- Heim, F. & Fahr, A. (1940). *Arch. exp. Path. Pharmacol.* **195**, 59.
- Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Mendel, B., Mundell, D. B. & Rudney, H. (1943). *Biochem. J.* **37**, 473.
- Mendel, B. & Rudney, H. (1943). *Biochem. J.* **37**, 59.
- Mik'helson, M. Y. (1941). *Bjull. eksp. Biol. Med.* **11**, 230. Cited in *Chem. Abstr.* (1947), **41**, 6339.
- Mik'helson, M. Y. (1943). *Farm. & Toksikol.* **6**, 49. Cited in *Chem. Abstr.* (1945), **39**, 129.
- Mik'helson, M. Y. (1946). *J. Physiol. U.S.S.R.* **32**, 635.
- Ord, M. G. & Thompson, R. H. S. (1950). *Biochem. J.* **46**, 346.
- Plattner, F. & Galehr, O. (1928). *Pflug. Arch. ges. Physiol.* **220**, 606.
- Richter, D. & Croft, P. G. (1942). *Biochem. J.* **36**, 746.
- Seidell, A. (1941). *Solubility of Organic Compounds*, 3rd ed., vol. 2. New York: D. Van Nostrand, Co. Inc.
- Stearn, A. F. (1949). *Advanc. Enzymol.* **9**, 25.

Composition of Ox Lens Capsule

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The behaviour and constitution of the ox lens capsule has been investigated to see whether it is made up of a single protein containing carbohydrate or of protein mixed with polysaccharide.

The lens capsule completely encloses the lens and is a non-cellular laminar membrane thought to be secreted by the lens epithelium. It is elastic and when cut rolls back on itself as if the outer layers were under tension. Mammalian lens capsules can easily be stripped off like a cellophane wrapper and are tough and withstand handling. The adult lens has a single layer of epithelial cells on the front face only and it is here that the capsule is thickest. The posterior capsule is thinner, being laid down in embryonic life when the lens epithelium extends to the back surface. The evidence that the capsule is secreted by the epithelium comes from the work of Shirmer (1889), who showed that injury to the capsule and epithelium of the lens in the adult rabbit was followed by repair and a thickening of the capsule over the site of injury where the epithelial cells proliferate. This was confirmed by Bakker (1936-7) who, after injuring the rabbit lens *in situ*, removed it and watched the repair of the capsule in a tissue culture of the whole lens.

Mörner (1894) showed that the ox lens capsule contained about 10% of carbohydrate. Krause (1933) found N, 14%, S, 0.84%, and considered that the capsule was a distinct insoluble protein unlike the other widely distributed insoluble proteins of the body. Hertel (1933) took X-ray diffraction photographs of the capsule and found the picture was like that of unoriented collagen. Washed lens capsule was used in all these analyses and was considered to be a single protein. If this assumption is correct the lens capsule protein is unique in giving the X-ray diffraction pattern of collagen and yet containing this large amount of carbohydrate. It is also peculiar if it is a type of collagen and yet is produced by epithelial cells.

METHODS

Preparation of lens capsule. Ox eyes were dissected within 1 hr. of death. The lens was removed in its capsule and this was then stripped off. The capsules were suspended in water and stirred for several days with many changes of water, in order to remove attached epithelial cells and lens fibres. Such treatment did not alter the appearance of the capsules, but would presumably remove any soluble substances present. The capsules were then either acetone-dried or used fresh.

Total nitrogen. This was estimated by micro-Kjeldahl followed by distillation and titration, as described by Markham (1942).

Hexosamine. Elson & Morgan (1933).

Total carbohydrate. The orcin colorimetric method described by N. W. Pirie (1936) was used.

Reducing sugars. Hagedorn & Jensen (1923).

Partition chromatography of amino-acids. The upward method described by Williams & Kirby (1948) was used.

Partition chromatography of sugars. The upward method was used with the developers and spraying solutions described by Jermyn & Isherwood (1949) and Partridge (1949).

RESULTS

Ox lens capsule, prepared as described, contained 14% N, 9-10% carbohydrate, estimated colorimetrically, or 9% reducing sugars after hydrolysis. It also contained about 1% hexosamine. This small amount is difficult to estimate owing to charring during hydrolysis. No phosphorus was found.

