.) extract was applied to a 1 × 14 cm. silicic acid column and eluted according to Hirsch and Ahrens (1958). The cholesterol-containing fraction was taken to dryness, redissolved in chloroform, and divided into two equal aliquots. Retention times with underivatized, di-, and trimethylsilyl ethers prepared from this sample according to Horning, van den Heuvel, and Creech (1963) coincided with corresponding standards on columns of 1 per cent. OV-1* and 3 per cent. OV-17 run at 200 and 225 °C. respectively in a gas-liquid chromatograph. No other peaks

*OV-1 and OV-17 are respectively, methyl and phenyl, methyl derivatives of silicone polymers which are important liquid phases for the separation of steroid and sterol derivatives by gas-liquid chromatography.

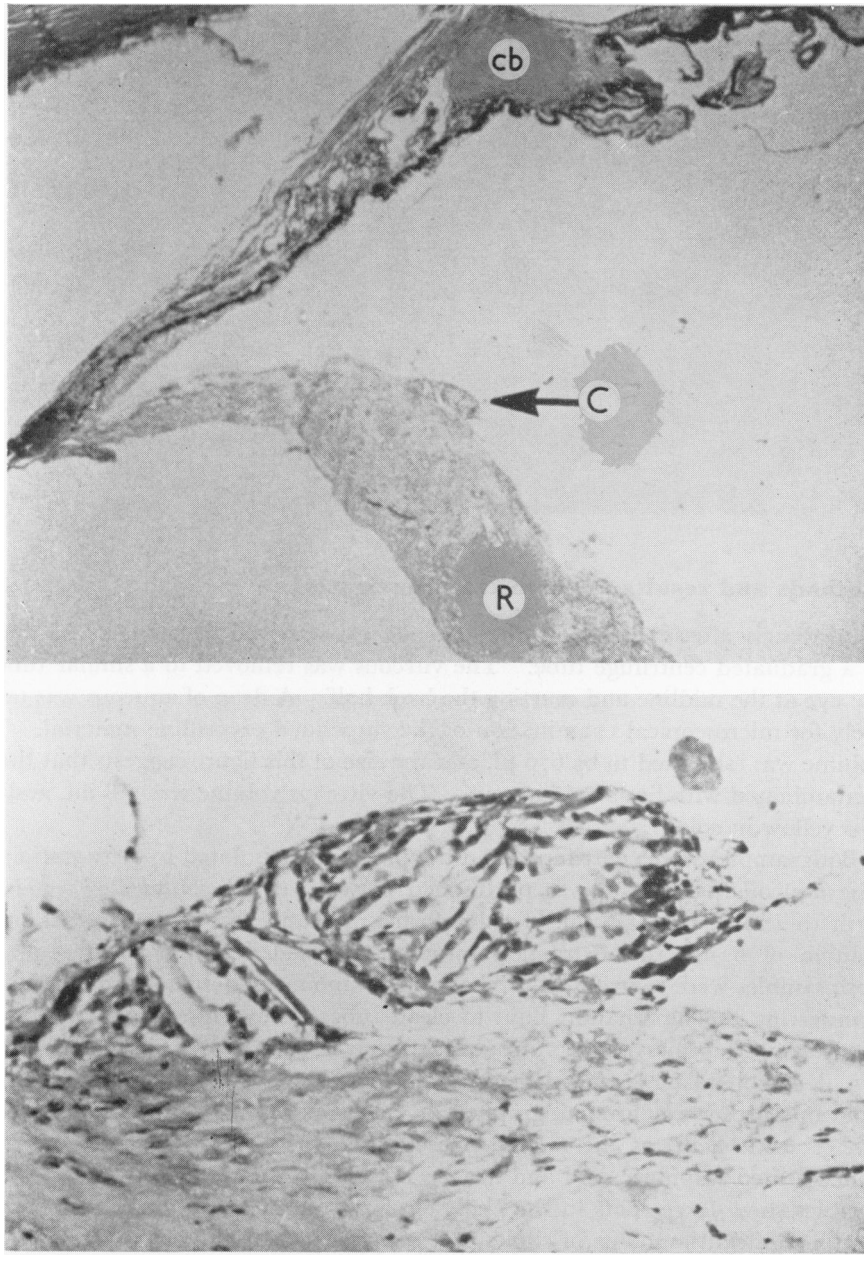

FIG. 2 *Sections of removed eye showing typical pathology*
Upper: Low-power photomicrograph, showing gliotic, detached retina (R), cholesterol clefts (C), and ciliary body (Cb)
Lower: High-power view of area indicated by arrow, showing cholesterol clefts in retino-vitreous interface. Retina shows extensive gliosis and disorganization. Haematoxylin and eosin

were detected. Thus, it seems clear that the crystalline material observed in cases of cholesterosis bulbi is indeed cholesterol—not a related but structurally different sterol.