General properties. The properties of the capsule were studied to see how far they tallied with those of collagen. Lens capsule is insoluble in boiling water, but goes into solution readily at about pH 3. Such solutions, after dialysis, set to weak jellies at a concentration of about 1%. The capsule does not gel as readily as gelatin derived from skin or tendon.

collagen, but the fact that it will form a gel after slight hydrolysis is in favour of it containing a protein like collagen. It also resembles collagen (Theis, 1946) in having the same definite marked shrinkage temperature between 60 and 65°.

Lens capsule is also soluble in 0.1N-sodium hydroxide or 40 % urea at 100°. Formamide, lactic acid, 40 % urea, 10 % calcium chloride or 90–95 % (w/v) phenol do not dissolve it at 37°. Formic acid dissolves the capsule at 37°. Considerable swelling of the capsule occurred in phenol, lactic acid and urea solutions at 37°, although no solution took place. Within this range the solubility and swelling of the capsule is the same as that of collagen. Many types of collagen are insoluble in boiling water, but dissolve in weak acid.

X-ray diffraction photograph. The X-ray diagram of dried stretched lens capsule was studied by Dr D. M. Crowfoot, and the results of Hertel (1933) were confirmed and extended. The picture conformed in general to that given by partly oriented collagen. The characteristic meridional spacing of 2.85 Å. was present, together with the equatorial spacing of 4.3 Å., but the longer spacings differed from those of typical collagen. This leaves open the possibilities that the lens capsule contains another protein besides typical collagen, or that it consists of an atypical collagen. The chemical analysis is more in favour of the second possibility. Attempts to decide the question were made by examining the amino-acid composition of the lens capsule and by trying to separate the carbohydrate by extraction and digestion methods.

Composition of lens capsule. A comparison of the constituent amino-acids with those of collagen was made. Sections of the eye stained with Millon's reagent showed that the lens capsule stained an intense red, far stronger than other parts of the eye. Estimation of tyrosine in lens capsule and cornea collagen by the method described by Lugg (1938) showed that the lens capsule contained 2.1 % and cornea collagen 1.0 % tyrosine, calculated on a moisture- and ash-free basis. Tristram (1946) found 1.4 % tyrosine in skin collagen.

A general survey of amino-acid composition was then made using paper partition chromatography. Lens capsule, cornea collagen, gelatin and sometimes casein were hydrolysed with 6N-hydrochloric acid overnight at 110° and the acid removed *in vacuo* in the usual way. Upward chromatography, as described by Williams & Kirby (1948), was used. Water-saturated phenol, sometimes with additions of 3 % ammonia or cupron were used as developers. The dried papers were sprayed with ninhydrin.

The general pattern of the lens capsule chromatogram was the same as that of cornea collagen and gelatin and quite unlike that of casein. The glycine spot was very prominent and proline and hydroxy-

proline spots were present. There seemed a larger spot corresponding to the leucines and phenylalanine in the lens capsule chromatogram. The difference in tyrosine content did not show up. All that one can say from this result is that the capsule showed some similarities to, and no marked difference from, collagen and gelatin.

Fig. 1. X-ray photograph of dried ox lens capsule. Stretched preparation showing some orientation of fibres. Photograph taken with cylindrical camera, radius 3.0 cm.

In order to try and separate the carbohydrate, capsules were collected and extracted with 10 % (w/v) sodium chloride followed by 0.1M-dipotassium hydrogen phosphate, with phenol 90–95 % (w/v), 40 % urea, or 10 % calcium chloride at 37°, extractives that have been used to separate polysaccharides from protein in other cases. Sodium chloride, followed by alkaline phosphate extraction brought out very little, the other extractives removed 10–20 % by weight of the capsule. The carbohydrate content of the extracted material was, however, usually rather less (5–7 %) than that of the original capsule. The undissolved residue had 10–11 % carbohydrate. Removal of carbohydrate was therefore not achieved by these methods. Mörner (1894) found that 0.1 % potassium hydroxide failed to extract the carbohydrate from lens capsule.

Extraction of carbohydrate from intact capsule having failed, the effect of enzyme digestion followed by fractionation was next tried. Pepsin, trypsin and collagenase dissolve lens capsule, leaving only a trace of insoluble material. Native skin collagen is dissolved by pepsin and collagenase, but is only very slightly digested and not then taken into solution by trypsin. Bidwell & van Heyningen (1948), who

described the properties of collagenase, found that it hydrolysed only collagen, gelatin and reticulin of all proteins investigated. It is, therefore, interesting that this enzyme digests the lens capsule, and collagenase digestion was used in an attempt to separate carbohydrate from protein.

Lens capsule (200 mg.) was digested with collagenase at 37° for several weeks and the digest was then dialysed. About half was dialysable and contained only 5–6% carbohydrate while the non-dialysable part had 13%. The non-dialysable fraction was fractionated by precipitation with ammonium sulphate. Precipitates having 10, 12 and 9% carbohydrate came down with 25, 50 and 100% saturation. The dialysed supernatant from complete saturation with ammonium sulphate contained 5.3 mg. material having 22% carbohydrate and 4% hexosamine.

Collagenase digestion does not, therefore, separate protein from carbohydrate, which appears distributed unevenly among the protein split products.

Digestion with either crystalline trypsin (Armour Chemicals) or with pancreatin (British Drug Houses Ltd.) led to the same result. Ethanol fractionation of a digest was no better than ammonium sulphate precipitation, the carbohydrate being distributed among the products of hydrolysis, the best fraction being that precipitated by 10 vol. ethanol and having 19.4% carbohydrate and 7.9% N. No hydrolytic method was successful in separating carbohydrate from protein, so the next step seemed to be to find out what type of sugar was present in the carbohydrate.

Identification of the carbohydrate. The constituent sugars of the lens capsule carbohydrate were identified by paper partition chromatography using the upward method. Lens capsule (150 mg.) was hydrolysed by heating for 3 hr. in a boiling-water bath with 0.5N-hydrochloric acid. This time of hydrolysis gave a maximum value of 9–11% reducing sugar. The acid solution was dialysed and the dialysate dried and redissolved in a small volume of water and brought to pH 6. This was applied to a column containing 0.5g. washed Zeo-Karb 215. The dried effluent (95 mg.) was dissolved, brought to pH 4 and again passed through a column. The dried effluent (72 mg.) had 14% total carbohydrate. It was extracted with ethanol at room temperature and the extract dried. This material (37 mg.) had a total carbohydrate content of 18% while the material not soluble in ethanol had 11% carbohydrate. The material soluble in ethanol was analysed chromatographically using the ethyl acetate-pyridine-water mixture (2:1:2 or 5:2:5) described by Jermyn & Isherwood (1949) as developer, and spraying the dried papers with aniline hydrogen phthalate (Partridge, 1949) to show up the spots. Repeated

upward runs were done in order to get good separation of spots, so that R_F values could not be measured. Marker spots of different sugar solutions were always used.

The lens capsule fraction gave two not completely separated spots corresponding in position to glucose and galactose. The glucose spot was much the larger of the two. This result was confirmed using a lens capsule fraction got by direct ethanol extraction of the dried acid hydrolysate followed by Zeo-Karb treatment of an aqueous solution of this material. No trace of any other sugar appeared on the chromatogram, but the final yield of carbohydrate usually only accounted for between 40 and 50% of the carbohydrate of the lens capsule. Zeo-Karb treatment removed considerable carbohydrate.

Colorimetric estimations agreed in showing the presence of a small amount of galactose. N. W. Pirie (1936) showed that galactose gave a red colour with orcin at 55° within 3 min. of heating, whereas other sugars gave yellow colours and only at higher temperatures. If lens capsule was heated with orcin at 55° a definite pink colour appeared in 3 min. and was much intensified and became orange when the solution was heated to boiling point. The colour given by the lens capsule with the carbazole reagent of Gurin & Hood (1939) also corresponded to a mixture of glucose and galactose.

DISCUSSION

No separation of constituents from the lens capsule has been achieved, and the conclusion still remains valid that it is probably composed of a single major constituent, an insoluble protein which contains about 10% of carbohydrate. It has many characteristics of collagen, but the two are not identical. From the point of view of the physiology of the eye the difference between the lens capsule protein and the closely associated fibrillar protein of the vitreous body is interesting. The protein of the vitreous body can be entirely separated from carbohydrate, and Pirie, Schmidt & Waters (1948) identified it with normal collagen from a study of its physical properties and chemical analysis. The cells of origin of the fibrillar protein of the vitreous body are not known.