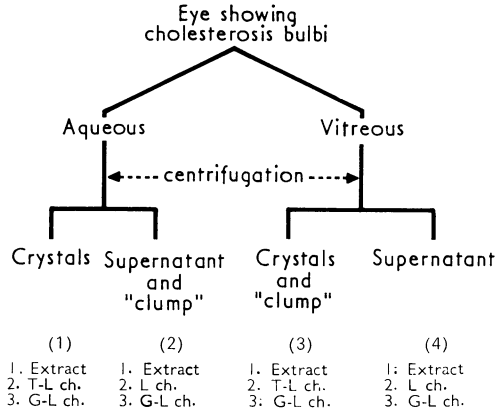

FIG. 3 *Sample preparation and analysis. T-L = thin layer; G-L = gas liquid; L = liquid; ch. = chromatography.*

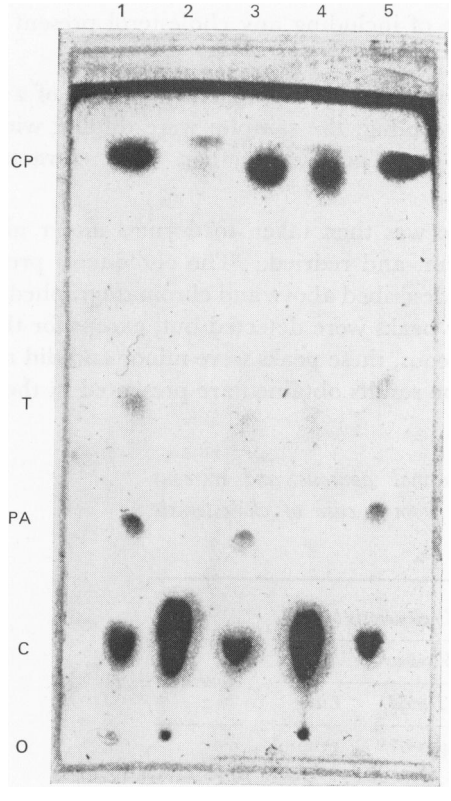

FIG. 4 *Thin-layer chromatography of chloroform methanol extracts of aqueous and vitreous crystals Lane 1, 3, and 5 Standard samples containing equal weights of cholesterol, palmitic acid, tripalmitin, and cholesterol palmitate*
Lane 2 Extract of aqueous crystals
Lane 4 Extract of vitreous crystals
Chromatography conditions described in text
CP = Cholesterol palmitate; T = Tripalmitin; PA = Palmitic acid; C = Cholesterol; O = Origin

The aqueous and vitreous supernatants from the low-speed centrifugation were extracted with 20 volumes of chloroform: methanol (2 : 1) and washed according to the method of Folch, Lees, and Stanley (1957). Since the vitreous supernatant had separated into a turbid upper phase and a clear lower phase (1·5 and 1·0 ml. respectively); each phase was extracted individually. Concomitantly, a normal aqueous sample (150 µl.) and a normal vitreous sample (300 µl.) were extracted for purposes of comparison.

Aliquots of the extracts were chromatographed on thin-layer plates as described above. The normal aqueous aliquot was faintly positive for cholesterol but negative for cholesterol ester. The normal vitreous aliquot, however, demonstrated the presence of both forms of cholesterol. Similar procedures with the aqueous and vitreous samples from the patient's eye revealed spots corresponding to cholesterol and cholesterol ester in all samples. Since thin-layer chromatography had revealed the presence of two forms of cholesterol in some samples and fatty acids in all samples, the remainder of the extracts from normal ocular fluids and aliquots of the extract from the pathological vitreous samples were chromatographed on a 900 mg. Florisil column (4 × 800 mm.) and eluted with 7 ml. of a mixture of petroleum ether: diethyl ether (6 : 1) followed by 7 ml. of a mixture of the same solvents but in a ratio of 2 : 1. Florisil was used because it acts as an ion exchanger and releases fatty acids only when eluted with dilute acetic or formic acid (Carroll, 1961). Since thin-layer chromatography of the Folch extract from the pathological aqueous had demonstrated the presence of a number of lipids, a larger (6 g.) Florisil column (1 × 14 cm.) was used and a separation of the entire extract according to Carroll (1961) was conducted. In this case, three major fractions, one before and one after the cholesterol fraction, were pooled in order to be absolutely sure of including any cholesterol present in the pathological aqueous.