The lens capsule is deeply stained by the Hotchkiss (1948) method for the detection of polysaccharide in tissue sections. Other structureless membranes such as Descemet's membrane of the cornea, Bruch's membrane of the retina also stain deeply. A basement membrane lining the whole vascular tree can also be demonstrated by this method (Day, 1950). It is therefore possible that insoluble proteins rich in carbohydrate are present in many membranes of the body, and that the lens capsule is not a unique structure. Such proteins would be difficult to detect

if they occurred in small amounts together with collagen. It is noticeable that the mixture of sugars in the lens capsule carbohydrate, glucose and galactose, are those which have been found in the 0.65 % of carbohydrate which it is difficult to remove from skin collagen (Schneider, 1937). The capsule also probably contains a small amount of hexosamine. Hydrolysates of whole capsule give a value of about 1 % and this might not be thought significant, but fractions prepared by enzyme digestion have been found to contain 2-4 % hexosamine, which makes it likely that a small amount is present in the capsule itself.

SUMMARY

1. Further analysis of ox lens capsule has failed to separate it into separate constituents.
2. It appears to be composed of a protein similar to collagen in many of its physical and chemical properties, but containing about 10 % of carbohydrate.
3. The carbohydrate consists mainly of glucose with a small amount of galactose.

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REFERENCES

- Bakker, A. (1936-7). *v. Graefes. Arch. Ophthalm.* **136**, 333.
 Bidwell, E. & van Heyningen, W. E. (1948). *Biochem. J.* **42**, 140.
 Day, R. (1950). *Amer. J. Ophthalm.* **33**, 224.
 Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
 Gurin, S. & Hood, D. B. (1939). *J. biol. Chem.* **131**, 211.
 Hagedorn, H. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.
 Hertel, E. (1933). *Arch. Augenheilk.* **107**, 259.
 Hotchkiss, R. D. (1948). *Arch. Biochem.* **16**, 131.
 Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
 Krause, A. C. (1933). *Arch. Ophthalm., N.Y.*, **9**, 617.
 Lugg, J. W. H. (1938). *Biochem. J.* **32**, 775.
 Markham, R. (1942). *Biochem. J.* **36**, 790.
 Möerner, C. T. (1894). *Hoppe-Seyl. Z.* **18**, 213.
 Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
 Pirie, A., Schmidt, G. & Waters, J. W. (1948). *Brit. J. Ophthalm.* **32**, 321.
 Pirie, N. W. (1936). *Brit. J. exp. Path.* **17**, 274.
 Shirmer, A. (1889). *v. Graefes Arch. Ophthalm.* **35**, 220.
 Schneider, E. (1937). *Collegium, Haltingen*, no. 809, 522.
 Theis, E. R. (1946). *Trans. Faraday Soc.* **42** B, 244.
 Tristram, R. G. (1946). *Biochem. J.* **40**, 721.
 Williams, R. J. & Kirby, H. (1948). *Science*, **107**, 481.

The Use of Radioactive Isotopes in Immunological Investigations

6. SOME OBSERVATIONS ON PRECIPITIN INHIBITION TESTS AND ON ANTIBODY VALENCE

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Landsteiner (1920) showed that the precipitation of azoproteins by their antisera could be partially or completely inhibited by simple substances structurally identical with, or very similar to, the compound which had been linked to the protein. This type of competitive inhibition has been used extensively by Landsteiner and his colleagues, and by many other immunologists, for the study of the serological specificity of conjugated proteins and other antigens (see the reviews by Marrack, 1938; Landsteiner, 1945; Boyd, 1947). It has also been shown that the immunological specificity of iodinated proteins is due to their 3:5-diiodotyrosine groups (Wormall, 1930), since 3:5-diiodotyrosine (and to a lesser degree the corresponding bromo-compound) specific-

ally inhibits the reaction between iodinated proteins and their antibodies; further evidence of this specific inhibitory action of 3:5-diiodotyrosine, and compounds of similar constitution and structure, was obtained by Jacobs (1932), and Snapper & Grunbaum (1936). Landsteiner suggested that the inhibitor specifically combined with the serologically active groups of the appropriate antibodies, thus preventing union of antibody and antigen to give precipitation. Proof of this union of specific inhibitor and antibodies was obtained by Marrack & Smith (1932) and Haurowitz & Breinl (1933). Further support was given by the observation that relatively simple azo dyes may (a) give precipitates with antisera to the corresponding dye-azoprotein,