Cholesterol ester samples were saponified under nitrogen in 1·0 ml. of 2·5 M KOH in methanol at 60°C. for 3 hrs. After cooling, the samples were diluted with four vols of H_2O and extracted four times with 3 ml. petroleum ether. The extracts were pooled within samples.

Each cholesterol-containing sample was then taken to dryness under nitrogen, transferred to a microtube with chloroform, and redried. The cholesterol present was then converted to a trimethyl silyl ether as described above and chromatographed on 3 per cent. OV-17 at 225°C. In all cases, other peaks were detected but, except for the unesterified cholesterol from the pathological aqueous, these peaks were minor and did not complicate the quantification of cholesterol. The results obtained are presented in the Table.

Table *Cholesterol concentrations in normal aqueous and vitreous and in aqueous and vitreous supernatants from a case of cholesterosis bulbi*

Eye	*Normal*		*Cholesterosis bulbi*		
Material	*Vitreous*	*Aqueous*	*Vitreous*		*Aqueous*
			Turbid	*Clear*	
Cholesterol ester	105*	—	480·0**	1100·0	4·0
Cholesterol	0·4	19·0	56·0	10·0	0·6

* All values are in μg./ml.
** The volumes of the turbid and clear vitreous subsamples were 1·5 and 1·0 ml. respectively

Discussion

The presence of cholesterol ester in the extract of the crystalline isolate from vitreous probably represents artefactual inclusion of the clearly visible "clumps" of material in this

isolate as a result of the gel-like vitreous centrifugation. The crystals isolated from the aqueous sample were centrifuged at lower speed and did not include the "clump" also present in the original sample. In addition, the presence of cholesterol ester in the extract of the aqueous supernatant, which did not include the "clump", was noted. However, contamination of the pathological aqueous by liquefied vitreous seems likely and it cannot be assumed that the cholesterol ester observed in the aqueous supernatant sample is due entirely to the presence of a water-insoluble "clump". In any case, it seems apparent that cholesterol ester is not a significant component of normal aqueous.

The dissolution of the isolated crystals in an organic solvent mixture, followed by complete transfer of the dried residue with chloroform, excludes the possibility that these crystals can belong to any class of compounds other than neutral lipids. Subsequent chromatographic analysis demonstrated only cholesterol or cholesterol ester, with some reservations in the latter case (*vide supra*). Thus, although the crystals were isolated from a complex solution, there was little contamination and there can be no doubt as to their identity.

Calculation of the concentration of suspended crystals in the aqueous leads to a figure of 26·3 mg. per cent. cholesterol. Analysis of the vitreous before centrifugation, however, would yield an even higher figure of approximately 35 mg. per cent. cholesterol. Corresponding figures for cholesterol ester may be calculated from the data presented in the Table.

The separation of the vitreous from the eye with cholesterosis bulbi into two distinct phases during centrifugation cannot be explained on the basis of their respective cholesterol contents. The lower phase contained the higher total cholesterol concentration.

The presence of low but significant quantities of cholesterol in normal aqueous is important, since it represents a means of transport of this most difficult-to-metabolize chemical. The subnormal level of cholesterol observed in the pathological supernatant aqueous is puzzling and leads to speculation that a carrier protein is necessary for cholesterol solubility in human ocular aqueous.

It seems apparent that, in a case of cholesterosis bulbi, the vitreous acts as a cholesterol sink. Indeed, the increased levels of cholesterol ester may represent a detoxification mechanism in the vitreous, since esterification would effectively bind fatty acids which are known to have toxic effects on mammalian tissues.

Although the presence of cholesterol clefts in the degenerating tissues suggests a source of cholesterol crystals, it is difficult to overlook the sudden influx of cholesterol caused by haemorrhage as an equally likely source of ultimately crystalline cholesterol. It seems probable that both processes contribute to the presence of cholesterol crystals in the aqueous and vitreous of eyes with cholesterosis bulbi.

Summary

Chromatographic analysis of the crystalline and supernatant fluid fractions of both the aqueous and vitreous obtained from an eye with cholesterosis bulbi were conducted. The results show that the crystals are pure cholesterol. Furthermore, a comparison with normal aqueous suggests that the normal eye has a mechanism for maintaining cholesterol in solution which is impaired in the eye with cholesterosis bulbi. The results also suggest that in cholesterosis bulbi the vitreous may act as a cholesterol sink, since abnormally high levels of cholesterol ester were observed.

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